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Antimicrobial Activity of Terpenoids Extracted from *Annona muricata* Seeds and its Endophytic *Aspergillus niger* Strain SH3 Either Singly or in Combination

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Keywords: Annona muricate; Antimicrobial Activity; Combined extract; Endophytic Fungi and Terpenoids

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Abstract

BACKGROUND: *Annona muricata* (Soursop) has an antimicrobial activity toward various pathogenic microorganisms which support its ethnomedicinal for the treatment of many infectious diseases.

AIM: Aim of the present study to evaluate the relation between antimicrobial activities of terpenoids extracted from different soursop parts with the isolated endophytic fungi.

METHODS: Endophytic fungal species of pulp and peel of Annona fruit along with those of seeds were isolated. Salkowski test was used for qualitative screening of terpenoids in plant and the isolated endophytic *Aspergillus niger* strain SH3.

RESULTS: Endophytic *A. niger strain* SH3 and Annona seed extract showed high terpenoid content indicated by the high intensity of reddish-brown colour. GC/Mass analysis revealed six compounds of terpenoids from endophytic *A. niger* strain SH3 extract and four compounds from seed extract with different retention times. The antimicrobial assay was performed using *A. niger* strain SH3 extract and Annona seed extract singly or in combinations against *S. aureus, P. aeruginosa, E. coli* and *C. albicans*.

CONCLUSION: The results revealed the significant antimicrobial activity of both extracts. However, the combined extract showed some reduction in antimicrobial activity which could be attributed to the antagonistic effect exhibited by their constituents.

Introduction

Annona muricata (Annonaceae) is a tropical plant species known for its edible fruits. It is called soursop, which has some medicinal merits and some toxic effects. Extracts of *A. muricata* have been famous for their antimicrobial, anti-inflammatory, anti-protozoan, antioxidant, insecticide, larvicide, and cytotoxic activities. Mechanisms of action of some pharmacological effects have been declared, such as cytotoxic, antioxidant, antimicrobial and anti-hypertensive activities [1]. *Annona* extracts from its leaves, roots, and seeds have shown antibacterial activity against a plethora of microorganisms. Endophytic fungi from medicinal plants can be considered as a reservoir of bioactive metabolites which include terpenoids, alkaloids, flavonoids, phenolic acids, quinones, steroids, tetralones and xanthones [2], [3].

Dicotyledonous plants such as soursop are proposed to have endophytic microorganisms which are a potential medicinal source. Endophytic microorganisms usually create symbiotic interactions with plant tissues. Several plant endophytic fungi have been shown to have antimicrobial activity [4]. The present study was performed to evaluate the antimicrobial activity of terpenoids in *Annona* seed and its fungal extracts. The combination of *Annona* seed extract and endophytic *A. niger* strain *SH3* has studied also.

Material and Methods

Annona muricata (Fruits and seeds) were collected from Abo-Rawash farms, Egypt. The seeds were surface-sterilised then air-dried before grinding into powder at room temperature and weighed.

Isolation and identification of endophytic fungi from seed, pulp and peel of Annona fruit

Plant parts were separated, two fresh fruits were washed thoroughly with tap water, surface sterilised with 70% ethanol for 1 min, 4% sodium hypochlorite for 3 min and again with 70% ethanol for 1 min, then rinsed twice with sterile distilled water. Samples were dried with sterile filter paper and cut into small pieces with sterile forceps and sterile gloves worn [5]. The fruits were then peeled, and small strips of each were obtained aseptically and plated on potato dextrose agar medium (PDA) containing chloramphenicol to suppress bacterial growth. Plates were then incubated at 25-27°C until the outgrowths of fungi from the explants were observed. The fungal growths were subcultured to produce pure culture on Cazpex-dox's plates. All isolates were maintained in Cazpex-dox's slants and kept at 4°C. The same procedure was applied to pulp and peel of rotten fruits where the fruits were placed in sterile polyethylene bags and stored for one week to allow deterioration. Isolation of endophytic fungi from *Annona* seeds was done by adding seed powder on the plate's surfaces, then was incubated. The endophytic fungal isolates were identified morphologically and microscopically according to Moubasher [6].

Extraction of terpenoids from Annona seeds

Terpenoids extraction from seeds were performed by agitation with ethanol (250 mL / 20 g of seed powder) three times for 48 hours on an orbital shaker. The extract was concentrated under vacuum till dryness. The concentrated extract was then stored in a vacuum desiccator at room temperature for further use.

Extraction of terpenoids from Annona endophytic fungi

The isolated fungi were grown in 2-litre standard flasks containing 500 ml of Potato Dextrose Broth. After 3 weeks of culturing at 25°C, the culture fluids were passed through four layers of cheesecloth to remove solids. To the culture filtrate, 0.25 g sodium carbonate was added with frequent shaking to reduce the number of fatty acids that may contaminate the culture; then terpenoids were extracted with two equal volumes of ethyl acetate solvent. Ethyl acetate layers were collected, concentrated and evaporated to dryness. Residues were stored for subsequent analysis [7].

Qualitative assay of terpenoids

Test for terpenoids (Salkowski test): Five ml of each extract was mixed with 2 ml of chloroform, then concentrated H_2S0_4 (3 ml) was carefully added to form a layer. A reddish-brown colouration at the interface was formed, indicating positive results for the presence of terpenoids [8].

The GC-MS analysis of fungal and seed terpenoids

The analysis was carried out using a GC/MS (Agilent Technologies 7890A) interfaced with a mass-selective detector (MSD Agilent 7000) equipped with an apolar Agilent HP- 5 ms (5%-phenyl methyl polysiloxane) capillary column (30 m × 0.25 mm i.d. and 0.25 µm film thickness). The carrier gas was helium with a linear velocity of 1 ml/min. The identification of components was based on a comparison of their mass spectra and retention time with those of the authentic compounds and by computer matching with NIST and WILEY library as well as by comparison of the fragmentation pattern of the mass spectral data with those reported in the literature [9].

Assay of antimicrobial activity

Antimicrobial activities of the terpenoids extracted from fungal *Aspergillus niger* SH3 and seed extract singly and in combination were tested against one Gram +ve bacterial species (*Staphylococcus aureus*), two Gram -ve bacterial species (*Escherichia coli* and *Pseudomonas aeruginosa*) and one fungal species (*Candida albicans*). The assay was performed using the Kirby- Bauer disc diffusion method [10], [11]. Nutrient agar (NA) medium was used for testing of bacteria, while candida agar (CA) medium was used for fungi.

The pathogens were inoculated by streaking over the surface of the sterilised media. Fungal and *Annona* extracts were applied at the surfaces of plates at 5 mg/disc dissolved in DMSO. Petri dishes were incubated at 37°c for 48 h for bacterial species and 25°C for 72 h for *Candida*. Sensitivity was then determined by measuring the mean diameter of the inhibition zones in mm. Ampicillin (5 mg/disc) was used as a positive control for bacteria. Amphotericin B (5 mg/disc) was used as a positive control for *C. albicans*. A control test for the solvent only was also performed.

Determination of relative activity

The relative activity of the tested extract concerning positive control was calculated by using

the following formula [12].

$$\text{Relative activity of the test extract} = \frac{100 \times (x-y)}{(z-y)}$$

Where,

x: total area of inhibition of the test extract;

y: total area of inhibition of the solvent;

z: total area of inhibition of the standard drug.

The total area of the inhibition was calculated by using area = πr2; where, r = radius of zone of inhibition.

Statistical analysis

The results were expressed as mean ± standard deviations (mean ± SD). Data were analysed by one-way analysis of variance (ANOVA).

Results

Isolation and identification of endophytic fungi

In the current study, endophytic fungi were isolated from different parts of *A. muricata* (Fresh, rotten fruits and seeds) (Table 1). A total of 65 fungal isolates were detected in *A. muricata* plant constituting 6 endophytic fungal species represented by 3 genera. *Aspergillus* was the most frequent genus represented by 37 isolates and 2 species, followed by *Penicillium* with 20 isolates and 3 species. The least dominant genus was *Rhizoctonia* which constituted one species with 8 isolates.

Table 1: Isolation of endophytic fungi from different parts of A. muricata

Source	Fruit				Seed	TC and Fr (%)							
	Pulp		Peel										
Fruit nature	F	R	F	R	S	TI	Fr	TI	Fr	TI	Fr	TC	Fr
Organism						(F)	(%) (F)	(R)	(%) (R)	(S)	(%) (S)		(%)
Aspergillus niger	0	8	1	3	0	1	3.33	11	39.28	0	0	12	18.4
Aspergillus niger strain *SH3*	5	6	4	6	4	9	30	12	42.86	4	57.14	25	38.5
Penicilium glabrum	3	0	1	0	2	4	13.33	0	0	2	28.57	6	9.23
Penicillium jensenii	2	0	1	0	0	3	10	0	0	0	0	3	4.61
Penicillium sclerotium	2	2	4	3	0	6	20	5	17.86	0	0	11	16.9
Rhizoctonia solani	5	0	2	0	1	7	23.33	0	0	1	14.28	8	12.4
Total count	17	16	13	12	7	30	46.15	28	43.08	7	10.77	65	100

F = fresh fruit; R = rotten fruit; S = Seed; TC = total count (cfu/ml); TI = total isolates; Fr (%) = frequency.

The fresh fruit was colonised with the highest endophytic count with frequency (46.15%) followed by the rotten fruit (43.08%) while seeds reported a frequency of only 10.77%. Concerning fungal species, *A. niger* strain *SH3* was the most dominant species represented by 25 isolates with frequency 38.5 % of the total isolates. *A. niger* came in the second rank with 12 isolates and 18.4 % frequency. *P.sclerotium, R. solani, P. glabrum* and *P. jensenii* came next with frequencies 16.9%, 12.4%, 9.23% and 4.61%, respectively (Table 1).

Terpenoids determination

Salkowski test was used for qualitative screening of terpenoids for both plant parts and endophytic fungal extracts. *A. niger* strain *SH3* and seed extract showed the high intensity of reddish-brown colour indicating high terpenoids concentration. Furthermore, the combination between seed and *A. niger* strain *SH3* extracts showed high terpenoids (Table 2).

Table 2: Qualitative assay of terpenoids produced by endophytic fungal species isolated from *A. Muricata* and Seed extract

Extract Salkowski test	Colour intensity
Pulp extract	-
Seed extract	++
Fungal extract	
A.niger	++
A. niger strain *SH3*	+++
P. glabrum	-
P. jensenii	+
P. sclerotium	-
R. solani	+
Combined extract *(A. niger* strain SH3 extract and Seed extract)	+++

+ = mild amount; ++ =moderate amount; +++= intense amount; - completely absent.

GC-MS of terpenoids in A. niger strain SH3 and Annona Seed extracts

Ten terpenoid compounds were detected among them 6 compounds from the extract of *A. niger* strain SH3 with different retention times (Table 3).

Table 3: GC-MS analysis of terpenoids in A. niger strain SH3 and Seed extracts of A. muricata

Terpenoids	Area %	Retention time (min)
A.niger strain *SH3* extract	1.55	30.98
1',1'.Dicarboxy.1á,2á.dihydro.3'H.cycloprop[1,2]cholesta.1,4,6.trien.3.one		
Tetra.tert.but	1.82	31.98
2,6.di(3.propenyl).3,7.imethoxybiycyclo(3.3.0)o cta.3,7.diene.2,4,6,8dicarboxylate		
25-Norisopropyl-9,19-cyclolanostan-22-en-24-one,3-acetoxy-24-phenyl-4,4,14-trimethyl	2.17	34.35
Silane,[(3a',5a',11a',20S)-pregnane-3,11,17,20,21-pentayl]pentakis(oxy)]pentakis.trimethyl	1.77	38.59
Anodendroside G, monoacetate (CAS)	1.38	39.29
3-[(Z)-2-Phenylethenyl]cholestan-2-one	1.54	45.70
Seed extract 2,4,6,8,10-Tetradecapentaenoic acid,9a(acetyloxy)-1a,1b,4,4a,5,7a,7b,8,9,9a-decahydro-4a,7b-dihydroxy-3-(hydroxymethyl)1,1,6,8-tetramethyl-5-oxo-1H- Cyclopropa[3,4]benz[1,2-e]azulen-9-yl.ester	0.24	33.50
4,6,8(14)-Cholestatriene	0.14	39.00
4-O-Methylphorbol 12,13-didecanoate	0.23	45.01
Pregnan-18-oic acid 3,9,11,20-tetrol, 3,11-diacetate, 18,20-lactone	0.23	46.75

Antimicrobial activity

Antimicrobial activity of seed and fungal extracts singly or in combination were assayed. Data

in Table 4 revealed that the two extracts had antimicrobial activity against gram +ve bacterial species and gram -ve bacterial species, but *C. albicans* was resistant towards any of them singly and in combination. *P. aeruginosa* was strongly susceptible to the inhibitory action of both extracts. Moreover, *E. coli* and *S. aureus* were very sensitive, respectively. The combined extract showed a reduction in antimicrobial activity which could be attributed to the antagonistic effect between both seed and endophytic *A. niger* strain *SH3* extracts. It is worth noting that the inhibitory action of the seed endophytic fungal species was more than that of the seed extract itself which clarifies that the endophytic microorganisms may be the source of the biological activity of the higher plant by its 2ry metabolites or at least intensify these activities.

Table 4: Antimicrobial activities of extracts of A. niger strain SH3 isolated from A. muricata and seed extract (singly and in combination)

Pathogenic	Inhibition zone diameter (mm)				
Microorganism	Negative Control	Positive Control	*A. niger* strain *SH3* extract	Seed extract	Combined Extract
Gram +ve bacteria					
S. aureus	$0^{a} \pm 0.0$	$21^{a} \pm 0.1$	$12^{b} \pm 0.5$	$11^{a} \pm 0.0$	$10^{a} \pm 0.0$
Gram –ve bacteria					
E. coli	$0^{a} \pm 0.0$	$25^{b} \pm 0.0$	$14^{a} \pm 0.0$	$12^{a} \pm 0.0$	$11^{b} \pm 0.5$
P. aeruginosa	$0^{a} \pm 0.0$	$25^{a} \pm 0.1$	$15^{a} \pm 0.0$	$13^{b} \pm 1.5$	$12^{a} \pm 0.0$
Yeast					
C. albicans	$0^{a} \pm 0.0$	$21^{b} \pm 0.0$	$0^{a} \pm 0.0$	$0^{a} \pm 0.0$	$0^{a} \pm 0.0$

The study supports the ethnomedicinal use of *A. muricata* for treatment of many infections. The results of antimicrobial activity of *A. niger* strain *SH3* extract, seed extract and in combination were compared with positive control either Ampicillin or Amphotericin B for evaluating their relative percentage inhibition, where *A. niger* strain *SH3* extract exhibits maximum relative percentage inhibition against *P. aeruginosa* (36%), (32.65%) against *S. aureus* (31.37%) against *E. coli* followed by seed extract and combined extract showed the least percentage (Table 5).

Table 5: Relative activity compared to the standard positive control

Pathogenic	Relative activity (%)		
Microorganism	*A. niger* strain *SH3* Extract	Seed Extract	Combined Extract
S. aureus	32.65	27.43	22.67
E. coli	31.37	23.04	19.36
P. aeruginosa	36.00	27.05	23.05
C. albicans	0.00	0.00	0.00

Mathew *et al.,* [20] proved the effectiveness of *A. muricata* leaf extract as an antibacterial agent against *Enterococcus faecalis*.

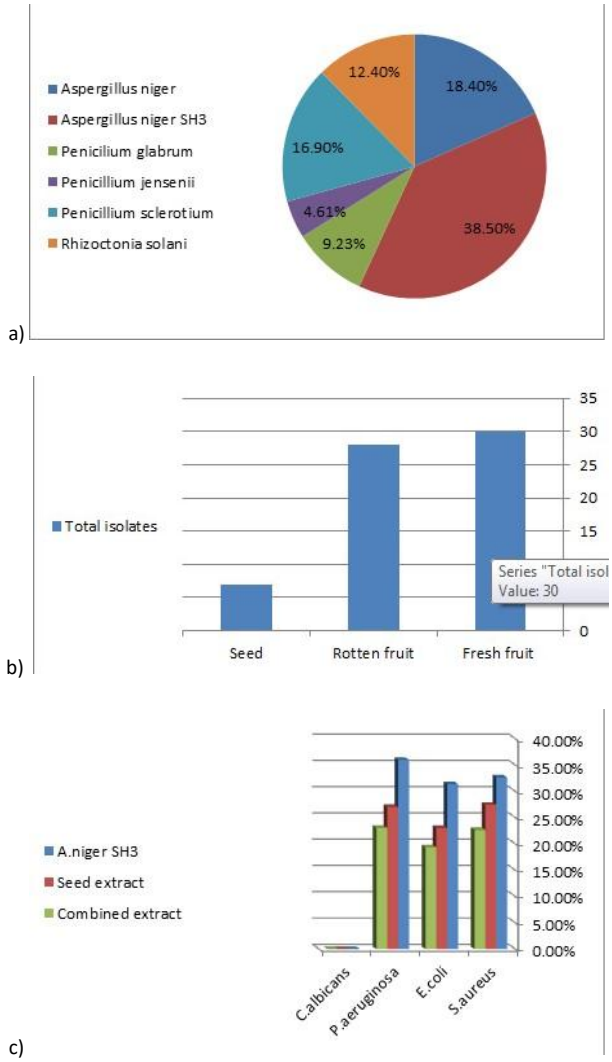

Figure 1: a) Frequency percentage of endophytic fungal species isolated from different parts of A. muricate; b) Total isolates of endophytic fungi isolated from different parts of A.muricata; c) Relative activity % of A. niger strain SH3 and seed extracts either singly or in combination

Discussion

In relations to our study, the fungal genera *Aspergillus, Penicillium, Mucor* and *Rhizopus* were isolated from *A. muricata* as endophytes [13], [14]. We can conclude that among the endophytic flora, *Aspergillus* was found to be the core group fungus from *Annona* with a colonisation frequency of 56.9%.

Salkowski test was more precise for checking terpenoids [15]. The appreciable amount of terpenoids may be due to the precise extraction process which influences the number of secondary metabolites [16].

Abdelhamid *et al*., [17] reported that GC-MS chromatogram of the ethanolic extract of Nelumbo nucifera seed showed thirty-eight peaks which indicates the presence of thirty-eight phytochemical

constituents including [[(trimethylsilyl)oxy] methyl]ethyl ester, Anodendroside E2 monoacetate, Betulin and Cholestan-3-one,cyclic 1,2-ethanediylaetal, (5á) exhibited various biological activities including Antiinflammatory, Antitumor, Antiviral, Cytotoxic and Hypolipemic [18]. Venkatachalam and Jyothiprabha, 2016 also reported antimicrobial activity of cinnamon (*Cinnamomum Verum*) extracts against Vancomycin-Resistant Enterococcus due to the presence of thirty major antimicrobial compounds identified by GC-MS analysis including Anodendroside F.

In this field, *Annona muricata* leaf extracts at a potency 20 mg/ml showed antimicrobial activity when tested against *P. aeruginosa, E. coli, S. aureus* and *C. albicans* with inhibition zones ranging from 20 to 42 mm [19].

The synergism of flavonoids, terpenoids, and alkaloids found in the extracts of *A. muricata* explains its antibacterial activity [1], [21]. It is reported that endophytic fungi from the same host plant could contain identical bioactive compounds but showed different activity [22]. Also, the combination of ethanolic extract of sour soup with antibiotic treatment increased the effectiveness of the antibiotic against multidrug-resistant strains of *E. coli* and *S. aureus* [2], [16].

In conclusion, *Annona muricata* with its endophytic fungi have an important role as antimicrobial agents against certain microorganisms. So, *Annona muricata* can be used for treatment of many infections which could be attributed to presence of terpenoids.

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Nitric Oxide Gene Polymorphism is a Risk Factor for Diabetic Nephropathy and Atherosclerosis in Type 1 Diabetic Patients

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Keywords: Nitric oxide; Genotype; Nephropathy; Atherosclerosis; Type 1 diabetes

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Abstract

AIM: To assess the risk factor for diabetic atherosclerosis nephropathy and diabetic nephropathy in type 1 diabetic patients.

PATIENTS AND METHODS: Thirty healthy volunteers age and sex-matched and Sixty-five type 1 diabetic patient were in rolled in the study. The mean age of patients was 17.99 ± 2.59 years, mean age of onset of diabetes was 7.00 ± 3.28 years, mean duration of diabetes was 10.91 ± 3.54 years. Glycosylated sex-matched (HbA1c) was assessed in blood samples, serum lipid profile was determined, and serum level of oxidised low-density lipoprotein (OxLDL), and nitric oxide was evaluated by enzyme-linked immunosorbent assay (ELISA) technique. Nitric oxide 894G > T genotype was analysed by (PCR-RFLP) method and confirmed by Sequencing. Assessment of the albumin / creatinine ratio was done in urine samples. Renal Doppler and Carotid intima-media thickness (cIMT) via ultrasound was also performed.

RESULTS: OxLDL, lipid profile, albumin/creatinine ratio, cIMT and resistivity index were significantly higher in diabetic patients while nitric oxide was significantly lower. Nitric oxide genotype shows no significant difference between diabetic's patients and controls. Diabetic patients with homozygous NO had a significantly lower serum level of Nitric oxide, a significantly higher OxLDL, albumin / creatinine ratio and lipid profile.

CONCLUSION: diabetic patients are liable for the occurrence of early diabetic nephropathy and atherosclerosis as a result of the presence of low level of nitric oxide. Nitric oxide gene polymorphism 894G > T in diabetic patients is a risk factor for diabetic nephropathy and atherosclerosis.

Introduction

Several Pathophysiological changes that lead to diabetic nephropathy are triggered by oxidative stress, advanced glycation end products and hypertension [1], [2]. Suppression of vascular dilatation factors with a subsequent reduction in the release or production of Endothelium-derived relaxing factor (EDRF) contribute to the initiation and augmentation of diabetic nephropathy. Decrease the production of nitrous oxide (NO) that is released by vascular endothelium as a vasodilatation factor also have a role in this regard. Reduction in nitrous oxide synthase (NOS) production may cause a decrease in NO level and vascular dilatation [3]. The enzyme Endothelial nitrous oxide synthetase (ENOS) is important for the contribution of vascular homeostasis and eNOS gene has 26 exons and is located on chromosome 7 [4], [5].

Angiotensin-Converting Enzyme gene polymorphism plays a pivotal role in diabetic nephropathy [6]. DD allele of the ACE gene has a role in the development and affect the severity of diabetic nephropathy with more rapid progression to end-stage renal disease [7]. For example, a positive association has been exemplified between proteinuria, and the D allele of ACE Polymorphism in a study enrolled 109 types 2 diabetic patients [8]. As there is a disagreement in the results of studies, some studies with large sample size done on specific races could not find this correlation [9]. In other studies, the correlation between diabetic nephropathy and its severity and polymorphism of some alleles of the eNOS gene are reported [10], [11], [12].

We are aiming to evaluate the risk factor for diabetic nephropathy and early atherosclerosis in type 1 diabetic patients.

Patients and Methods

Patients

The study included 65 adolescent type 1 diabetics among those attending to the endocrine clinic, Medical Center of Excellence, National Research Centre. The control group consisted of 30 healthy normal volunteers with age and sex-matched. Control group was the healthy friends or relatives of our patients.

Children with type 1 DM, duration of disease > 5 years, patients age > 14 and < 19 yrs old were included in the study. We selected this age group of patients with short duration of diabetes, firstly, to explore whether early atherosclerotic changes start in short duration of diabetes or needs longer exposure to the diabetic milieu and secondly because in younger age group (< 14 yrs old) atherosclerotic lesions are expected to be in the form of microscopic intimal fatty streaks that is too minute to be resolved by ultrasonography.

Patients during acute diabetic complications, e.g. diabetic ketoacidosis (DKA) or hypoglycemia, patients suffering from cardiac diseases, e.g. congenital, rheumatic heart, left ventricular dysfunction, patients on metformin or multivitamins and smokers were excluded from the study.

Study design and protocol

It is a cross-sectional observational study done after obtaining approval from the ethical committee of the National Research Centre, Cairo, Egypt. The registration number is 11052. Written informed consent was obtained from all patients or their parents and controls after the full discussion about the aim of the study. This study is a part of a project done in the National Research Centre for evaluation of cardiac, vascular and endothelial function in adolescent type 1 diabetic patients.

All the studied patients were subjected to: History taking include: age of patients, sex, age of onset of diabetes, duration of diabetes, type and dose of insulin therapy, family history of diabetes, presence of any symptoms of cardiac, renal, neurological affection or presence of any autonomic dysfunction and history of taking drugs other than insulin.

Clinical examination

I. Patients and controls were subjected to general, cardiac, chest and neurological examination.

II. Blood pressure was measured three times for patients and controls after 5-minute rest in the sitting position on both upper limbs with the use of automatic manometer (Omron M4 Plus, Omron Health care Europe, Hoof drop, and Holland). The mean value of the second and the third measurement was calculated. The measurements taken on the dominant limb were analysed.

III. Anthropometric measurements in the form of weight, height, waist circumference (WC), and hip circumference (HC) were taken for each participant. The weight and height of the participants were measured up to 0.01 kg and 0.1 cm using a Seca Scale Standing Balance and a Holtain Portable Anthropometer (Holtain, Ltd, Crymych, Wales, U.K.). Body mass index (BMI) was calculated as weight (in kilograms) divided by height (in meters) squared. Waist circumference was measured at the level of the umbilicus with the participant standing and breathing normally; hip circumference was measured at the level of the iliac crest, using non-stretchable plastic tape to the nearest 0.1 cm. The waist / hip ratio and waist / height ratio (cm / cm) were calculated. Each measurement was taken as the mean of three consecutive measurements, using standardised equipment [13], [14]. The landmarks, instruments used, and techniques followed were those recommended by the international biological program [13], [14].

Laboratory investigation

All patients and controls underwent the following tests: For cholesterol measurements, venous blood was sampled after a 12 h fast. Serum total cholesterol was determined by a commercial kit (Boehringer-Mannheim, Germany) [15]. High-density lipoprotein (HDL) cholesterol was separated from the serum by precipitation of the other lipoproteins with a heparin / manganese procedure [16]. Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald equation. The concentration of triglycerides (Tg) was measured in a TechnoCon AutoAnalyzer II (TechnoCon Instruments, Tarrytown, NY, USA). Glycosylated haemoglobin (HbA1c) was done every 3 months, and the mean value was calculated per year. It was measured using high-pressure liquid chromatography (Nichols Institute, Van Nuys, CA, USA) [17].

Screening for microalbuminuria was assessed in fresh morning urine samples by measuring albumin/creatinine ratio by enzyme-linked immunosorbent assay (ELISA) kit provided by Orgentec Diagnostika, Gmbh (Mainz, Germany) [18].

Nitric oxide production in sera was measured using enzyme-linked immunosorbent assay (ELISA) using a kit from Quantikine; R & D Systems, Minneapolis; USA. The assay was conducted as per the manufacturer's instructions using NO control with a standard curve plotted, and samples were measured at a 450 nm wavelength.

Serum concentrations of oxidised low-density lipoprotein (OxLDL) were detected by commercially available solid-phase two-site enzyme immunoassay

kit (Mercodia AB, Uppsala, Sweden). Measurements of the OxLDL levels in the sera were performed according to the recommendations of the manufacturer. The intra and interassay coefficients of variations were 5.5% – 7.3% and 4.0% – 6.2%, respectively, and the sensitivity was < 1 mU/L.

Analysis of Nitric oxide G894T SNP by RFLP

DNA Extraction: Three ml of blood was collected from each subject in a sterile EDTA vacutainer. Samples were stored at -20°C till DNA extraction. Genomic DNA was extracted using the QIAamp DNA Mini isolation kit (QIAGEN, # 51304) following the manufacturer's instructions and was stored at -20°C until the analysis. The DNA concentration was determined at a 260 / 280 nm absorbance ratio by NanoDrop 2000c Spectrophotometer (Thermo Fisher).

PCR Analysis of G894T SNP

Genomic DNA was amplified for determination of the eNOS G894T SNP using polymerase chain reaction (PCR), in a 25 µl reaction mixture containing 150 ng genomic DNA, 12.5 *µ*L master mix (QIAGEN, Germany), 5 pmol each forward and reverse primers : forward primer 5_TCC CTG AGG GCA TGAGGC T-3 and reverse primer 5_TGA GGGTCA CAC AGG TTC CT-3_ (QIAGEN, Germany). DNA was initially denatured at 95°C for 5 min before amplification. PCR amplification was accomplished using 30cycles, consisting of 2 min denaturation at 95°C, 45-sec annealing at 62°C, and 1 min extension at 72°C and the final extension included a 1min extension at 72°C. The reaction was carried out in BioRad thermal cycler.

Restriction Enzyme Analysis

The amplified product (10 µl) was digested in a 20 µl final reaction volume using 2 µl of Reaction Buffer, 8 U of BanII restriction enzyme (Life Technologies, USA), samples were then incubated for 5 hrs at 37°C, digested PCR products were separated by 1.2% agarose gel electrophoresis and visualized after ethidium bromide staining by UV spectrophotometry (Biometra).

Confirmation of PCR by Direct DNA Sequencing

PCR amplified fragments were completely sequenced in both directions using primers and a BigDye Terminator Cycler Sequencing Kit v1.1 (Applied Biosystems, Warrington, UK), reactions were analysed on 3100 Genetic Analyzer capillary sequencer (Applied Biosystems). Sequences were compared using BLAST [Basic Local Alignment Search Tool] (www.ncbi.nlm.nih.gov).

Carotid intima-media thickness (cIMT) assessment

A single experienced vascular sonographer, who was blind to the clinical and laboratory data of the study subjects, performed all imaging studies. The images were obtained using (General Electric medical ultrasonographic machine model: Vivid 7 Pro, GE Vingmed ultrasound AS-Nl90, Horton-Norway equipped with 7.5-10 MHz linear-array transducers). Imaging of the carotid arteries is performed in the cardiovascular ultrasound laboratory with the subject resting in the supine position with his / her neck extended, and the head turned 45° toward the contralateral side. Care was taken to have the vessel as perpendicular as possible to the plane of ultrasound beam to ensure optimal imaging of the vessel wall in its longitudinal axis with the least possible pressure in order not to compress the overlying jugular vein and to allow expansion of the carotid artery in all directions. A longitudinal section of the common carotid artery 1 cm proximal to the carotid bulb was imaged to achieve the consistent site of measurement, and a resolution box function was used to magnify this part of the artery. Three maximal IMT measurements of the far wall of the artery at 3 mm intervals were obtained starting at 1 cm proximal to the bulb and moving proximally. The reported IMT for each side is the average of these 3 measurements, and the reported IMT for each subject is the average of the 6 measurements (3 measurements from the right and 3 from the left common carotid artery). Generally, images are recorded in the plane where the maximal cIMT can be visualised. Magnification of the vessel wall allows easy identification of the intimal-medial complex, defined by the border between the echolucent vessel lumen and the echogenic intima and the border between the echolucent media and echogenic adventitia. Image frames are selected based on areas where the intima-media complex is best visualised and appears the thickest, irrespective of the cardiac cycle, with manual assessment by the sonographer using electronic callipers online [19].

Renal Doppler

Renal colour duplex ultrasound scans at baseline & after three years, using 3-6 MHz convex array transducer (Toshiba, Xario ultrasound machine). Patients were scanned in the supine position. The transducer was placed in a longitudinal position just to the Lt. of the midline, recording colour flow & Doppler spectrum from the abdominal aorta where peak systolic velocity of the abdominal aorta was recorded. Then, the transducer was placed in transverse position just distal to the origin of superior mesenteric

artery, to achieve transverse view of the aorta at the origins of both renal arteries where peak systolic velocity of both renal arteries was recorded, and renal artery stenosis was ruled out in all patients by tracing and examining different segments of both renal arteries from origin to renal hilum. Then, resistivity indices were recorded in the segmental, interlobar and arcuate arteries, on both sides

Statistical Analysis

Statistical analysis was conducted using Statistical Package for Social Science (SPSS) program version 17.0 (Chicago, Illinois, USA), t-test or Mann Whitney-U (for non-symmetrically distributed data) for independent variables was done. Chi-square was used for the analysis of NO genotype in both patients and controls. One-way ANOVA was used for comparing NO genotype about different other parameters followed by post HOCC test for detection of significance.

Results

Sixty-five type 1 diabetics (33 males and 32 females) and 30 healthy volunteers (15 males and 15 females) were in rolled in the study. The mean age of patients was 16.3 ± 1.5 yrs and mean duration of diabetes were 9.4 ± 2.9 yrs. HbA1c, albumin/creatinine ratio, cholesterol, triglyceride, LDL, OxLDL, cIMT and resistivity index were significantly higher, on the contrary, serum level of nitric oxide was significantly lower in diabetic patients (Table 1).

Table 1: Comparison between demographics, laboratory data, carotid intimal medial thickness and resistivity index of diabetic patients and controls

	Patients		Controls		
Variables	Mean	SD	Mean	SD	P-value
Age (yrs)	16.32	1.52	16.13	2.63	0.70
Anthropometric data:					
BMI (kg/m^2)	24.91	4.20	24.76	5.67	0.8
BMI (SDS)	24				
Waist circumference (cm)	83.60	9.39	84.78	12.25	0.60
HIP circumference (cm)	91.69	8.37	91.20	11.93	0.80
Waist / hip ratio	0.91	0.06	0.93	0.05	0.20
Waist / height ratio	0.51	0.06	0.52	0.08	0.90
Blood pressure:					
Systolic blood pressure (mmHg)	119.35	12.53	118.21	14.42	0.70
Diastolic blood pressure (mmHg)	81.94	9.20	78.57	6.51	0.05
Laboratory data:					
HbA1 (%)	9.55	1.90	5.43	0.65	0.0001
	28.4		10.7		
Albumin / creatinine ratio (µg/g creatinine)	78.33 ± 100.65 (5.8 – 384.2)		11.28 ± 4.23 (5.4 – 23.2)		0.0001#
Total cholesterol (mg/dl)	188.81	63.77	100.54	20.41	0.0001
Triglyceride (mg/dl)	103.46	78.29	68.89	28.39	0.03
HDL-c (mg/dl)	51.77	20.58	52.21	11.12	0.90
LDL-c (mg/dl)	118.66	47.53	62.50	19.88	0.0001
Nitric oxide (µmol/l)	28.42	7.06	40.33	6.32	0.0001
OxLDL (mg/l)	17.56	6.45	9.06	3.92	0.0001
Image analysis:					
Carotid intimal medial thickness (mm)	0.49	0.08	0.40	0.05	0.0001
Resistivity index	0.60	0.04	0.5	0.01	0.004

t-test for independent variables; # Mann Whitney U test was used; Median, mean ± SD (range); BMI: body mass index; HbA1: glycosylated haemoglobin; LDL: Low-density lipoprotein; HDL: high-density lipoprotein; OLDL: oxidised low-density lipoprotein.

No significant difference was found in NO genotype in diabetic patients and controls (Table 2).

Table 2: Comparison between nitric oxide genotype in diabetic patients and controls

Genotype	Patients		Controls		P-value
G894T:	N	%	N	%	
GG (normal)	39	62.9	15	53.6	0.7
GT (hetero)	18	29	10	35.7	
TT (homo)	5	8.1	3	10.7	

GG: Normal genotype; GT: heterozygous genotype; TT: Homozygous genotype.

Albumin/creatinine ratio, total cholesterol, triglyceride, LDL-c, OxLDL and nitric oxide revealed a significant difference with nitric oxide genotype. On the other hand, cIMT and resistivity index had no significant difference (Table 3 and Table 4).

Table 3: Comparison between nitric oxide genotype and demographic, anthropometric, laboratory data and image study of diabetic patients included in the study

Variables	Normal (GG) (group 1)		Hetero (GT) Group 2		Homo (TT) Group 3		P-value
	Mean	SD	Mean	SD	Mean	SD	
Age (yr)	16.29	1.65	16.68	1.03	15.24	1.57	0.2
Insulin dose (u/kg)	1.40	0.47	1.49	0.44	1.42	0.32	0.80
Waist/ hip ratio	0.91	0.07	0.93	0.06	0.88	0.04	0.30
Waist / height ratio	0.51	0.07	0.53	0.06	0.52	0.07	0.40
BMI (kg/m^2)	24.95	3.75	25.81	5.32	23.42	4.25	0.50
HbA1c (%)	9.20	1.87	10.22	1.69	9.70	2.30	0.20
Albumin / creatinine ratio (µg/g creatinine)	52.32	72.41	109.86	116.81	174.06	159.05	0.01 1 vs. 2,3
Cholesterol (mg/dl)	171.13	56.35	217.38	65.26	217.60	86.30	0.03 1 vs 2,3
Triglycerid (mg/dl)	83.59	37.88	141.88	125.62	128.80	76.06	0.03 1 vs 2
HDL-c (mg/dl)	49.63	21.40	54.38	17.41	57.80	23.91	0.6
LDL-c (mg/dl)	101.96	31.69	134.64	43.00	110.28	33.02	0.01 1 vs 2
Oxldl (ng/ml)	16.44	5.06	18.29	7.82	23.82	8.51	0.02 1 vs 3
Nitric oxide	31.28	5.49	25.13	6.67	17.88	4.19	0.0001 1 vs. 2,3 2 vs. 3
Uric acid (mg/dl)	4.63	0.75	4.60	0.74	4.70	0.52	0.90
cIMT	0.50	0.07	0.47	0.09	0.47	0.07	0.4
Resistivity index	0.60	0.04	0.60	0.03	0.60	0.04	0.80

BMI: Body mass index; cIMT: carotid intimal medial thickness; LDL-c: low density lipoprotein cholesterol; HDL-c: High density lipoprotein cholesterol.

Discussion

In the current study, HbA1c, albumin / creatinine ratio, cholesterol, triglyceride LDL, OxLDL, cIMT and resistivity index were significantly higher while nitric oxide was significantly lower in diabetic patients.

Type 1 diabetic patients in our study had significantly higher carotid artery IMT (cIMT) compared with normal control. These findings are in agreement with the findings of postmortem studies that have indicated a relation between early atherosclerotic lesions and diabetic state [20]. Järvisalo et al., [21], had reported that increased rate of subclinical atherosclerosis in a very young age is related to type 1 diabetes as a risk factor and it can be

detected by the presence of an increase in cIMT [21]. Several previous studies demonstrated that cIMT is increased in adults with type 1 diabetes [22], [23], [24], [25], [26], [27].

Table 4: Comparison between allele of nitric oxide genotype and demographic, anthropometric, laboratory data and image study of diabetic patients included in the study

Variables	Normal genotype (GG)		Allele (GT or TT)		P-value
	Mean	SD	Mean	SD	
Age of patients (yrs)	16.29	1.65	16.37	1.28	0.8
Duration of disease (yrs)	9.15	3.09	9.74	2.64	0.5
Insulin dose (U/kg)	1.40	0.47	1.47	0.41	0.5
Systolic blood pressure (mmHg)	121.03	10.46	116.52	15.26	0.2
Diastolic blood pressure (mmHg)	83.08	9.15	80.00	9.17	0.2
BMI (SDS)	1.31	0.93	1.33	1.11	0.9
BMI (kg/m^2)	24.95	3.75	25.29	5.12	0.8
Waist/ hip ratio	0.91	0.07	0.92	0.06	0.6
Waist/ height ratio	0.51	0.07	0.53	0.06	0.2
HbA1c (%)	9.20	1.87	10.11	1.79	0.06
Albumin/ creatinine ratio (µg/g creatinine)	52.32	72.41	124.45	126.38	0.006
Cholesterol (mg/dl)	171.13	56.35	217.43	68.44	0.007
Triglycerid (mg/dl)	83.59	37.88	138.76	114.12	0.008
HDL (mg/dl)	49.63	21.40	55.19	18.55	0.3
LDL (mg/dl)	101.96	31.69	128.84	41.45	0.007
VLDL (mg/dl)	14.37	6.66	28.70	9.38	0.003
Oxldl (mg/dl)	16.44	5.06	19.55	8.13	0.07
Nitric oxide	31.28	5.49	23.48	6.85	0.0001
Uric acid (mg/dl)	3.91	1.01	4.10	9.78	0.7
cIMT (mm)	0.50	0.07	0.47	0.08	0.2
Resistivity index (RI)	0.60	0.04	0.60	0.03	0.5

BMI: Body mass index; CIMT: carotid intimal medial thickness; LDL-c: low-density lipoprotein cholesterol; HDL-c: High-density lipoprotein cholesterol.

On the contrary, Singh et al., [19], reported that impairment in the endothelial function occurs in the first decade of the onset of type 1 diabetes, and after considerably longer exposure to the diabetic milieu the increase in cIMT occur. Our study revealed that diabetic children with impaired endothelial function also had increased cIMT. This discrepancy may be explained by differences in study populations and methodology. Additional genetic risk factors have been suggested to have the potential influence on atherosclerosis burden [19], but no supporting data exist.

Our study revealed that diabetic patients had a significantly higher resistivity index (RI) (P = 0.004). Our results are comparable with results seen in type II diabetic patients [16], [19] and also comparable with the results of many previous studies in type I diabetic patients, which demonstrated that diabetic children with no clinical evidence of renal dysfunction have RI values significantly greater than in age-matched healthy controls; therefore, suggesting a preclinical stage of DN [28], [29], [30].

No significant difference was found between nitric oxide genotype of diabetic patients and controls in our study. On the contrary, in type 2 diabetes as compared to normal population NOS synthase gene polymorphism was more common, but no correlation was not found between this gene polymorphism and retinopathy or proteinuria [3].

In the present study, albumin / creatinine ratio was significantly higher in patients with heterozygous and homozygous nitric oxide genotype. An association between diabetic nephropathy and 3 eNOS polymorphism (894G > T, 27-bp-VNTR and -786T > C) was found in a study on 400 diabetic patients, [31]. In the Chinese population, and not in non-Asian populations Ze-jun Ma et al., in a meta-analysis, found a significant association between diabetic nephropathy and the eNOS-4b / a polymorphism [32)]. Khamaisi showed that at the progressive phase of diabetes decreasing renal NOS activity is accompanied by a decline in neuronal NOS activity and protein expression [33]. Rippin et al., [34] and Momeni et al., [3], found no correlation between diabetic nephropathy and NOs polymorphism in their study done on type 1 diabetic patients. El-Din and Hamdy [35], reported that risk of end-stage renal disease in type 2 diabetic patients was associated with TT genotype of eNOS, so it may be a useful marker to identify diabetic patients with high risk. In a systemic review, the association of DN with eNOS T-786C gene polymorphism and 4b / a gene polymorphism was reported by Dellamea et al., [36]. While Bernhard et al., concluded that eNOS gene polymorphism doesn't play a significant role in the development of diabetic nephropathy in their study on either type 1 or type 2 diabetic patients [37].

eNOS gene polymorphisms that lead to decreased NO expression have been implicated with DN. The mechanism responsible for the potential association between risk of DN and *eNOS* polymorphisms is not known yet. However, variants of the *eNOS* gene may cause defective NO synthesis and decreased NO levels, which enhance the susceptibility to glomerular disease and deteriorate the renal function [38].

Cholesterol, triglyceride, LDL, OxLDL were significantly higher, while nitric oxide was significantly lower in nitric oxide genotype polymorphism (heterozygous or homozygous). In a study by Corapciogluin on 102 controls and 97 Turkish diabetic foot ulcer patients, it was reported that eNOS *G894T* polymorphism, GT-TT alleles were significantly higher than the GG alleles in patients with atherosclerotic heart disease [39].

It has been shown that atherosclerosis in animal models is accelerated by eNOS inhibition and that endothelial NO pathway abnormalities are present in humans with atherosclerosis [40]. This evidence suggests that several key steps may be inhibited by NO in the atherosclerotic process and that an alteration of NO production within the vascular endothelium could contribute to the pathogenesis of atherosclerosis. Thus, eNOS could be a candidate gene for atherosclerosis [41]. In human endothelial nitric oxide synthase (eNOS) gene, a single base exchange (G894 →T) in exon 7 results in a Glu → Asp substitution at residue 298 of the eNOS gene [42].

Limitation of the study is the number of patients small, and from our knowledge, no

researches are done in type 1 diabetes in this topic before.

We conclude that diabetic patients are liable for the occurrence of early diabetic nephropathy and atherosclerosis as a result of the presence of low level of nitric oxide. Nitric oxide gene polymorphism in diabetic patients is a risk factor for diabetic nephropathy and atherosclerosis.

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Comparison of Fixation Methods for Preservation Cytology Specimens of Cell Block Preparation Using 10% Neutral Buffer Formalin and 96% Alcohol Fixation in E-cadherin and Ki-67 Immunohistochemical Examination

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Abstract

BACKGROUND: Cytological and molecular examinations are among the most important examinations in cancer diagnosis. 96% alcohol is a fixative solution commonly used by clinicians for cytological samples because of its accessibility and affordability. Cellblock preparation from cytology specimen may increase morphology detail and may be used for further biomarker analysis. E-cadherin is an adhesion protein expressed in the cell membrane of most carcinoma. Ki67 is a protein expressed in nuclei of malignant cells that used as a proliferation marker.

AIM: This study was designed to investigate the effect of fixation duration in 96% alcohol on protein preservation for immunohistochemistry (IHC) evaluation compared to 10% neutral buffered formalin (NBF) as the gold standard.

METHODS: Twenty-five fine-needle aspiration biopsy (FNAB) specimen diagnosed as carcinoma were fixed in 10% NBF and 96% alcohol for 1 hour, 6 hours, 24 hours, 48 hours and 72 hours. Cell blocks preparation were made from those 6 groups of specimens. E-cadherin and Ki67 IHC were done to cell blocks section and evaluated. The data were statistically analysed using the Friedman test with p-value < 0.05 of a significant level.

RESULTS: There were significant differences between E-cadherin and Ki67 expression in cell block preparation from 96% alcohol-fixed cytology specimen for 1 hour, 6 hours, 24 hours, 48 hours and 72 hours to 10% NBF (p = 0.0001).

CONCLUSION: The result indicated that 96% alcohol is not suitable as a fixative solution for cell block preparation in E-cadherin and Ki-67 IHC examination.

Introduction

Cytological and molecular examinations are among the most important examinations in cancer diagnosis. Recently, cytology testing is becoming more frequent as the less invasive sampling technique develops. With the development of personalised medicine in the treatment of cancer, a molecular examination is a very important examination. The cell block (CB) offers many advantages, over other cytological preparations, particularly for diagnostic and immunohistochemical testing. One of the important points to making a good cell block is a fixation. Ten percent NBF is universal fixative for optimal preservation of cellularity, cytomorphology, and architecture in the cell block. It also provides optimal fixation for FNAB material for cell block sample [1]. 10% NBF as a gold standard. However, there are also disadvantages to using NBF as a fixative, including the handling of formalin, since formaldehyde is considered a carcinogen and crosslinking agent [2].

The most commonly used fixatives for diagnostic pathology and cytologic specimens are 10% NBF and 95% ethanol [3]. In our institution, 96% alcohol used by clinicians as a fixative for cytology specimens because of its accessibility and affordability. To our knowledge, no study has examined the effect of 96% alcohol fixative agent in cell block preparations from FNAB sample. So, the

author wants to know the duration of 96% alcohol fixation that will greatly affect the preservation of protein molecules.

In this study, to see the effect of fixation, we were using IHC to analyse how the preservation of protein molecules. To analyse the preservation of antigens in cell block preparations, IHC was carried out using E Cadherin, for proteins located in the cell membranes, and expressed in most carcinomas [4]. Other IHC examination was Ki67 as a marker of proliferation in tumour cells and expressed in proteins located at nuclei and generally associated with tumour cell proliferation and malignant potential of the tumour [5].

Our study assessed alcohol 96% with fixation time: 1 h, 6 h, 24 h, 48 h and 72 h interacted with tumour cell block, which might cause protein denaturation. Based on experience, where cytology samples obtained from clinicians, 96% alcohol had been fixed within a few hours, due to a late and delayed transportation or laboratory sample process from late Friday surgery during the weekend, and the waiting time for a long holiday weekend. Yamashita-Kashima *et al.* discovered that the time to and length of fixation of tumour specimens could affect HER2 IHC and fluorescence in situ hybridisation (FISH) scores [6].

This study was designed to investigate the effect of fixation duration in 96% alcohol on protein preservation for immunohistochemistry (IHC) evaluation compared to 10% neutral buffered formalin (NBF) as the gold standard.

Material and Methods

This research has ethical clearance from the Health Research Ethics Committee Padjadjaran University with number 1150/UN6.KEP/EC/2018.

Cytologic Specimens and Cell Block Preparation

In total, we used 25 fresh surgical specimens; all tumours are carcinoma (ovarian carcinoma, invasive breast carcinoma of no special type (NST), Papillary thyroid carcinoma). FNAB was performed in a tightly controlled manner in the surgical pathology gross room at Dr Hasan Sadikin Hospital / RSHS. For each specimen, 6 separate FNABs were performed, sampling the same area. Because we were working with large surgical pathology specimens, we were able to sample the same general area of the tissue without sampling the same needle track in with the tissue that has been disrupted by prior needle pass. We used 23-gauge needles from Terumo medical corp., with a 10-cc slip-tip syringe and using the standard FNAB technique [7]. The first FNAB was rinsed and fixed with 10% NBF centrifuge for 7 minutes at 3000 revolutions per minute, decant supernatant, add 10% NBF then the specimen was submitted for processing. The five FNABs fixed with 96% alcohol were processed according to the duration of fixation 1 hour, 6 hours, 24 hours, 48 hours, 72 hours then centrifuged for 7 minutes at 3000 rpm respectively. After that, the cells block was prepared from residues.

IHC of Cell Block Section

Immunohistochemical (IHC) techniques were performed according to Agustina *et al., [8]*. IHC staining on the samples was performed manually using a labelled streptavidin-biotin immunoperoxide complex method, using the Starr Trek Universal HRP Detection system (Biocare Medical, Concord, CA, USA). Each cell block was cut into 4-µm slices and examined on L-lysine coated glass slides and baked at 60°C for one hour on a standard histology hotplate. Deparaffinized using xylene and rehydrated using an alcohol solution than brought to water. Antigen retrieval used a decloaking chamber (DC2008INTL, Biocare Medical, USA) in EDTA (pH 8.0), followed by cooling at room temperature for 20 minutes. Sections were then treated to block endogenous peroxidase, stained with primary antibodies, and incubated for 1 hour at room temperature. Detection was done by horseradish peroxidase polymer-based detection system (Biocare Medical) and diaminobenzidine chromogen and counterstained with haematoxylin. The primary antibodies were E-cadherin (G10) sc-8426 from Santa Cruz Biotechnology, inc (Santacruz, CA) and KI-67(SP6) from Cell Marque (Rocklin, CA, USA).

IHC Analysis and Interpretation

To analyse antigen preservation in the cell block, we used immunostaining E-cadherin that represent antigen in membrane and Ki-67 in nuclei. The expression for E-cadherin in the membrane of cancer cells was score with histologic score (also known as histoscore) scheme [9]. The intensity of staining was categorized as 0 (negative), 1 (weak), 2 (moderate), or 3 [10]. The percentage of positive cells were scored as 0 (negative), 1 (< 20%), 2 (20% < 50%), 3 (≥ 50%-80%), 4 (> 80%). A histoscore was generated as the product of the intensity and the area of the staining. The histoscore was than dichotomised into weak expression (histoscore 0-4) and strength of expression (histoscore 6-12). All procedures were done by 2 assessors pathologist (BSH and TI). Both of whom were blind to the fixative used.

To analyse immunostaining of Ki-67 for the antigen located in nuclei. The number of Ki-67-positive cells was counted using image analysing

software QuPath according to Zhong, *et al.,* and Laurinavicius *et al*., [11], [12]. The image-analysis software automatically counts the nuclei of cells that have an intensity that exceeds the predetermined threshold level. The advantages of using quantitative analysis are a time-saving alternative to manual counting method, reduce the variability of the pathologist in counting the tumour cell [13]. Histoscore was calculated with 40 cut-off points, ≤ 40 was weak, > 40 was strong [14].

Statistical Analysis

The quantitative comparative analysis method is applied in this study, six paired groups with an experimental design to obtain a good preservation cell and an optimal Immunohistochemistry (IHC). P ≤ 0.05 is considered statistically significant. The data obtained were recorded on a special form and then processed using SPSS program ver. 22.0 for Windows (IBM Corp., Armonk, NY, USA).

Results

Tumour Characteristic

The clinical characteristics of the carcinomas summarised in Table 1. In total, we used 25 fresh surgical specimens; all tumours are diagnosed as carcinoma.

Table 1: Characteristic sample from each tumour

Samples	N = 25
Ovarian Carcinoma	10
Invasive breast carcinoma of no special type (NST)	5
Papillary Thyroid Carcinoma	10

Immunohistochemistry of E-cadherin

E-cadherin is a membrane protein expressed on the cell membrane, commonly known as epithelial cell marker [15]. We evaluated the E-cadherin expression on different tumour tissues through IHC examination, on cell blocks fixed with 10% NBF, compared to cell blocks fixed with alcohol 96% in different fixation time. E-cadherin histoscore showed that 10% NBF fixation gives strong result 100%. Meanwhile, alcohol 96% fixation tends to exhibit a histoscore decrease, as shown in Table 2. P-value showed a significant difference between 10% NBF and alcohol 96% fixation.

From the results of statistical tests on the E-cadherin Histoscore in Table 2, information was obtained that P-value = 0.0001 was smaller than 0.05 (P-value < 0.05) which meant that it was significant or statistically significant thus it could be explained that there were differences between E-Cadherin Histoscore in 10% NBF fixation group and all of 96% alcohol duration fixation.

Table 2: Histoscore comparison of E-cadherin expression between 10% NBF fixation and alcohol 96% with a various fixation time

Variable	10% NBF N = 25	96% Alcohol					P-value
		1 h	6 h	24 h	48 h	72 h	
		N = 25	N = 25	N = 25	N = 25	N = 25	
Histoscore E-cadherin							0.0001**
Weak	0	2	7	11	16	16	
Strong	25	23	18	14	9	9	
P-value		0.157	0.008**	0.0001**	0.0001**	0.0001**	

Description: Categorical data on p values are calculated (with) Friedman test. **Significant difference was determined by p < 0.05.

From the results of statistical tests on the E-cadherin Histoscore in Table 2, information was obtained that P-value = 0.157. The P-value in the Histoscore was greater than 0.05 (P-value > 0.05) which means it was not statistically significant so it could be explained that there was no difference between E-Cadherin Histoscore in 10% NBF fixation group and 1 hour of 96% alcohol fixation.

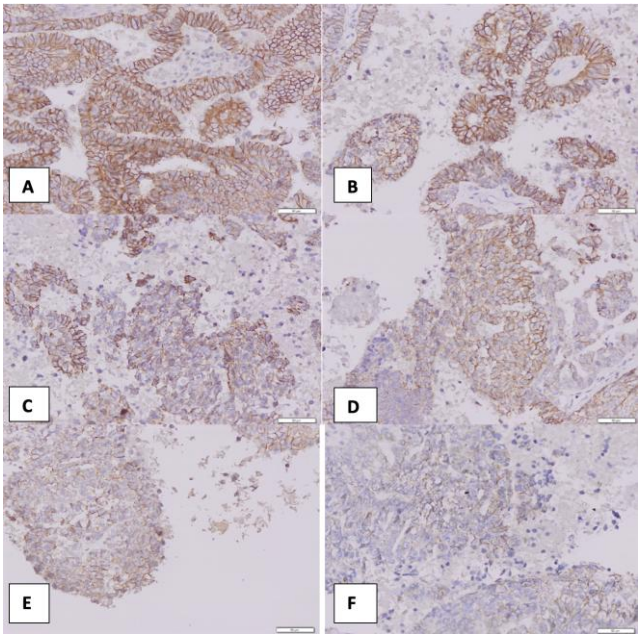

Figure 1: Representative images of E-cadherin expressions on immunohistochemistry staining; A) Cellblock section fixed with 10% NBF; B), C), D), E) and F) Cellblock sections fixed with 96% alcohol for 1 h, 6 h, 24 h, 48 h, and 72 h respectively (x 200)

From the results of statistical tests on the E-cadherin Histoscore in Table 2, information was obtained that P-value = 0.008 was smaller than 0.05 (P-value < 0.05) which meant that it was significant or statistically significant thus it could be explained that there were differences between E-Cadherin Histoscore in 10% NBF fixation group and 6 hours of 96% alcohol fixation.

We used McNemar statistical analysis test to reveal the significant difference between 10% NBF and alcohol 96% fixation in each fixation time. The results showed that short duration of fixation time has no significant difference between both fixation method

(p > 0.05) meanwhile longer duration exhibit significant difference (6 h, 24 h, 48 h, 72 h).

E-Cadherin expressions on IHC staining represent antigen in the membrane. The strong of expression (Figure 1A and 1B) and weak expression (Figure 1C, 1D, 1E and 1F).

Immunohistochemistry Ki-67

To analyse the preservation of antigen in cells block, we also performed immunostaining for the nuclear protein Ki-67 (shown in Figure 2) [5]. The histoscore comparison on Ki-67 expression in FNAB samples fixed with 10% NBF and 96% alcohol with different fixation time showed a significant gradual decrease in a time-dependent manner for both fixation methods (Table 3; P-value = 0.0001).

Table 3: Histoscore comparison on Ki-67 expression between 10% NBF fixation and 96% alcohol with a various fixation time

Variable	10% NBF	96% Alcohol					P-value
		1 h	6 h	24 h	48 h	72 h	
	N = 25	N = 25	N = 25	N = 25	N = 25	N = 25	
Histoscore Ki-67							0.0001**
Weak	6	12	20	24	25	25	
Strong	19	13	5	1	0	0	
P-value		0.003	0.0001**	0.0001**	0.0001**	0.0001**	

Description: Categorical data on p values are calculated (with) Chi-square test; **Significant difference was determined by p < 0.05.

Statistical analysis indicated that Ki-67 histoscore fixed with 10% NBF compared to 1h and 6h of 96% alcohol showed a significant difference as well as to longer alcohol fixation time. IHC staining result on (96% alcohol-fixed) FNAB samples demonstrated the gradual reduction of Ki-67 staining, as listed in Figure 2.

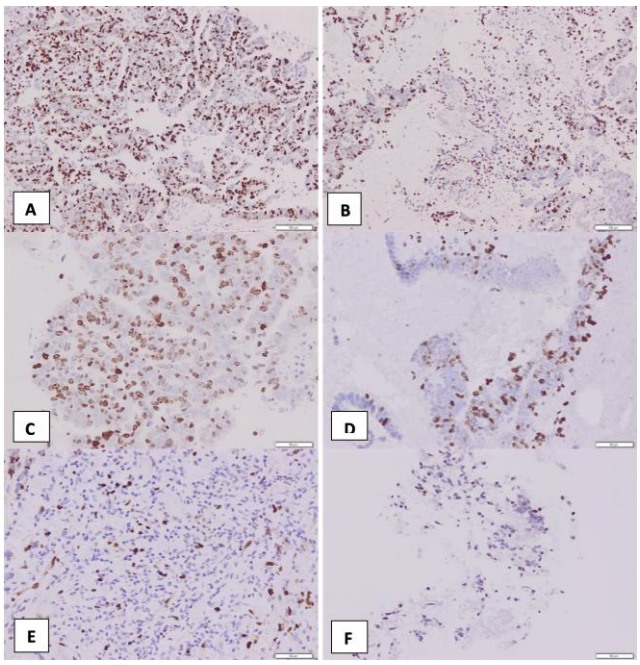

Figure 2: Representative images of Ki-67 expression on immunohistochemistry staining; A) Cellblock section fixed with 10% NBF; B), C), D), E) and F) Cellblock sections fixed with 96% Alcohol for 1 h, 6 h, 24 h, 48 h, and 72 h respectively (x 200)

Immunohistochemical expression of Ki-67 shows a strong association with tumour cell proliferation growth.

Discussion

Fixative agents are essential in diagnostic pathology. Ten percent NBF is commonly used in histopathology to fix tissue because it provides optimal fixation for FNAB material for cellblock samples. However, NBF has carcinogenic property as it has been categorised as 'carcinogenic to humans' (group1) by the International Agency for Research on Cancer (IARC, 2005) [16]. It has also become a major burden on the environment, and it crosslinks molecular groups [2]. Most laboratories in Indonesia use 96% alcohol as a fixative agent for cytology specimens because of its accessibility, and it's relatively inexpensive. Based on the literature, alcohol work as a coagulating agent which precipitates protein by breaking hydrogen bonds in the absence of protein cross-linking [17]. This research aims to determine the effect of 96% alcohol as a fixative agent in making cell blocks from cytology samples for further use in E-cadherin and Ki-67 immunostaining analysis. However, the effectiveness of using 96% alcohol as a fixative agent in making a cell block has not been evaluated and documented, and not supported by the literature.

Different fixation method can compromise the stability of protein expression in IHC staining. In our study, we compare the histoscore on E-cadherin as well as Ki-67 expression fixed with 10% NBF and 96% alcohol with various fixation time: 1 h, 6 h, 24 h, 48 h, and 72 h. Surprisingly we observed a gradual decrease of histoscore result corresponded to different 96% alcohol fixation time.

According to the previous study, Essen et al., discovered that tissues fixed in non-crosslinking alcohol-based fixatives could successfully be immunohistochemically stained for most antibodies following the usual NBF based protocols. Recently, alcohol-based fixative such as RCL2 and Boonfix have been proposed. Nonetheless, NBF-fixed tissues still provide significantly better immunostaining results (84% good staining) compared to RCL2 (66% good) and Boonfix-fixed tissue (60% good staining). The application of alcohol-based fixative may have additional benefits for molecular techniques, as they are expected to preserve DNA and RNA to a larger extent [2].

Moelans et al. found that alcohol-based fixative can replace NBF as the standard fixative agent, by saying that alcohol-based non-crosslinked fixative gives a better outcome in terms of preserving DNA and RNA, as well as providing quality and

applicability in molecular diagnostics. Nonetheless, despite the argument, NBF still provides a better result compared to alcohol-based fixative, and alcohol is unlikely to replace NBF universally [18]. This argument agrees with our findings, which 96% alcohol application showed a histoscore decrease in time-dependent fixation manner. In contrast, NBF fixation exhibited 100% and 76% histoscore on E-cadherin and Ki-67 immunostaining respectively.

Our results are consistent with Essen et al., the study that alcohol denatures proteins, showed by the decrease in histological results when fixed with different fixation time in a time-dependent manner.

The histoscore comparison of E-cadherin immunostaining showed no significant difference in the strong-weak category between fixation with 10% NBF and 96% alcohol for 1-hour. This result indicates that 1 hour might be optimum for fixation time to generate cell block.

Our results correspond with the study performed by Matsuda et al., compared the morphology and the quality and quantity of ribonucleic acid [19] and protein in paraffin-embedded tissues of nude mice implanted with human uterine cervical cancer cells, followed by fixation with commonly used fixatives, including 4% paraformaldehyde (PFA), 10% neutral buffered formalin (NBF), 20% NBF, and 99% ethanol (EtOH). The assay was then continued for IHC staining on E-cadherin, Ki-67, VEGF-A, HLA class 1, AE-1 protein expression. This study indicated that formalin fixation is better than alcohol fixation for RNA preservation in paraffin-embedded cancer cell implantation models. Their results showed that 90% of cells fixed by ethanol 99% showed that ethanol 99% cause cell shrinkage due to cell dehydration. Fixation with NBF 10% and NBF 20% showed good results on cell morphology quality. The 99% EtOH-fixed samples showed marked decreases of Ki-67 immunostaining compared with the formalin-fixed samples and showed a decrease of E-cadherin immunostaining to a lesser extent [20]. Su et al. found that formalin-based fixation is preferable to compare to ethanol 99% in analysing cell morphology. Gong et al., demonstrated significantly lower detection rates of Ki-67, PCNA, and p53 with the ethanol-based fixative ThinPrep as compared with formalin-fixed cell-block slides in malignant cases [21]. This is consistent with our study that IHC staining result on 96% alcohol-fixed FNAB samples demonstrated the gradual reduction of Ki-67 immunostaining. Our findings indicated that Ki-67 immunostaining results were incompetent for further analysis even from the 1hour fixation time with 96% alcohol.

Different from previous research, Denda et al., revealed ethanol-fixed smears, that Ki67 could be immunostained successfully with heat-induced antigen retrieval. The optimal antigen retrieval condition for each antibody must be individually determined. For the nuclear antigens, heat-induced antigen retrieval may allow access of the antibody to the DNA-binding protein epitopes, partly hindered by steric effects, because it can denature double-stranded DNA into single-stranded DNA. However, the role of antigen retrieval in the immunostaining of cytologic specimens is currently unclear and not yet optimised [3].

Ten percent NBF as the gold standard in the fixation process is routinely used in histology samples, as well as in IHC staining. This study shows that cell blocks fixated with 10% NBF showed good consistent results and were able to preserve cells obtained from cytology samples. Similarly, JH Williams suggests that some types of fixation include 10% normal saline, 10% NBF and 10% formalin showed consistent results both for cell preservation and immunohistochemistry [22]. According to Engel et al., specimens fixed with 10% NBF showed excellent results in preserving antigens and showing consistent results for immunohistochemistry [23].

In summary, we discover that the management of cytology samples using 96% alcohol fixation was not recommended as a fixative agent for making cell blocks and followed by IHC examination. Fixation with NBF 10% as the gold standard showed good results, and optimal histoscore values, hence it is recommended for sample fixation when making cell blocks, before IHC examination.

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Basic Science

Analysis of *NPHS2* Gene Mutations in Egyptian Children with Nephrotic Syndrome

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Keywords: NPHS2 mutation; Steroid resistant nephrotic syndrome; R229Q polymorphism

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Abstract

BACKGROUND: Mutations in the *NPHS2* genes are the main aetiology of early-onset and familial steroid-resistant nephrotic syndrome (SRNS). The pathogenic *NPHS2* mutation together with the p.R229Q variant has been less described among Egyptian children.

AIM: This study aims to determine the mutation of *NPHS2* in children with NS and discover the role of p.R229Q variant in SRNS

METHODS: The study included 53 children with NS, and 53 healthy volunteers matched in age and sex controls. The median age at disease onset was 7.3 years. Among NS cases, 31 cases had steroid-sensitive nephrotic syndrome (SSNS) and 22 children with steroid-resistant nephrotic syndrome (SRNS). Polymerase chain reaction amplification of the whole coding region of *NPHS2* gene was carried out for its mutational analysis. Restriction digestion testing was carried out after PCR to determine the presence of R229Q polymorphism. Randomly selected samples were re-genotyped by two independent technicians for assessment of Quality control

RESULTS: NS patients showed a significant higher frequency of heterozygous genotype GA (89.5%) compared to control group (10.5%) with increased risk of NS (OR, 12.04; 95% CI, 2.61 to55.38; $p < 0.0001$). Moreover, SRNS showed a significant higher frequency of GA genotype (68.2%) than the SSNS group (6.5%). The GA genotype was associated with increased risk of SRNS (OR, 31.1; 95% CI, 5.73 to 168.48; $P < 0.001$) and the A allele was associated with increased risk of SRNS (OR, 15.52; 95% CI, 3.325 to 72.422; $P < .001$).

CONCLUSION: R229Q polymorphisms are associated with SRNS, and any child with SRNS should be searched for mutations in the *NPHS2* gene.

Introduction

The most common primary glomerular disease in children is nephrotic syndrome (NS). Nephrotic syndrome in children is classified as having steroid-sensitive (SSNS) or steroid-resistant (SRNS) [1].

NPHS2 gene mutation has been reported in 10–30% of children with sporadic SRNS children [2]. The frequency of SNP (*p.*R229Q) which is the most frequently reported polymorphism among SRNS is 5% in the European population as compared to healthy one [3]. Progressive damage of the glomerular filtration barrier occurs in these patients resulting in the development of end-stage renal disease (ESRD) [4]. An autosomal recessive form of SRNS with an early onset of the disease occurs due to mutations in the NPHS2 gene. One of the major glomerular diseases in Egyptian children is idiopathic NS and about 30% of these NS children is resistant to treatment by steroids [5].

In contrary, in other parts of the world , resistance to steroid therapy is prevalent in10–20% of

cases of NS [6]. Therefore, the aim of this study is to analyze the mutation of *NPHS2* gene in Egyptian children by applying next generation sequencing (NGS) and determine the role of SNPs in Egyptian children, concentrating our analyses on SRNS children carrying the p.R229Q variant.

Subjects and Methods

The current case control study was conducted on 53 patients with nephrotic syndrome (NS) and 53 healthy volunteers with matched age and sex during the period from January 2017 to June 2018.The patients were referred from the Pediatric Nephrology Units, Al Zahra and Al – Hussein hospitals, Al-Azhar University, Cairo, Egypt. The clinical records of all the subjects were reviewed for age at onset, gender, family history, treatment modalities and response to therapy.

All patients were diagnosed as nephrotic syndrome according to their clinical presentation and laboratory studies. The patient's group was categorized into 2 groups: 21 children with SRNS and 32 children with SSNS. Steroid responsive NS (SSNS) was regarded as complete remission achieved with steroid therapy. Steroid-resistant NS (SRNS) was regarded as failure to achieve remission following 4-week prednisone 60 mg/m2 followed by three methylprednisolone pulses [7], [8].

The informed consent forms were given by caregiver of patients.

The study protocol was approved by the medical Research Ethical Committee of AL-Azhar University.

Genomic DNA was extracted from peripheral leukocytes of whole-blood samples using standard laboratory protocols.

The 5 mL blood samples were drawn into tubes containing EDTA. Genomic DNA was extracted from peripheral leukocytes using standard techniques. NPHS2exon 5 was polymerase chain reaction (PCR)-amplifiedusing the following primers: F 5'-AGGATTTACCACAGGATTAAGTTGTGCA – 3' and R 5'-TAGCTATGAGCTCCCAAAGGGATGG – 3'. Three microliters of unpurified PCR product were diluted to 10 lL in recommended restriction buffer containing 5 U of ClaI and digested at 37°C overnight. The PCR products were visualized by electrophoresis in a 3% agarose gel with ethidiumbromide and stored in digital form. Quality control for these assays was assessed by randomly selecting 50 samples to be re-genotyped by two independent technicians. Each batch of restriction digestion contained a positive control (confirmed G allele) to avoid mistyping. 10% of the samples were randomly picked and re-genotyped to give consistent results. The products were resolved on 2% agarose gel and viewed in a gel documentation unit (Bio-Rad). Quality control for these assays was assessed by randomly selecting 30 samples to be regenotyped by two independent technicians.

Statistical Analysis

The SPSS version 21 was used in this research. Chi-square test (χ^2) was used for comparison of frequencies between patients and controls and t test was used for comparing means. The association between case-control status and each polymorphism, measured by the odds ratio (OR) and its corresponding 95% confidence interval (CI). Hardy-Weinberg equilibrium was checked using χ^2 test to compare the observed genotype frequencies with the expected frequencies among the case and control subjects.

Results

Table 1 shows distribution of genotypes of R229Q polymorphism in NS patients compared to control group. NS patients showed significant higher frequency of heterozygous GA genotype (89.5%) compared to control group (10.5%); ($\chi 2$ = 14.43; p < 0.001), and increased risk of NS (OR, 12.04; 95% CI, 2.61 to 55.38; p < 0.0001).

Table 1: Genotypes distribution of R229Q polymorphism in NS cases and controls

Genotype	NS (n = 53)	Controls (n = 53)	Odds ratio (95% Confidence Interval)	*P* value
Genotype				
	36 (41.4%)	51 (58.6%)		
GG			1	
GA	17(89.5%)	2 (10.5%)	12.04 (2.61-55.38)	< 0.0001
AA	0	0	--	--
		$\chi 2$= 14.43; P < 0.001		

Table 2 shows comparison of the genotype's distribution of R229Q polymorphism between SRNS and SSNS patients. Significant increase of heterozygous genotype (GA) was observed in SRNS (68.2%) as compared to SSNS patients (6.5%) $\chi 2$ = 22.5; P < 0.001.

Table 2: Genotypes and allele frequency distribution of R229Q polymorphism in children with SRNS and SSNS

Genotype	SRNS (n = 22	SSNS (n = 31)	Odds ratio (95% Confidence Interval)	*P* value
GG	7 (31.8%)	29 (93.5%)	1	
GA	15 (68.2%)	2 (6.5%)	31.1 (5.73-168.48)	< 0.0001
AA	0	0		
x2= 22.51 p < 0.001				
Allele				
G	29 (32.9%)	60 (67%)	1	
A	15 (88.2%)	2 (11.8%)	15.52 (3.325-72.422)	< 0.0001

The GA genotype was associated with

increased risk of SRNS (OR, 31.1; 95% CI, 5.73 to 168.48; P < 0.001) and the A allele was associated with increased risk of SRNS (OR, 15.52; 95% CI, 3.325 to72.422; P < 0.001).

Amplified PCR products in 6 healthy subjects with wild genotype (GG) were illustrated in Figure 1(left). Moreover, PCR product of exon 5 of *NPHS2* gene for three patients and two healthy controls with wild genotype (GG) of R229Q and for one patient with the heterozygous genotype (GA) of R229Q was illustrated in Figure 1(right).

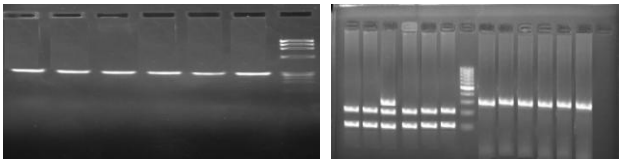

Figure 1: Amplified PCR products in 6 healthy subjects (left); Agarose gel stained with ethidium bromide illustrating PCR product of exon 5 of NPHS2 gene before and after digestion with ClaI endonuclease enzyme for patients and healthy controls with wild genotype (GG) of R229Q and for one patient with the heterozygote genotype (GA) of R229Q (right)

Figure 2(left) shows sequence chromatogram of exon 5 of *NPHS2* gene, illustrating wild pattern GG. Figure 2(right) shows sequence chromatogram of exon 5 of NPHS2 gene and heterozygous pattern (p.Arg229 Gln) (c.686G > A). Site of mutation is denoted by the arrow.

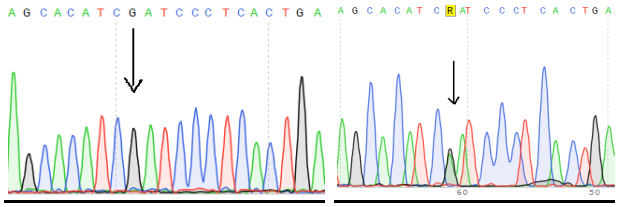

Figure 2: Sequence chromatogram of exon 5 of NPHS2 gene showing wild pattern (p.Arg 229Gln) (c.686G > A). Site is denoted by the arrow (left); Sequence chromatogram of exon 5 of NPHS2 gene showing heterozygous pattern (p.Arg229 Gln) (c.686G > A). Site of mutation is denoted by the arrow (right)

Discussion

The R229Q polymorphism (c.686G > A; rs61747728) is considered a non-neutral polymorphism and it has also been associated with glomerular disease. It is a podocin variant resulting in an amino acid substitution from arginine to glutamine in~1 – 2% of European populations; the R229Q variant is being present and is associated with the development of microalbuminuria**.** The adult-onset FSGS is the result of compound heterozygosity of the R229Q variant with a pathogenic podocin mutation [9]. The role of *NPHS2* mutations in adult onset disease have limited confirmation because of identification of few cases [10], [11], [12]. Few studies reported the effect of p.R229Q and p.P20L in causing disease in European, North American Caucasian and South American populations [13], [14]. A non-conservative substitution was caused by p.P20L in a previous study [15]. In the Czech population, the highest frequency of p.R229Q has been reported (12%) [16]. An increased (2 – 3 folds) risk of micro albuminuria with progression gradually to ESRD at the age of thirty and forty years is caused by p.R229Q, with tendency to have later-onset disease (i.e. typically FSGS) [17].The single heterozygous mutation (p.R229Q) could not by itself be considered as a causative mutation because a previous study predicted 2 *NPHS2* gene SNPs, a heterozygous 1082T > C and a homozygous 954T > CA [4]. A low prevalence of the two genes in Japanese and Chinese NS patients was reported in several studies [18], [19]. There are a number of other genes make a significant contribution to the spectrum of disease-causing mutations beside the *NPHS1* and *NPHS2* genes. In familial autosomal-recessive steroid-resistant nephrotic syndrome *NPHS2* mutations was detected for the first time by [20]. Also, in sporadic cases of steroid-resistant nephrotic syndrome [2], [15], [21] and late-onset focal segmental glomerulosclerosis (FSGS) [12], *NPHS2* mutations were identified thereafter. Individuals with the same genotype revealed a wide range of phenotypic variability [19]. In a previous study the mutation detection rate in familial autosomal-recessive and sporadic steroid-resistant nephrotic syndrome was 43% and 10.5% respectively [22]. The *NPHS2* mutations were present in patients of Italian, French, German, and Israeli-Arab origin, but it is not present in children of Israeli-Jewish and Japanese origin [2], [15], [21].

In conclusion, *NPHS2* mutations were observed in the studied Egyptian children with nephrotic syndrome and significant higher frequency was detected in SRNS cases. Therefore, every child with SRNS should be searched for mutations in *NPHS2* gene by target-oriented next generation sequencing analysis. Moreover, mutational analysis of *NPHS2* genes should be included in the diagnosis of NS among Egyptian patients.

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Basic Science

Gambir Extract (*Uncaria Gambir*) Decreases Inflammatory Response and Increases Gastric Mucosal Integrity in Wistar Rats - Model Gastritis

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Keywords: Gambir extract; Uncaria gambir; TNF alpha; Prostaglandin E2; Gastritis

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Abstract

BACKGROUND: *Uncaria gambir* (local name: gambir) is a plant native to Sumatera, Malaya and Borneo. This plant is potential as local wisdom for therapeutics. In Sumatera, gambir was used as a traditional treatment for fever, diarrhoea, diabetics and wound healing.

AIM: To explore the efficacy of gambir extract on TNF alpha level, prostaglandin E2 level, lesson area, body weight, lipid profile and leptin level in Wistar rat-model gastritis.

METHODS: This study was an experimental study, with a pre-post-test control group design. The subjects in this study were 30 male rats, 8 weeks old, weight 150-200 gram. Rats were administered with gambir extract at the dose of 20, 40 and 80 mg/kg BW/day for 3 days. Gambir was extracted by maceration methods. Statistical analysis was performed by SPSS 18.

RESULTS: Gambir extract at the dose of 80 mg/kg BW exhibited the highest efficacy in reducing TNF alpha level, lesion area and increasing prostaglandin E2 level compared to gambir extract at doses of 20 mg/kg BW, 400 mg/kg BW, negative control, and positive control.

CONCLUSION: Gambir extract was effective in reducing TNF alpha level, lesson area, and increasing prostaglandin E2 level in Wistar rat-model gastritis.

Introduction

Gastritis is an inflammatory condition that occurs in the gastric mucosa. The symptoms resulting from gastric mucosa inflammation are burning sensation and discomfort in the stomach. Gastritis is one of the serious health problems in the world [1]. The incidence of chronic gastritis increases with age. In Western countries, the prevalence of gastritis sufferers is almost 80% in the elderly population of sixty years old and predicted to become 100% in the population of seventy years old. Chemical irritants of gastric mucosae, such as alcohol and aspirin, induce chemical injury in gastric mucosa. It is one of the serious exogenous factors that induces gastric mucosa inflammation that will lead to gastritis [2]. Anti-gastritis drugs, such as antacids, PPI or H2 antagonists, are oral medications commonly used in the management of gastritis. The undesirable side effects of using oral anti-gastritis drugs are considerable, therefore exploring new drug compounds from natural ingredients is expected to be a solution to the discovery and development of new drugs with better efficacy and safety [3], [4], [5].

Indonesia is one of the countries with a rich

biodiversity of medicinal plants. Gambir (*Uncaria gambir*) from the Rubiaceae family is one of Indonesia native medicinal plants [6]. Wide varieties of the study had been conducted to explore the potential effect of Gambir [7], [8], [9]. This study aimed to assess the potential of gambir extract in treating gastritis in vivo, which would further explore the effect of extracts on TNF-α and prostaglandin E2 so that new standardised herbs could be obtained in the management of gastritis.

Material and Methods

The study design was an experimental study, with post-test control group design. The study had been approved by the Bioethics Committee, Faculty of Medicine, Universitas Sriwijaya (189/kpt-fkunsri/rsmh/2017).

Gambir were provided by Indonesia Traditional Herbal Research Center, Tawangmangu, Central Java, Indonesia. Gambir was washed, dried and drilled, followed by maceration method by ethanol 96%, evaporated and gambir extract was obtained. Phytochemical analysis through Thin Layer Chromatography (TLC) was performed to obtain the component information of gambir extract.

Thirty rats (Eureka Research Laboratory, Indonesia) were used in this study. Inclusion criteria were male Wistar rats, eight weeks old, weight 150-200 gram and healthy. Rats were divided into 5 groups; every group consisted of 6 rats. Gastritis in rats was induced by administrating ethanol 96% for 3 days. Rats were treated, group 1: aqua dest 1 mL (negative control), group 2: ranitidine 20 mg/kg BW (positive control), group 3: gambir extract 20 mg/kg BW, group 4: gambir extract 40 mg/kg BW, and group 5: gambir extract 80 mg/kg BW. Treatment was carried out for 3 days.

Following the performed treatments, rats were killed by anaesthesia. Rats gastric organ was evacuated, washed and assayed for lesion area size by callipers, and the tissue was obtained for homogenisation by centrifugation 3000 rpm, at -4°C, for 20 minutes. The supernatant was collected and used to assay TNF alpha and prostaglandin E2 level using ELISA methods. The procedure of ELISA was based on the assay protocol on the manual (Cloud-Clone Corp®, Texas, USA). The sample solution was bottled using the capillary tube on Silica GF silent phase 254 which was activated by heating at 105°C-110°C for 1 hour then eluted with methanol: chloroform phase (1: 39) v/v. Chromatogram results were observed in UV254 nm. Spotting was detected by H_2SO_4 spray.

The statistical analysis was done by SPSS 18.0 (SPSS Inc., Chicago, Illinois, USA). Data were assessed for bivariate and multivariate analysis. T-test was used for bivariate analysis and pos hoc test for multivariate analysis.

Results

As shown in Table 1, gambir extract at the dose of 80 mg/kg BW was the most effective in reducing lesion area size, TNF alpha level and increasing prostaglandin E2 level compared to gambir extract at the dose of 20 mg/kg BW, 40 mg/kg BW, negative control and positive control. Gambir extract showed dose-dependent manner efficacy in reducing lesion area size, TNF alpha level and increasing prostaglandin E2 level. Gambir extracts at the dose of 80 mg/kg BW possesses higher efficacy to reduce lesion area size, TNF alpha level and increase prostaglandin E2 level compared to gambir extract at doses of 20 mg/kg BW and 40 mg/kg BW. The increasing doses of the extract were positively related to the efficacy to reduce lesion area size, TNF alpha level and increase prostaglandin E2 level, thus affecting the gastric mucosal lesion.

Table 1: The Efficacy of Gambir Extract on Lesion Area, TNF Alpha and Prostaglandin E2

Variable	Group	Mean ± SD	p-value*
Lession area (mm)	Negative control	2.72 ± 0,83	0.01
	Positive control	0.42 ± 0,13	-
	Extract 20 mg/kg BW	0.58 ± 0.22	0.01
	Extract 40 mg/kg BW	0.43 ± 0.24	0.56
	Extract 80 mg/kg BW	0.29 ± 0.10	0.02
TNF alpha (pg/mL)	Negative control	212 ± 10.09	0.01
	Positive control	170 ± 13,76	-
	Extract 50 mg/kg BW	198 ± 20.53	0.01
	Extract 100 mg/kg BW	169 ± 10.78	0.43
	Extract 200 mg/kg BW	149 ± 10.84	0.01
Prostaglandin E2 (pg/mL)	Negative control	28.78 ± 7.87	0.01
	Positive control	60,65 ± 11.09	-
	Extract 50 mg/kg BW	49.23 ± 7.34	0.01
	Extract 100 mg/kg BW	61.23 ± 5.87	0.34
	Extract 200 mg/kg BW	73.13 ± 6.35	0.02

*Paired T test; VS positive control; p = 0.05.

Phytochemical Analysis

Qualitative test of phytochemical component performed on gambir extract exhibited that gambir contained an alkaloid, steroid/ternoid (essential oil), and flavonoid components.

Figure 1: Thin Layer Chromatography Analysis of Gambir Extract

Discussion

Gambir extract showed dose-dependent manner efficacy in reducing lesion area size, TNF alpha level and increasing prostaglandin E2 level. Gambir extracts at the dose of 80 mg/kg BW possesses higher efficacy to reduce lesion area size, TNF alpha level and increase prostaglandin E2 level compared to gambir extract at doses of 20 mg/kg BW and 40 mg/kg BW. The increasing doses of the extract were positively related to the efficacy to reduce lesion area size, TNF alpha level and increase prostaglandin E2 level, thus affecting the gastric mucosal lesion in rats [10], [11]. Based on the phytochemical analysis, gambir extract contained an alkaloid, steroid and flavonoid. Quercetin (one of flavonoid) supplementation was reported to reduce stress oxidative in hypertensive patients [12]. Its antioxidant activity may also suppress the elevation of oxidant, MDA in diet-induced obesity rat models [13], [14]. Quercetin was reported to downregulate the expression of NfKB, TNF alpha and other proinflammatory cytokines. Flavonoid had the potential to reduce the damaged of the gastric mucosal membrane by inhibiting apoptosis and reducing anti-inflammatory activity. Flavonoid had the efficacy to inhibit apoptosis in 3T3-L1 preadipocytes by decreasing the mitochondria membrane potential, downregulating expression of B-cell lymphoma 2 (Bcl-2) and poly (ADP-ribose) polymerase (PARP), and activating Bcl-2 homologous antagonist/killer (Bak), Bcl-2-associated X protein (Bax), and cysteine-dependent aspartate-directed proteases 3 (caspase 3) [15], [16]. Gambir extract was the potential to inhibit inflammation in the gastric mucosal membrane by decreasing the inflammatory process through increased prostaglandin E2. Prostaglandin E2 plays a pivotal role to protect gastric mucosal membrane [17].

Prostaglandin E2 is a prostaglandin with complex biological activity, which is involved in GI-RI inflammation [18]. PGE2 synthase is the last key enzyme in the synthesis of prostaglandin E2. Prostaglandin E2 is the prostaglandin with the highest abundance and greatest distribution in the human body. It serves a major role in inflammation as a pain and fever mediator during inflammation. Additionally, it may induce vasodilation and microvascular leakage [19]. As a type of unsaturated fatty acid, prostaglandin E2 is predominantly composed of 20 carbon atoms. It has the basic structure of one five-carbon ring and two side chains. Arachidonic acid is synthesised into prostaglandin E2 under the catalysis of cyclooxygenase (COX) and prostaglandin synthase [20]. Prostaglandin E2 escapes through facilitated diffusion and binds to E-prostanoid 1 – 4 in an autocrine or paracrine manner. In this way, it may alter the levels of intracellular second messengers, and send signals to cells, causing a series of physiological or pathophysiological changes [18].

Cyclooxygenase prevents the conversion of arachidonic acid into prostaglandin E2. Therefore, it may quickly alleviate active substance-induced inflammation. As a result, it may reduce the excitability of the peripheral and central pain-sensing conduction system [21]. Thus, COX-2 serves anti-inflammatory and analgesic functions. Prostaglandins may promote the secretion of gastric fluid and bicarbonate. In this manner, they can protect the gastric mucosal barrier, promote the renewal of gastric mucosal cells and improve mucosal blood flow. Furthermore, COX-2 may stimulate the active transport process of cells, activate adenylate cyclase and stabilise the lysosome. It may also maintain the level of mucosal thiol compounds and stimulate surface-active phospholipids in gastric mucosa [22].

TNF-α, the major pro-inflammatory cytokine released from the migrated macrophages, plays an important role in the pathogenesis of gastric ulcers through stimulation of intercellular adhesion molecule (ICAM)-1 expression on vascular endothelial cells which increases leukocyte adhesion to the endothelial surface on post-capillary venules and promotes transendothelial migration of leukocytes to inflammatory sites [23]. TNF-α also increases intracellular oxidative stress and up-regulation of cytokine-induced neutrophil chemoattractant (CINC)-1 mRNA and protein in gastric epithelial cells [24].

In conclusion, the gambir extract exhibited efficacy in increasing gastric mucosal integrity through reducing lesion area size, TNF alpha level and increasing prostaglandin E2 level.

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Basic Science

Experimental, Clinical and Morphological Analysis of H-Ras Oncoproteins for Locally Advanced Breast Cancer

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Abstract

BACKGROUND: Activated forms of Ras are enhanced in both breast cancer as well as the cell lines with EGFR and HER2 expression. Therefore, H-Ras could be activated in breast tumours in the absence of direct mutational activation of Ras itself and could contribute to 20-50% of the cases. Expression inhibition, signal transduction interruption from H-Ras to the nucleus could become a promising therapeutic target.

AIM: The aim of this study was to investigate the clinical and morphological criteria of locally advanced breast cancer and the expression of H-Ras oncoprotein in patients who have been subjected to different regimens of farnesyltransferase inhibitor.

METHODS: H-Ras status was assessed by immunohistochemistry (IHC).

RESULTS: An association between the expressions of H-Ras and Her2/neu ($p = 0.001$) as well as the tumour proliferation index Ki-67 ($p = 0.001$) in patients with breast cancer was established. Analysis of the relationship between H-Ras expression showed a relatively strong association with progression-free survival both before the treatment ($V = 0.47$; $p = 0.001$) and after the treatment ($V = 0.45$; $p = 0.001$). These results may indicate the clinical applicability of H-Ras as a prognostic factor or serve as a therapeutic target for breast cancer treatment.

CONCLUSION: These results could indicate the potential clinical application of H-Ras as a prognostic factor or a therapeutic target for breast cancer treatment.

Introduction

High heterogeneity of breast cancer highlights molecular nature of malignant cells as a fundamentally important aspect, which is connected to the biological behaviour of the tumour and characterises their growth rate, ability to invade and metastasise and influences disease prognosis [1], [2].

It has been proven that mutations and certain genes' rearrangements lead to the activation of signalling system both at the level of growth factors and their receptors, as well as on the downstream level of signal transmission along the protein cascade into the cell nucleus. As a result, activation of the signaling system on the downstream level occurs regardless of the involvement of the ligand and the receptor [3].

Mutational activation of RAS genes contributes to the formation of malignant processes in more than 30% of cases, which makes them one of the most frequent oncogenic mutations [4]. Three isoforms – KRAS, HRAS and NRAS, are among the most studied genes of the RAS family. Mutations in

KRAS oncogenes occupy the highest percentage of occurrence in colorectal cancer patients, making up to 21,6% of cases, while NRAS makes up to 8.0% and HRAS is the least frequent making up to 3.3% of cases [5]. Spandidos D.A. showed for the first time that malignant breast tumours have an increased expression of HRAS oncogene compared to the corresponding samples of the normal tissue [6]. Further research identified an association between the high expression of p21 Ras oncogene in breast cancer and the aggressive course of the disease [7]. In another study, comparative analysis of HRAS oncogene expression of breast cancer and stomach cancer with regular clinical and pathological parameters was conducted, which revealed that high expression of p21 Ras oncogene in breast cancer patients is often associated with the tumour aggressiveness [8], [9], [10].

Although Ras rarely mutates in case of breast cancer, Ras is activated by various upstream regulators, including the epidermal growth factor receptors family, in particular, ErbB1 and ErbB2 [11].

Previous studies have shown that c-Ha-Ras protein expression could be used as a prognostic marker for the breast cancer progression as well as patients' stratification based on the expression status and risk of development of metastasis for choosing preoperative chemotherapy courses [12].

Blocking Ras signalling and H-Ras inhibition in breast cancer is quite promising. Considerable efforts have been made to develop pharmacological agents that block the function of Ras. One of them is a development of effector signalling inhibitors-Raf-MEK-ERK and PI3K-AKT-mTOR pathways inhibitors in particular, which have Ras mutations. The second step is to inhibit the association of the Ras membrane alternative prenylation, whereby they are modified by the addition of another isoprenoid lipid, geranylgeraniol. Geranylgeranylated Ras-proteins remain functional and get transformed in the presence of farnesyltransferase inhibitors. Therefore, farnesyltransferase inhibitors have demonstrated anti-tumor activity in breast cancer [13], [14], [15], [16], [17].

Another mechanism by which Ras could be activated in breast cancer is associated with decreased expression of RasGAP regulatory protein. Mutations in the NF1 gene in neurofibromatosis contribute to the decreased formation of RasGAP neurofibromin. Hence women with this pathology have a higher risk of breast cancer development [18], [19].

A relatively recently discovered mechanism of increased H-Ras expression in breast cancer showed that the expression of miRNA let-7 – a negative regulator of the expression of H-Ras protein, is reduced in cancer stem cells and clinical samples. Studies have also shown that restoration of the expression of let-7 reduces the expression of H-Ras, cells proliferation and metastatic spread [20]. Thus, understanding the mechanisms of action on signalling pathways contributes to the detection of novel therapeutic targets for breast cancer treatment. We suggest that H-Ras could become a target for farnesyltransferase inhibitor, as well as in combination with other immunohistochemical factors, will contribute to the breast tumour progression.

This study aimed to investigate the clinical and morphological criteria for locally advanced breast cancer and H-Ras oncoprotein expression in patients who were subjected to the different treatment regimens with farnesyltransferase inhibitors.

Material and Methods

The present study was conducted after ethical approval from the Institutional Ethics Committee (17/9/10/KSMU/IEC/2017). In the present study morphological samples of patients with histologically verified stage II or III of the disease, T2N1-2M0, T3N0-2M0 served as inclusion criteria for the study. A group of 100 female patients with locally advanced breast cancer were recruited from June 2012 to February 2014. Their age ranged from 29 to 78 years, averaged at 59 ± years. The clinical staging was determined by the International TNM classification system (7^{th} edition). The study was approved by the Ethics Committee of Karaganda Medical University (Karaganda, Kazakhstan).

Immunohistochemical determination of H-Ras oncoprotein expression was done on archival histological material samples of patients with breast cancer before and after the treatment. For immunohistochemical studies histological paraffin slices up to 5 μm thick were prepared, followed by dewaxing, then the slices were dehydrated and washed in sodium citrate buffer (PBS, sc-294091, Santa Cruz). Immunohistochemical staining of prepared slices was done using the avidin-biotin system of antigen detection ImmunoCruz® ABC Kit (sc-516216) by the manufacturer instructions. To visualise the positive reaction 3,3-diaminobenzidine tetrachloride (DAB, sc-24982) was used as a chromogen.

Mouse monoclonal anti-IgG_1 antibodies to H-Ras protein (sc-29, Santa Cruz) of murine, rat and human origin characterised by the positive reaction in the cytoplasm of the tumour cells were used in this study. Primary antibodies against H-Ras were substituted with a buffer (PBS) or non-immune anti-IgG_1 as a negative control. Two independent researchers were involved in the expression assessment. Positive staining on the tumour cells membrane and cytoplasm was considered as a positive test. The percentage of positively stained cells and the intensity of staining were evaluated.

Percent of positively stained cells: < 10%-0; 10-50%-1; 51-80-2%; > 81%-3. The intensity of the staining: no reaction-0 points, weak reaction-1 point; moderate reaction-2 points, pronounced reaction – 3 points. IRS rating scale: 0-2 points – negative reaction; 3-4 points – weak reaction; 6-8 points – moderate reaction; 9-12 points – pronounced reaction.

Statistical analysis. The corresponding data are presented as a mean ± standard deviation (N = 3); confidence interval was calculated using Wald's methods. The analysis of the significance of relationships between the qualitative variables was performed using Pearson's chi-square criteria. Kramer's correlation analysis was used to assess the relationship between two qualitative variables. To assess the relationship between the two quantitative variables, Spearman's correlation method was performed. Statistical analysis was carried out using Statistica 10. P < 0.05 was considered to be an indicator of statistical significance.

Results

All 100 patients were females with the mean age of ± 59 years. The average follow-up period was 12 months. The majority (68%) of patients had stage II of the disease. In 65% of cases, regional metastatic spread in one axillary lymph node was detected, 9%-in two lymph nodes, 4%-in three.

H-Ras expression was evaluated in 200 tumour tissue samples before and after the neoadjuvant therapy for breast cancer. Immunohistochemical staining of H-Ras was performed (Figure 1). The immunohistochemical reaction was described as no reaction, a weak reaction and an intense reaction. According to the classification criteria, positive H-Ras expression was identified in 45 tumours (45%) before the treatment and in 35 tumours (35%) after the treatment.

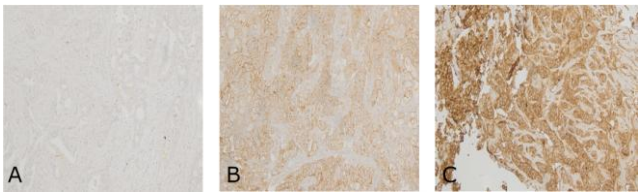

Figure 1: Immunohistochemical staining for the expression of H-Ras in human breast tissues of locally advanced cancer. The intensity of staining was described as A) no reaction; B) a weak reaction and C) an intense reaction (x 200 magnification)

Analysis of the relationship between the H-Ras expression before and after the treatment with clinical and pathological variables is summarised in Table 1.

Table 1: Relationship between the H-Ras expression and clinical and morphological criteria of 100 breast cancer patients (p = 0.05)

Features	N	H-Ras expression before the treatment p level		χ^2. p level	H-Ras expression after the treatment		χ^2. p level
		Negative (n = 55) [CI, %]	Positive (n = 45) [CI. %]		Negative (n = 65) [CI. %]	Positive (n = 35) [CI, %]	
				Age			
≤ 50	29	16 [37.54; 71.60]	13 [28.40; 62.46]	2.87; 0.41	18 [43.95; 77.36]	11 [22.64; 56.05]	2.71 0.25
> 50	71	39 [43.40; 65.95]	*32 [34.05; 56.60]*	4.19 0.37	47 [54.59; 76.15]	24 [23.85; 45.41]	5.05 0.16
				Depth of tumour invasion			
T2	65	34 [40.38;63.98]	31 [36.03; 59.62]		41 [50.90; 73.79]	24 [26.21; 49.10]	
T3	17	7 [21.56;64.05]	10 [35.95; 78.44]	8.06 0.62	10 [35.95; 78.44]	7 [21.56; 64.05]	5.12 0.69
T4	18	13 [48.80;87.83]	5 [12.17; 51.20]		14 [54.25; 91.53]	4 [8.47; 45.75]	
				Regional metastatic spread			
N0	16	8 [28.00;72.00]	8 [28.00; 72.00]		8 [28.00; 72.00]	8 [28.00; 72.00]	
N1	66	34 [39.71;63.15]	32 [36.85; 60.29]	2.08	43 [53.08; 75.55]	23 [24.45; 46.92]	4.74
N2	11	9 [51.15;96.01]	2 [3.99;48.85]	0.83	8 [42.89; 90.80]	3 [9.20; 57.11]	0.19
N3	7	4 [24.98;84.25]	3 [15.75; 75.02]		6 [46.65; 99.47]	1 [0.53; 53.35]	
				Estrogen receptors			
Negative	52	26 [36.89;63.11]	26 [36.89; 63.11]	10.80 0.05	30 [44.18; 70.14]	22 [29.86; 55.82]	5.31 0.14
Positive	48	32 [52.49;78.38]	16 [21.62; 47;51]		35 [58.89; 83.54]	13 [16.46; 41.11]	
				Progesterone receptors			
Negative	64	30 [35.17;58.93]	34 [41.07; 64.83]	5.17 0.39	40 [50.23; 73.35]	24 [26.65; 49.77]	1.38 0.70
Positive	36	25 [53.03;82.11]	11 [17.89; 56.97]		25 [53.03;82.11]	11 [17.89; 56.97]	
				Her-2\neu status			
Negative	54	49 [79.67;96.40]	5 [3.60; 20.33]	61.68 0.001	51 [84.30; 96.68]	3 [1.32; 15.70]	51.42 0.001
Positive	46	6 [5.74; 26.04]	40 [73.96; 94.26]		14 [19.00; 44.89]	32 [55.11; 81.00]	
				Ki-67 proliferation index			
< 15	34	1 [0.78; 16.22]	33 [83.78;99.78]	265.81 0.001	1 [0.78; 16.22]	33 [0.78; 16.22]	155.04 0.004
≥ 15	66	11 [9.39; 27.61]	45 [56.17; 78.19]		21 [21.81; 43.83]	35 [41.16; 64.57]	
				Therapy			
Arglabin	31	14 [29.15;62.24]	17 [37.76;70.85]		20 [46.88; 78.95]	11 [21.05; 53.12]	
AC	38	23 [44.69;74.43]	15 [25.57; 55.31]	11.96 0.28	23 [44.69; 74.43]	15 [25.57; 55.31]	7.86 0.44
AC + Arglabin	31	18 [40.74;73.61]	13 [26.39; 59.26]		22 [53.25; 84.06]	9 [15.94; 46.75]	

AC – treatment regimen adriablastin + cyclophosphan.

As a result of the performed comparative analysis, a statistically significant difference between HRAS and clinical and pathological features such as Her-2/neu (p = 0.001), Ki-67 proliferating index (p = 0.001) of the patients with a verified breast cancer diagnosis were found.

A study of the efficacy of the therapy showed a strong relationship between the sum of the diameters of the tumour according to the RECIST 1.1 and percentage reduction of the tumour size (r = 0.87; p < 0.05) (Figure 2).

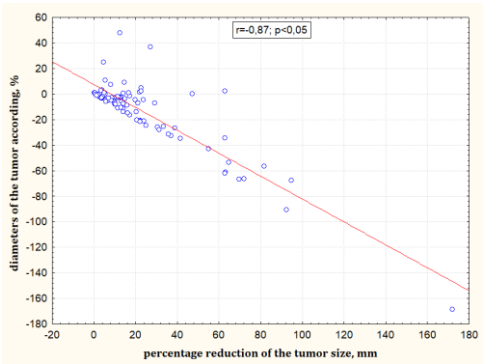

Figure 2: Relationship between the sum of the diameters of the tumour according to the RECIST 1.1 and percentage reduction of the tumour size

According to the results of the association analysis of the expression ability of H-Ras oncoprotein with clinicopathological factors in breast cancer, a strong correlation with the expression of Her-2/neu was determined, where Kramer's correlation coefficient of V = 0.78 (p = 0.001) before the treatment and V = 0.67 (p = 0.001) after the treatment. In addition, the strong correlation with the proliferative activity index Ki-67 and H-Ras before the treatment with V = 0.57 (p = 0.001) and relatively-strong correlation with H-Ras after the treatment with V = 0.53 (p = 0.001) were identified.

When analyzing the relationship between the H-Ras expression and progression-free survival, Kramer's correlation coefficient before the treatment was V = 0.47 (χ^2 = 68.92, p = 0.001) and V = 0.45 (χ^2 = 62.11, p = 0.001) after the treatment, which indicates the presence of relatively strong correlation. Also, the correlation between the percentage of positively stained for H-Ras cells and progression-free survival (time to progression) was investigated. Spearman's correlation coefficient was calculated: r = -0.78 before the treatment and r = -0.72 after the treatment.

The relationship between H-Ras and progesterone receptors turned to be less pronounced and accounted for V = 0.32 (p = 0.06) before the treatment and V = 0.25 (p = 0.17) after the treatment. Correlation between H-Ras and estrogen receptors was found to be V = 0.26 (p = 0.15) before the treatment and V = 0.23 (p = 0.11) after the treatment, which is considered to be moderate, however considering p value, the relationship was not significant.

When studying the relationship between H-Ras with effectiveness of anti-tumor therapy according to the RECIST 1 criteria, Kramer's correlation coefficient was V = 0.1 (p = 0.84) and V = 0.15 (p = 0.94), the obtained results require further study.

Discussion

Ras activation in breast cancer tumour could occur in the presence of EGFR or HER2 without direct mutation in Ras itself and could account for 20-50% of cases [21], [22]. Thus, expression inhibition and H-Ras to nucleus signalling interruption could become a promising therapeutic target. The immunohistochemical study had shown that in 45% of breast cancer cases before the treatment and 35% of cases after the treatment H-Ras expression was present. Previous research indicated that H-Ras was expressed in 60% of breast cancer cases, while a mutation in this gene attribute to only 5-10% of cases, which could be due to post-transcriptional regulation mechanisms [23], [24]. Moreover, it was shown that H-Ras positive breast cancer patients had a worse prognosis than H-Ras negative patients. Analysis of relationship between the H-Ras expression and progression-free survival indicated a relatively strong correlation both before the treatment (V = 0.47; p = 0.001) and after the treatment (V = 0.45; p = 0.001). Correlation between H-Ras and progression-free survival (time to progression) before the treatment was r = -0.78 (p = 0.03) and after the treatment r = -0.72 (p = 0.04).

The results of this study showed that there is a correlation between H-Ras expression and Her2/neu expression (p = 0.001) as well as with the tumour proliferation index Ki-67 (p = 0.001) in patients with breast cancer. These results could indicate the potential clinical application of H-Ras as a prognostic factor or a therapeutic target for breast cancer treatment.

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Basic Science

Dietary Ethanolic Extract of *Mangosteen pericarp* Reduces VCAM-1, Perivascular Adipose Tissue and Aortic Intimal Medial Thickness in Hypercholesterolemic Rat Model

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Keywords: Atherosclerosis; Dyslipidemia; Mangosteen Pericarp Ethanolic Extract; VCAM-1; PVAT; IMT

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Abstract

BACKGROUND: High-fat diet (HFD) is associated with dyslipidemia which is a risk factor for atherosclerosis. Dyslipidemia causes oxidative stress which induces vascular cell adhesion molecule-1 (VCAM-1). Oxidative stress also triggers the thickening of tunica intima-media (IMT) and Perivascular Adipose Tissue (PVAT). Xanthone compound in ethanolic extract of *Mangosteen pericarp* (EEMP) has an antioxidant property to overcome the oxidative stress.

AIM: The objective of this study is to investigate the effect of dietary EEMP administration on the expression of VCAM-1 and thickness of PVAT and IMT in atherosclerotic rat model fed with HFD.

METHODS: This experimental laboratory study uses 25 Wistar strain *Rattus norvegicus* which were divided into 5 study groups. Negative Control group (GT1) was given a normal diet, Positive Control group (GT2) was treated with HFD, and three treatment groups were each treated with HFD with *Mangosteen pericarp* extract of 200 mg/kg BW (GT3), 400 mg/kg BW (GT4), and 800 mg/kg BW (GT5). Measurements of VCAM-1 expression were performed using immunofluorescence. PVAT and IMT measurements were performed on rat aortic preparations.

RESULTS: One-way ANOVA test showed the addition of dietary EEMP significantly ($p < 0.05$) decreased the expression of VCAM-1 and decreased the thickness of PVAT and IMT in treatment groups as compared with both negative and positive controls. Tukey HSD test showed a dose of 800 mg/kg BW was the most effective dose for decreasing VCAM-1 level, PVAT and IMT.

CONCLUSION: Dietary EEMP significantly decreases the expression of VCAM-1, as well as the thickness of PVAT and IMT in Wistar strain *Rattus norvegicus* treated with HFD.

Introduction

Cardiovascular disease such as hypertension, stroke, coronary heart disease and heart failure, is the leading cause of global death. In 2015, it was estimated that 17.7 million people or about 31% of the world's deaths were due to this disease [1]. Coronary heart disease develops from the atherosclerotic process. The exact cause of atherosclerosis still unknown, but research suggests that atherosclerosis starts when certain factors damage the inner layers of the arteries [2].

Atherosclerosis is an irreversible process, causing disease by the slow development of narrowing of the arterial lumen, mainly located in the intima of middle-large size arteries [3], [4]. Atherosclerosis is progressive and leads to the formation of atherosclerotic plaques that causes obstruct in blood vessels. Atherosclerotic plaque formation begins from chronic inflammation which triggers oxidative stress. Oxidative stress is a state of

reactive oxygen species (ROS) and anti-oxidants imbalance [5]. Physiologically; ROS acts as a regulator in various processes of defence mechanisms, differentiation, proliferation, and migration of body cells. Conversely, an excess of ROS levels in the body is a sign of a pathological condition characterised by excessive inflammation and contributes to the pathogenesis of atherosclerosis [6], [7].

High ROS production can increase the production of PVAT and thickened IMT in dyslipidemia [8]. ROS can activate peroxisome proliferator-activated receptor-ɣ (PPAR-ɣ), which is a master regulator of adipogenesis, thus causing expansion and dysfunction of PVAT [9]. ROS also contribute to endothelial dysfunction characterised by increased expression of adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1) [10].

Garcinia mangostana L., or commonly known as mangosteen, is rich in phenol components, such as xanthones, tannins, and anthocyanins [11]. These components have been shown to have many biological activities, and in particular, mangosteen pericarp extract has anti-inflammatory, anti-cancer, anti-microbial, and anti-oxidant effects both *in vivo* and *in vitro [12]*. Xanthone has a role as a scavenger antioxidant capable of inhibiting ROS [13]. Based on these facts, ethanolic extract of mangosteen pericarp (EEMP) is expected to inhibit VCAM-1 expression and decrease PVAT and IMT thickness in the aorta of atherosclerotic rat model treated with high-fat diet (HFD).

Material and Methods

Laboratory Animals

This research used 8-week-old male Wistar strain *Rattus norvegicus* weighing about 1.5-2 kg which was kept in Pharmacology Laboratory of Faculty of Medicine, Brawijaya University, Malang, East Java, Indonesia. Before the treatment, the rats underwent an acclimatization process for 2 weeks, and later were divided into 5 treatment groups (n = 5), i.e. Negative Control with normal diet (G1), Positive Control with HFD diet (G2), the group with HFD and 200 mg/kg BW EEMP administration (G3), the group with HFD and 400 mg/kg BW EEMP administration (G4), and the group with HFD and 800 mg/kg BW EEMP administration (G5).

Feeding and Creating Dyslipidemic Rat Model

Rats were fed according to their respective treatment groups. The normal group was given a normal diet of PARS 62%. Groups with HFD were given 2% cholesterol, 0.2% cholic acid, and 5% lard oil supplements, 30 grams daily, *ad libitum [1]*.

EEMP (Ethanolic extract of mangosteen pericarp) Process

The EEMP was obtained through drying, extraction, and evaporation processes. *Mangosteen pericarps* were washed and then dried in an oven at 80 degrees until dry or free of water content. The dried mangosteen pericarp was refined evenly into 100 grams of dried sample, and placed into a 1-litre Erlenmeyer flask. It was soaked in 70% ethanol until the volume reached 1000 mL, shaken for 30 minutes, and settled for 1 night until a sediment form. The next step was the evaporation process using *Rotary Evaporator*. The evaporation process is completed when the ethanol solution is separated from the active ingredient. The EEMP were administered to the research animals through a feeding tube with doses of 200 mg/kg BW, 400 mg/kg BW and 800 mg/kg BW respectively.

Rat Dissection

Dissection was performed 2 months subsequent of EEMP administration. Surgery was performed after the animal had been given ketamine as an anaesthetic agent. Later, the aorta was retrieved and preserved using 10% formalin for subsequent aortic preparation with haematoxylin-eosin staining.

Measurement of IMT and PVAT Thickness

Measurement of IMT and PVAT thickness was conducted at Anatomy Pathology Laboratory, Faculty of Medicine, Brawijaya University, Malang, East Java, Indonesia. Aortic preparation was done using paraffin block and *HE*. The measurements were made by drawing perpendicular vertical lines of the intima tunica and outline of the media tunica. Measurements were made in 5 clockwise zones and then averaged. The measurement of PVAT thickness was performed by measuring the mean of the lowest, medium, and highest thickness of PVAT on aortic preparations. Measurements with 400 x magnification and were made using *scan dot slide Olyvia* software.

VCAM-1 Measurement

Aortic tissue was fixated with PHEMO buffer (Pipes 0.068 M, HEPES 0.0025 M, EGTA 0.005 M, MgCl 0.003 M, 10% DMSO, PH 6.8), which contained 3.7% formaldehyde, 0.05% glutaraldehyde, 0.5 triton x-100, for 10 minutes at room temperature. Coverslip was blocked by 10% goat serum/PBS for 10 minutes.

VCAM-1 expression on aortic tissue was identified through double-staining immunofluorescence using anti-rat VCAM-1 with rhodamine as the secondary antibody, and α-actin was coloured by fluorescein isothiocyanate as the secondary antibody, and then when observed with Confocal Laser Scanning Microscope (CLSM), the double-stained result would show VCAM-1 expression in smooth muscle cells. Lastly, the collected data were quantitatively analysed with Olympus fluoview 1.7A software.

Ethics

The medical research ethics committee of the Faculty of Medicine of Brawijaya University has stated that this research is ethically detailed with No.211 / EC / KEPK-S1-PD / 06 / 2017

Statistical Analysis

Statistical analysis was performed using SPSS version 16 software with significance level of 0.05 (p = 0.05) and 95% confidence interval (α = 0.05). This research used One Way ANOVA Parametric Test to determine the effect of EEMP in 5 groups of an atherosclerotic rat model. Data analysis was further conducted using *Tukey HSD* Test to understand the difference between groups.

The presentation of ANOVA and Post Hoc Tukey HSD data tables refer to Seng *et al.,* (2018) [15].

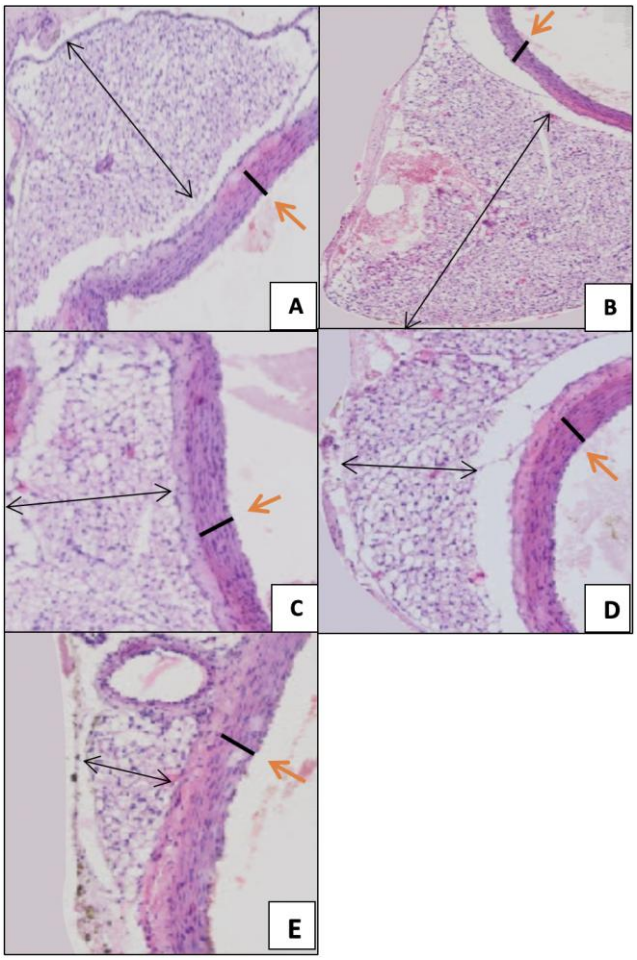

Figure 1: Images of aortic preparation with tunica intima-media and PVAT thickness measurement on each research animal treatment group; A) Normal diet group (GT1); B) HFD group (GT2); C) the group with HFD and 200 mg/kg BW EEMP administration (GT3); D) the group with HFD and 400 mg/kg BW EEMP administration (GT4); and (E) the group with HFD and 800 mg/kg BW EEMP administration (GT5)

Results

Histopathological Findings of IMT and PVAT

Measurements were done with 400 x magnification and were made using *scan dot slide Olyvia* software. Blackline that is pointed by red arrow shows the tunica intima-media thickness and black arrow of each image shows the PVAT thickness. Histopathological features of the aorta between 5 groups show a clear difference in tunica intima-media and PVAT thickness. Intima-media of picture D has a similar thickness to intima-media of picture A as the negative control. Tukey HSD test shows that the dosage of EEMP 200 mg/kg BW can lower the BMI, with the mean dosage value closest to negative control is 400 mg/kg BW. The thickness of PVAT in figure E is thinner than others, shown as the length of the black arrow. Tukey HSD Test shows that PVAT thickness in 800 mg/kg BW EEMP group was significantly different compared to the HFD group.

Immunofluorescence Findings of VCAM-1

On this study, statistically significant differences were seen in all of the parameters; IMT ($p < 0.001$), PVAT ($p = 0.008$), VCAM-1 ($p = 0.032$), HDL ($p = 0.010$), LDL ($p < 0.001$), TG ($p = 0.007$), and TC ($p < 0.001$. From the data presented in Table 1, it appears that there was a worsening of lipid profiles in the group of rats with HFD administration.

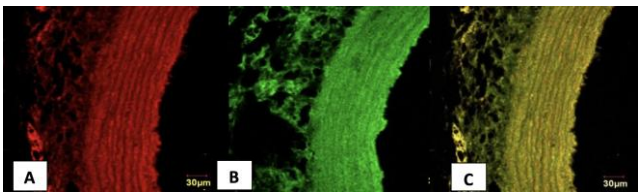

Figure 2: A) VCAM-1; B) α-actin expression; C) expression of VCAM-1 in Smooth Muscle Cell (SMC); Immunofluorescence findings of VCAM-1 in Smooth Muscle Cell (SMC), labelled with α-actin; A) VCAM-1 – rhodamine; B) α-actin – fluorescein isothiocyanate (FITC). The colour green shows α-actin expression (SMC marker); C) Double staining of VCAM-1 and α-actin showed the expression of VCAM-1 in smooth muscle cells of aortic tissue. The colour yellow shows expression of VCAM-1 in SMC

Then with EEMP treatment, there was a significant improvement of lipid profile from ONEWAY ANOVA test. Not only improved lipid profiles, but EEMP administration can also reduce IMT, VCAM-1, and PVAT thickness with significant ONE-WAY ANOVA test results.

Table 1: IMT, PVAT thickness, and VCAM-1 Expression Calculation, and Lipid Profile variables at Groups of Treatment (GT1-GT5) with ANOVA and Tukey HSD results

Variables	Groups of Treatment	Mean ± SD	F-statistics (df)	*P*	Tukey HSD	95% CI		adj p
IMT (μm)	GT1	61.05 ± 2.01	13.998 (24)	<0.001	GT1-GT2	0.98	3.21	< 0.001
	GT2	78.19 ± 6.33			GT1-GT3	-1.02	3.35	0.128
	GT3	62.76 ± 6.71			GT1-GT4	-0.67	1.59	0.468
	GT4	61.10 ± 3.35			GT1-GT5	-0.47	0.85	0.263
	GT5	60.59 ± 1.31			GT2-GT3	1.68	3.27	< 0.001
					GT2-GT4	1.22	3.14	< 0.001
					GT2-GT5	0.54	3.06	< 0.001
					GT3-GT4	-1.15	2.93	0.206
					GT3-GT5	-0.83	2.25	0.068
					GT4-GT5	-0.33	1.76	0.104
PVAT	GT1	547.48 ± 152.10	4.608 (24)	0.008	GT1-GT2	3.42	6.28	0.006
(μm)	GT2	744.24 ± 115.00			GT1-GT3	1.68	3.76	0.012
	GT3	737.00 ± 58.27			GT1-GT4	2.82	4.08	0.020
	GT4	711.64 ± 112.11			GT1-GT5	-4.42	1.36	0.075
	GT5	554.77 ± 33.95			GT2-GT3	-3.84	0.87	0.187
					GT2-GT4	-4.86	2.48	0.245
					GT2-GT5	1.27	5.08	0.046
					GT3-GT4	-2.63	1.34	0.591
					GT3-GT5	2.74	4.92	0.024
					GT4-GT5	1.04	3.86	0.012
VCAM-1	GT1	477.01 ± 81.35	3.542 (24)	0.032	GT1-GT2	0.38	2.74	0.026
(AU)	GT2	628.51 ± 18.00			GT1-GT3	-3.26	1.82	0.143
	GT3	497.52 ± 64.70			GT1-GT4	-2.84	2.49	0.281
	GT4	451.26 ± 100.84			GT1-GT5	-3.04	1.86	0.309
	GT5	447.71 ± 101.30			GT2-GT3	2.32	4.68	0.027
					GT2-GT4	2.28	5.86	0.018
					GT2-GT5	1.96	4.70	0.009
					GT3-GT4	-2.46	1.68	0.074
					GT3-GT5	-3.86	2.92	0.065
					GT4-GT5	-3.58	3.28	0.073
HDL	GT1	30.00 ± 7.52	4,865 (24)	0.010	GT1-GT2	-2.38	3.31	0.086
(mg/dL)								
	GT2	22.25 ± 6.50			GT1-GT3	-3.26	3.98	0.078
	GT3	28.75 ± 7.50			GT1-GT4	-3.53	3.74	0.056
	GT4	37.25 ± 7.13			GT1-GT5	1.64	2.32	0.035
	GT5	41.50 ± 5.25			GT2-GT3	-2.75	3.87	0.062
					GT2-GT4	2.51	3.04	0.020
					GT2-GT5	1.79	3.35	0.012
					GT3-GT4	-2.92	4.02	0.073
					GT3-GT5	1.81	3.69	0.009
					GT4-GT5	-2.86	3.63	0.086
LDL	GT1	60.25 ± 16.19	15.474 (24)	<0.001	GT1-GT2	1.44	4.82	< 0.001
(mg/dL)								
	GT2	126.25 ± 17.03			GT1-GT3	0.86	5.18	< 0.001
	GT3	89.50 ± 9.84			GT1-GT4	-0.98	4.32	0.012
	GT4	78.75 ± 12.94			GT1-GT5	-2.45	1.72	0.087
	GT5	65.75 ± 8.26			GT2-GT3	1.36	4.27	< 0.001
					GT2-GT4	0.46	2.62	< 0.001
					GT2-GT5	2.38	3.86	< 0.001
					GT3-GT4	1.26	2.76	< 0.001
					GT3-GT5	0.26	2.39	< 0.001
					GT4-GT5	-1.41	3.84	0.065
TG	GT1	101.75 ± 6.80	5,331 (24)	0.007	GT1-GT2	0.97	4.05	0.013
(mg/dL)	GT2	146.25 ± 33.38			GT1-GT3	-2.25	5.72	0.067
	GT3	102.50 ± 12.17			GT1-GT4	-1.36	3.84	0.248
	GT4	101.75 ± 11.70			GT1-GT5	-1.72	3.24	0.357
	GT5	97.50 ± 10.66			GT2-GT3	4.84	8.63	0.004
					GT2-GT4	4.81	8.97	0.002
					GT2-GT5	5.94	9.08	0.001
					GT3-GT4	-0.45	4.64	0.492
					GT3-GT5	-2.76	3.86	0.074
					GT4-GT5	-3.46	4.06	0.062
TC	GT1	107.75 ± 10.53	18.442 (24)	< 0.001	GT1-GT2	2.76	5.87	< 0.001
(mg/dL)	GT2	181.25 ± 23.72			GT1-GT3	-4.64	1.08	0.083
	GT3	113.25 ± 12.55			GT1-GT4	-5.26	2.21	0.074
	GT4	104.00 ± 18.31			GT1-GT5	-5.46	1.98	0.059
	GT5	102.50 ± 7.18			GT2-GT3	2.43	4.66	0.001
					GT2-GT4	1.24	3.57	< 0.001
					GT2-GT5	0.69	2.74	0.001
					GT3-GT4	-4.79	3.27	0.056
					GT3-GT5	-4.02	1.85	0.086
					GT4-GT5	-2.52	0.82	0.074

Footnote: GT1: Negative control with normal diet; GT2: Positive control with HFD diet; GT3: the group with HFD and 200 mg/kg BW EEMP administration; GT4: the group with HFD and 400 mg/kg BW EEMP administration; GT5: the group with HFD and 800 mg/kg BW EEMP administration. Abbreviation: HFD, High Fatty Diet; IMT, Intima-Media Thickness; PVAT, Peri Vascular Adipocyte Tissue; VCAM-1, Vascular Cell Adhesion Molecule, HDL, High-Density Lipoprotein, LDL, Low-Density Lipoprotein; TG, Triglyceride; TC, Total Cholesterol.

Tukey HSD test was performed on VCAM-1, IMT and PVAT showed that a dose of 400 mg/kg BW was a significant dose to decrease IMT and VCAM-1 thickness because approached with normal diet group and a dose of 800 mg/kg BW was a significant dose to decrease PVAT thickness approached with normal diet group.

Discussion

The peel of mangosteen fruit has long been used by residents in various countries as an ingredient in traditional medicine. Several experimental studies have proved that EEMP could be used as anti-tumour, anti-inflammatory, anti-bacterial, anti-viral, and anti-oxidant agents. In previous studies, the ethanolic extract of mangosteen pericarp was mentioned to have the highest antioxidant effect [14]. The EEMP as a source of antioxidants works by releasing electrons to free radicals to form stable products to prevent the generation of chain reactions [15]. Xanthone compound in the pericarp of mangosteen is needed by the body for the balance of pro-oxidants. Xanthone has binding properties towards unstable free oxygen. This free oxygen acts as free radical and destroying body cells; therefore, xanthone can inhibit the degeneration process of cell damage [15]. Several *in vitro* studies have shown that mangosteen pericarp extract can absorb free radicals [16]. Xanthones are divided into α-mangosteen, β-mangosteen, γ-mangosteen, and methoxy-betamangosteen, but the most common form is α-mangosteen [17]. The α-mangosteen antioxidant may increase lipoprotein lipase enzyme activity and improve very-low-density lipoprotein (VLDL) catabolism. As a result, the total cholesterol, triglyceride, and LDL levels fall, and HDL or good cholesterol level rises [18].

In Table, HFD administration has been shown to worsen the lipid profiles of research animals significantly. This worsening of lipid profile shows the presence of dyslipidemia. Dyslipidemia is one of the risk factors for atherosclerosis [17]. HFD causes dyslipidemia, and manifestations are elevated LDL level in the body; in a condition of excessive fat intake, LDL may accumulate in IMT and PVAT of the blood vessels. On the other hand, dyslipidemia will cause adipocyte cells hypertrophy. Adipocyte growth is associated with an increase of pro-oxidant enzymes, such as NADPH oxidase, and a decrease of antioxidant enzymes, such as catalase [19. These two conditions will increase the H_2O_2 production. H_2O_2 reacts with Fe_2^+ to release hydroxide (OH^-) via Fenton reaction. OH^- is a free radical that oxidises LDL accumulated in tunica intima; this process is called lipid peroxidation, and it will produce oxidised LDL (oxLDL); from this stage, the atherosclerosis process begins. OxLDL will later lead to an increase of proinflammatory cytokines, such as *Tumor Necrosis Factor-α* (TNF-α) [17]. Increased proinflammatory cytokines will trigger an increase in VCAM-1 expression [20]. Increased expression of VCAM-1 causes accumulation of monocytes in IMT. The adhesion of monocyte-endothelial cells to atherosclerotic lesions is affected by intercellular oxidative stress in Mononuclear Cells (MNC) and causes the thickening of IMT [21]. The administration of HFD which may cause thickening of PVAT will also

lead to an increase in PVAT proinflammatory property that is characterised by decreased expression of adiponectin and increased expression of IL-6, IL-8, and monocyte chemoattractant protein-1 (MCP-1). These parameters (VCAM-1, PVAT, and IMT) play a role in atherosclerosis pathogenesis.

VCAM-1 is also a molecule that plays a role in atherogenesis. One component in atheroma plaque is accumulated leukocytes. Adhesion molecules, such as VCAM-1, activates endothelial cell signal transduction, which in turn alters the form of endothelial cells to pave the way for leukocyte migration [22]. Increased expression of VCAM-1 in HFD group showed a positive tendency. This result is accordance with previous research by Huang *et al.,* reported that after treating rats with HFD for 12 weeks, VCAM-1 expression in the aorta and the levels of sVCAM-1 were increased significantly [23]. After EEMP administration, VCAM-1 expression displayed a significant decrease. Aside from being an anti-oxidant, xanthone also has anti-inflammatory properties. In an experiment conducted by Wihastuti *et al*., the administration of EEMP exhibit the ability to lower TNF-α and IL-1 levels in high-cholesterol diet-induced rats compared to the positive control group. The two cytokines are known to be related to the expression of VCAM-1 [24]. These findings will contribute to further improving our understanding of the underlying mechanism regulating adhesion molecule levels by exploring the effects of EEMP on the pathogenesis of atherosclerosis.

The thickness of the research animal's PVAT in the HFD group (seen in Table) experienced a significantly different increase, as evidenced by Tukey HSD *post-hoc* test results. HFD administration may induce a pro-inflammatory phenotype in PVAT with low adiponectin expression and high IL-6, IL-8, and MCP-1 expression [25]. This thickening of PVAT illustrates a condition of inflammation and endothelial dysfunction as a result of an increase in ROS [25]. Consistent with our studies, others reported that PVAT plays a role in the pathogenesis of vascular lesion formation. Ketonen *et al*. reported that obesity-induced endothelial dysfunction is caused by increased oxidative stress and enhanced expression of inflammatory cytokine in PVAT [26]. In this research, the administration of EEMP that contains xanthone as an anti-oxidant agent was proved by ANOVA parametric test to be able to decrease the thickness of PVAT significantly. The EEMP dose that significantly lowered PVAT thickness was 800 mg. This EEMP dose could decrease PVAT thickness almost until the level of normal group rats. PVAT thickness in 800 mg/kg BW EEMP group was significantly different compared to the HFD group. The increase of free radical and oxidative stress conditions in atherosclerosis leads to the thickening of adipocyte tissue that produces proinflammatory and anti-inflammatory cytokines. This event is characterised by an increase in PVAT thickness observed using histopathologic imaging. In addition to PVAT, oxidative stress is also evident from the increasing number of foam cells and narrowing of the lumen diameter of the blood vessel due to atherosclerotic plaque deposition [27].

Based on results, it can be concluded that administration of mangosteen pericarp ethanolic extract (*Garcinia mangostana Linn*) may decrease the expression of VCAM-1, IMT and PVAT thickness in research animals with the high-fat diet group. 400 mg/kg BW of EEMP was proved to exhibit IMT and VCAM-1 expression value, and 800 mg/kg BW of EEMP was able to exhibit the value of PVAT thickness which was equivalent to a normal physiological condition in research animal groups. The results of this study were similar to other studies which proved that the administration of anti-oxidants might reduce oxidative stress, therefore inhibiting atherosclerosis process by inhibiting LDL oxidation and decreasing ROS in endothelial cells [9].

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Basic Science

The Activity of Hydrolyzed Virgin Coconut Oil to Increase Proliferation and Cyclooxygenase-2 Expression towards on NIH 3T3 Cell Line in Wound Healing Process

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Abstract

AIM: This study aims to determine the effect of hydrolysed virgin coconut oil (HVCO) to increase cell proliferation, COX-2 expression of NIH 3T3.

METHODS: The sample used was Virgin Coconut Oil (VCO). VCO was partially hydrolysed using lipase from *Rhizomucor miehei* (active on sn-1,3 position) to produce hydrolysed VCO (HVCO) composed of free fatty acids, 2-monoglycerides. Then acid value was determined. The effect of HVCO on proliferation was evaluated using the MTT method. Wound healing assay was established by a cell migration method, and COX-2 expression was determined using RT-PCR.

RESULTS: Acid value is 135.89 ± 0.12 mg NaOH/g oil and free fatty acids (FFA) is 48.50 ± 0.06%. The effect of HVCO 62.5 µg/mL on cell proliferation after 24h, 48h, and 72h incubation found as viable cells are 109.24 ± 0.52%; 118.26 ± 0.91% and 106.59 ± 0.74%. Percent of wound closed after 24 h and 48 h incubation are 69.94 ± 0.54% and 100.00 ± 0.00%, and expression of COX-2 increased from 1 (control) to 1.83 (HVCO).

CONCLUSION: The results suggest that HVCO is effective to increase cells proliferation and hence wound healing process.

Introduction

Wound healing is a process involving many cells consisting of four stages, namely hemostasis, inflammation, proliferation, and remodelling [1]. In the hemostasis, stages are the beginning of the wound healing process by involving platelets [2]. During the inflammatory phase, fibroblasts function as cytokine secretions, and growth factors to activate the body's defence system [3]. During the proliferation and remodelling phases, fibroblasts are important for granulating and reorganising tissues of the extracellular matrix [3].

Wound healing is associated with bacterial contamination in the wound area. The ultimate goal of wound healing is to restore the functional properties of the skin and prevent infection [4]. The COX enzyme consists of 2 isoenzymes such as COX-1 (COX-3 = COX-1 variants), and COX-2. COX-2 plays a role in the process of angiogenesis [5]. The expression of COX-2 affects the process of angiogenesis, migration, and proliferation of fibroblasts which is very important in wound healing [6]. The expression of COX-2 affects the process of angiogenesis, migration, and proliferation of fibroblasts [6].

VCO is obtained from the flesh of mature fresh coconuts fruit (*Cocos nucifera*) processed at low

temperature or without heating [4]. VCO contains phytosterol that can be beneficial as anti-inflammation. Coconut oil as triglyceride does not have antimicrobial and antiviral activities, but when VCO is partially hydrolysed, it will generate free fatty acids and monoglycerides [7]. The combination of free fatty acids and monoglycerides are proved to be an antibacterial and antiviral agent, whereas diglycerides are not [8], [9]. Lauric acid and monolaurin (monoglyceride of lauric acid) are antibacterial and antiviral through several mechanisms including by liquefying and damaging the lipid layer structure in virus and cell membrane of bacteria [8], [9].

This study aims to determine the effect of HVCO in the wound healing process by measuring the activity of cell proliferation, COX-2 expression, and cell migration in NIH 3T3 cells.

Material and Methods

Materials

Virgin coconut oil (VCO) (Palem Mustika®, Indonesia), NIH 3T3 fibroblasts were purchased from Parasitology Laboratory, Faculty of Medicine, Gadjah Mada University. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal bovine serum and kept at 37°C with a CO_2 supply of 5%. Lipase from *R. miehei* 20.000 U/g (Sigma), and reagents used were buffer Tris-HCl, nuclease-free water, sodium hydroxide, concentrated hydrochloric acid, distilled water, n-hexane, sodium sulfate anhydrous, potassium hydrogen phthalate, phenolphthalein, ethanol, and demineralized water, DMSO, phosphate buffer saline (PBS), and fetal bovine serum (FBS). All chemicals and reagents commonly used in COX-2 expression assay.

Enzymatic hydrolysis of VCO

Thirty (30) g of oil was transferred into 250 ml Erlenmeyer, and then, 30 ml distilled water, 12.5 ml 0.063 M $CaCl_2$, 25 ml buffer Tris-HCl 1 M pH 8, and 3 ml lipase *R. miehei* were added. The mixture was incubated at 50°C for 10 h and stirred at 200 rpm for within every 1 h incubation time. At the end of the mixture incubation time, the mixture was transferred into the separating funnel and extracted, 50 ml n-hexane was added, and shaken at 5 minutes was done. The mixture was allowed to stand for some time until two layers were formed. The upper layer (n-hexane fraction) was separated as the first extract, while the bottom layer (water fraction) was extracted again with 50 ml n-hexanes above and separated as the second extract. The first and the second extracts were mixed, and then, 250 g sodium sulfate anhydrous was added to absorb the water residue. The combined extract was allowed to stand for 15 min, filtered, and the n-hexane was evaporated using the water bath and resulted in HVCO, and then the acid value was determined. HVCO was then used to determine biomarkers in the wound healing process [10], [11], [12].

Acid value determination

Five (5) g VCO was weighed, and procedure titration carried out, then the acid value and free fatty acid (FFA) percentage of HVCO was calculated as previously described [10], [12].

Proliferative Activity

HVCO (1000 µg/mL; 500 µg/mL; 250 µg/mL; 125 µg/mL; 62.5 µg/mL; 31.25 µg/mL; and 15.625 µg/mL in *co-solvent* DMSO (Sigma) was submitted for proliferative test. In that way, NIH 3T3 cell line (1×10^4 cells/mL was grown in DMEM complete medium. After 24; 48 and 72 h treatment, MTT assay was performed and cell viability was counted to determine the proliferative activity [13], [14], [15].

Wound Healing Migration Assay

The migration assay was carried out with NIH 3T3 cells seeded at 5×10^4 cells/well in 24-well plates and incubated for 24 h at 37°C. Cultured cells were washed with PBS and added culture media which containing 0.5% FBS and incubated for 24 h. Scratch was done in the bottom centre of the well within the cell layer using a yellow tip. Cell residues in the plate were washed with PBS and treated with HVCO and incubated for 48 h at 37°C and documented under the inverted microscope against cell migration rapidity after 0, 24, and 48 h. The space from scratch treatment between control and treatment culture cell was quantified using Image J software and defined as cell migration area [16].

Expression of COX-2

NIH 3T3 cells (5×10^4 cells/well) were seeded into 6-well plate and incubated for 24 h. After that, the cells were treated with HVCO and then incubated for 24 h. Both floating and adherent cells were collected in a conical tube using trypsin 0.025%. The cells were washed thrice with cold PBS and centrifuged at 2500 rpm for 5 min. The supernatant was separated and used for RNA extraction (Genaid, USA) and RNA concentration was determined by spectrophotometric method (Nanodrop) and stored at -80°C until used. Complementary DNA (cDNA) was synthesised from 3.0 µg total RNA using RT-PCR kit (Toyobo, Japan) in a final volume of 20 µL using random primers based on the manufacturer's instructions. RT-PCR was

carried out in Apply Biosystem Proflex. The reaction mixture consisted of GoTaq Green (12.5 µL) (Promega), 1.0 µL of cDNA 1 µL forward primers, 1 µL reverse primers, and 9.5 µL nuclease-free water to make a total volume of 25 µL. β-actin was used as internal reference control. The PCR primers were used for β-actin (F: 5'-gtc gta cca ctg gca ttg t-3'; R: 5'-cag ctg tgg tga agc t-3'), Cox-2 (F: 5'-cca gca ctt cac gca tca gt-3'; R: 5'-acg ctg tct agc cag agt ttc ag-3'). The PCR condition was comprised of first incubation at 95°C for 2 minutes, 95°C for 30 sec, annealing at 55°C 30 sec, extension at 72° for 1 minute, and 35 cycles. The PCR products were detected by electrophoresis in 2% agarose gels and added gel red 10 µL. Then, they were visualized with gel doc [13], [17], [18].

Statistic Analysis

The results were presented as means ± SD. The statistical analysis was carried out by using SPSS edition 21.

Results

Acid value and %FFA of HVCO

The acid value is 96.89 ± 0.12 mg NaOH/g oil and free fatty acids (FFA) is 48.50 ± 0.06%.

Wound Healing Migration Assay

A little wound repair was observed in wells with HVCO at 62.5 µg/mL after 24 and 48h incubation with 61.94 ± 0.54% and 100.00 ± 0.00% respectively closure area. The wound healing migration of HVCO is given in Figure 1.

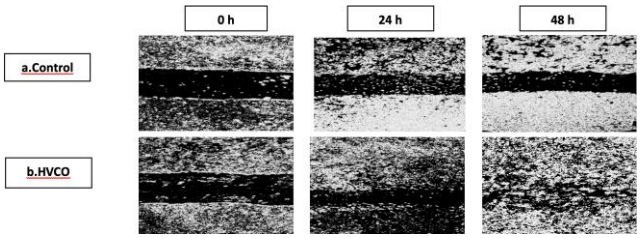

Figure 1: Wound Healing Migration Assay. NIH 3T3 cells were treated by HVCO 62.5 µg/mL for 0; 24 and 48 h and measured the closure area; A) control cells; B) HVCO 62.5 µg/mL. Proliferative Activity

The percentage of viable cells after treatment and incubation for 24 h, 48 h, and 72 h (109.98 ± 0.52; 118.26 ± 0.53; 106.59 ± 0.43) showed the stimulation effect of HVCO towards the proliferation of NIH 3T3 cells. The effect of HVCO is given in Figure 2.

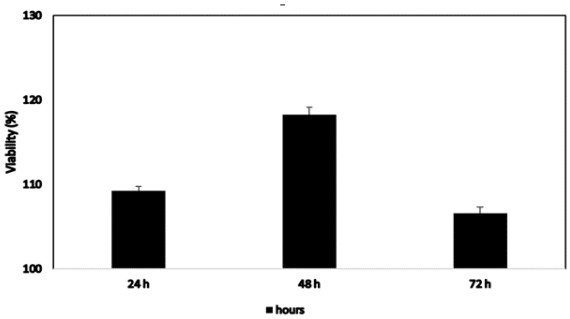

Figure 2: Percentage of viable cells of NIH 3T3 cells were treated by HVCO 62.5 µg/mL for 24; 48 and 72 h and measured viable cells

COX-2 Expression

HVCO were showed a significant up-regulatory effect on the expression of COX-2 (1.93 ± 0.49). COX-2 expression is given figure 3.

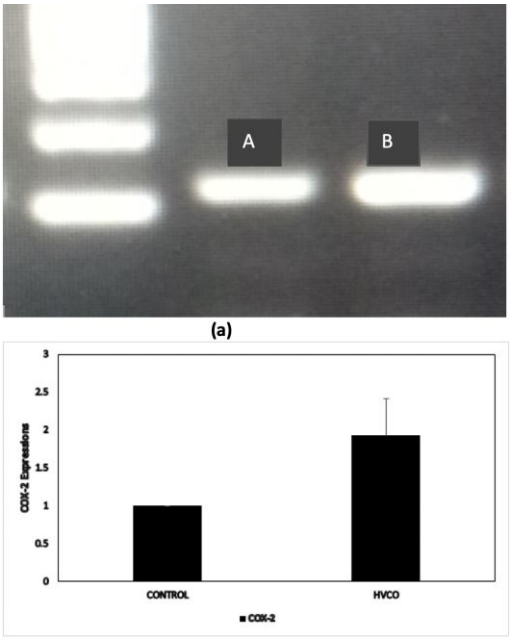

Figure 3: Representative figures showing COX-2 expression after treatment with HVCO 62.5 µg/mL (a) bands of COX-2 expressions (A = Control Cell; B = HVCO = 62.5 µg/mL), and (b) chart of COX-2 expressions

Discussion

Enzymatic hydrolysis of VCO was done using lipase from *R. miehei,* which is specific for acyl groups at sn-1 and sn-3 position in triglyceride molecule [10], [11]. At the temperature of 50°C, this enzyme hydrolyses fatty acids on sn-1 and sn-3 positions in triglyceride molecule which generates two free fatty acids (FFAs) and 2-monoglyceride in hydrolyzed VCO (HVCO) [10], [11]. Acid value is defined as mg NaOH used to neutralise FFA contained in 1 g of fats or oils to indicate the amount of FFA in one-gram fats or oils. HVCO composed of FFAs mainly as lauric acids and

2-monoglycerides mainly as 2-monolaurin [12].

Lauric acid and monolaurin are the antibacterial and anti-inflammatory agent that able to overcome skin problems [19]. Lauric acid and monolaurin decrease the time for complete epithelialization because lauric acid and monolaurin can increase proliferation cells and migration cells [4]. During the wound healing process, cells at the wound edges proliferate and migrate, leading to re-epithelialization of the wound surface [20].

Migration of NIH 3T3 fibroblasts was assessed using the wound healing scratch assay. Lauric acid and monolaurin increase proliferation cells and migration cells [4]. Cell migration activity in the HVCO group is faster than the control group. Lauric acid and monolaurin increase proliferation cells and migration cells. Lauric acid and monolaurin are found in HVCO that stimulate cells to migrate. So, the percentage of HVCO group cell migration is faster than the control group.

Cyclooxygenase-2 (COX-2) is an inducible enzyme which plays a critical role in multiple pathophysiological processes including inflammation, atherosclerosis, tissue injury, angiogenesis and tumorigenesis [6]. According to Futagami et al., study, COX-2 mRNA in the normal rat skin with a wider allocation than the COX-2 protein but with less intensity. After the injury, this COX-2 protein and mRNA were expressed primarily in the head and basal layers of the epidermal wound edges, which are structured of migratory and proliferative cells [6].

In the Ebeling et al., study, COX-2 is one of the wound healing parameters. Pentacyclic triterpene and botulin are an active compound of birch bark extract. They influence the inflammatory phase of wound healing by upregulating proinflammatory cytokines, chemokines and cyclooxygenase-2 (COX-2) in human primary keratinocytes. Exemplarily, Ebeling et al., confirm upregulation in the ex-vivo pig wound healing model for IL-6 and COX-2. They provide evidence for COX-2 and IL-6 that their mRNA increase is due to an mRNA stabilising effect, a process in which p38 MAPK and HuR (human antigen R) are essentially involved [21]. In this study, Lauric acid and monolaurin increase of COX-2 expression which mediated angiogenesis and migration NIH 3T3 cell.

In conclusion, HVCO increase cell proliferation after 24 hours incubation, constant at 48 and decreased after 72 hours incubation, cell proliferation decreases. Percent of the wound closed at 48 hours incubation, closing up to 100%. HVCO is also able to increase COX-2 expression. HVCO increase cell proliferation, percent of the wound closed, and COX-2 expression, so HVCO is useful as wound healing.

Acknowledgement

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Basic Science

Knockdown of CD-74 in the Proliferative and Apoptotic Activity of Breast Cancer Cells

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Keywords: CD 74; Knockdown; Proliferation; Apoptosis; Breast cancer; CAMA-1; MDA-MB-231 cells

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Abstract

BACKGROUND: The cluster of differentiation (CD) 74 is known for its immunological functions and its elevated level was reported in various cancer cells.

AIM: The aim of the present study was to investigate the expression and potential roles of CD74 in the proliferative and apoptotic activity of breast cancer.

METHODS: Expression of CD74, macrophage migration inhibitory factor (MIF) and CD44 was assayed in CAMA-1 and MDA-MB-231 cell lines using flow cytometry. CD74 was knocked down using CD74 siRNA-transfection in CAMA-1, and MDA-MB-231 cells and proliferation and apoptosis were determined in the transfected breast cancer cells.

RESULTS: The data showed that CD74, MIF and CD44 were expressed in breast cancer cell lines and were associated with cell proliferation and apoptosis. Correlation analysis revealed that CD74 was positively correlated and colocalised with MIF on the cell-surface of CAMA-1 and MDA-MB-231. The knockdown of CD74 significantly reduced CAMA-1 and MDA-MB-231 cell proliferation and increased the level of apoptotic cells.

CONCLUSION: We concluded that the interactions of CD74 with MIF and CD74 with CD44 could be a potential tumour marker for breast cancer cells. Moreover, the level of co-expression of MIF and CD74 or CD44 could be a surrogate marker for the efficacy of anti-angiogenic drugs, particularly in breast cancer tumours. In short, the study revealed the potential roles of CD74 in the proliferation and apoptosis of breast cancer which may serve as a potential therapeutic target for breast cancer.

Introduction

The role of a cluster of differentiation (CD) – 74 is a transmembrane glycoprotein, and its role has recently been reported in the pathogenesis of several cancers including breast cancer [1], [2]. Several studies have suggested that a small proportion of intracellular CD74 is modified by the addition of chondroitin sulfate (CD74-CS), a form of CD74 and chondroitin sulfate is a sulfated glycosaminoglycan usually found attached to proteins as part of a proteoglycan [3], [4]. CD74-CS is expressed on the surface of immune cells and can bind MIF, mediating MIF's signalling pathway [3], [4]. Cell-surface expression of CD74 is not strictly dependent on the expression of class II MHC molecules in term of antigen presentation [5], [6] and numerous non-class II positive cells express CD74 which functions as a receptor for the initiation of different signalling cascades [7], [8]. MIF is the natural ligand of CD74 and binds to the extracellular domain of CD74 with high affinity (KD = 1.40 Å ~ 10-9 M) and initiates a signalling cascade [9]. When bound to the extracellular domain of CD74, MIF promotes signalling pathways including cell proliferation and apoptosis [9], [10], [11], [12], [13]. The short cytoplasmic tail of CD74 lacks an intracellular signal-transducing domain, although serine phosphorylation takes place in the P35 variant of CD74, requiring CD44, a

polymorphic transmembrane protein with kinase activating properties [14], [15]. CD74 forms a complex with CD44 which is essential for the MIF-induced signalling cascade [10], [16]. This cascade induces phosphorylation of ERK1 and ERK2 and activates various effector proteins involved in inflammatory processes and cell proliferation. ERK1 and ERK2 remain phosphorylated for many hours and hence this cascade continues for up to 2 to 3 hours [17], [18], [19], [20], [21]. Concurrently, MIF binding to CD74 activates the P13K-Akt pathway leading to phosphorylation of BAD and BAX proteins which are involved in apoptosis [22]. In addition, this cascade augments Bcl-2 expression, further supporting cell survival [23], [24], [25]. Thus, the binding of MIF to the CD74 / CD44 complex initiates a pathway resulting in the proliferation of the mature B cell population and their rescue from cell death. In addition to activating the P13K-Akt pathway, MIF binding to CD74 also induces a signalling pathway which involves Syk tyrosine kinase [6], [16] and induces cleavage of intramembrane CD74 regional releases intracellular domain (CD74-ICD) [26], [27]. CD74-ICD translocates to the nucleus where it induces activation of transcription mediated by the NF-κB p65 / RelA homodimer and its co-activator, TAFII105, resulting in regulation of transcription of genes that control B cell proliferation and survival [3], [6], [16]. Therefore, the CD74-MIF-CD44 complex initiates a pro-survival signal leading to the increase of proliferation and inhibition of apoptosis.

Recently, we quantified colocalization of CD74 and CD44 in breast cancer cells through non-invasive and validated bioimaging procedure [28] and also determined several novel biomarkers involved in the pathogenesis of breast cancer [29]. Not only have these, but we also showed that treatment of human breast cancer cells with interferon-γ up-regulates the expression of CD74 along with MIF and CD44 [2], [30]. In continuation of these studies, the present study was hypothesised to find out the potential roles of CD74 in the proliferative and apoptotic activity in breast cancer. This was achieved by studying the colocalization of CD74 and MIF as well as CD74 and CD44 by two different techniques confocal microscopy and immunoprecipitation. The cells proliferation and apoptosis in CD74 siRNA transfected cells were also studied to address the hypothesis that blocking CD74 or MIF would affect apoptosis and cell proliferation.

Methods

Cell lines and cell culture

Two human mammary gland cell lines, CAMA- 1 and MDA-MB-231, were used, which were derived from a malignant pleural effusion. The CAMA-1 cell lines were maintained in RPMI 1640 medium (LONZA-Belgium), supplemented with 10% (v / v) fetal calf serum (FCS; Imperial Laboratories, Andover, UK). The MDA-MB-231 cell line was maintained in D-MEM (high glucose), supplemented with 10% FCS. Raji cells (human negroid Burkitt's lymphoma) and HeLa cells (human cervical cancer), expressing high levels of CD74, MIF, and CD44, respectively, served as additional positive controls. Raji and HeLa cells were cultured in RPMI 1640 (LONZA-Belgium) containing 10% FCS and cultured in a humidified atmosphere of 5% CO_2at 37°C. All media used for this study were purchased from PAA Laboratories GmbH (Pasching, Austria).

Flow cytometry analysis

Cell lines were lifted with Accutase (Sigma-Aldrich), and 1 x 10^6cells were used per sample. Monoclonal primary antibodies, By2 (anti-CD74), ab55445 (anti-MIF) and 156-3c11 (anti-CD44) were employed in indirect immunofluorescence staining. Cells were preincubated with saturating concentrations of primary antibody, followed by washing and labelling with FITC-conjugated goat anti-mouse IgG (Bio-legend). For cell surface staining, cells were fixed with 4% formaldehyde solution and washed with 1X phosphate-buffered saline (PBS). The cells were then blocked with blocking buffer (PBS / 0.1% BSA, bovine serum albumin) and washed in PBS. Primary and secondary antibodies were diluted with 0.1% BSA in PBS. Cells were sorted on a BD FAS Aria and analysed by FlowJo 8.8.6.

Immunofluorescence

In preparation for confocal immunofluorescence microscopy for studying colocalization between CD74 and MIF, CAMA-1 and MDA-MB-231 cells were cultured in LabTek 8-well chambers (Thermo Fisher Scientific) at a density of 6 x 10^3cells per well for two days. Following this, they were seeded. The cells were fixed with 4% paraformaldehyde for 20 min on ice. Cells were then blocked with 2% (w / v) BSA prepared in 1X PBS for 1 h at room temperature. For single staining of each antigen, cells were incubated with anti-CD74 (clone: By2) at a concentration of 1:500, (anti-MIF) (clone: ab55445) and 156-3c11 (anti-CD44) at a concentration of 1:400 for 1 h, followed by three washes with PBS. Secondary antibody, anti-mouse IgG conjugated with Alexa Fluor® 488 or Alexa Fluor® 555 (Invitrogen, Carlsbad, CA, USA), was used at a dilution of 0.25 µg / 100 ml for 1 h. For double staining, cells were blocked again with 2% BSA and the staining process was repeated for each desired pair. Cells were thoroughly washed with PBS, the chambers removed, and the slide was mounted with anti-fade mounting medium (Vector Shield) covered with a coverslip (Chance proper LTD, West Midlands, England) and sealed with rubber cement (Fixogum

Rubber Cement, Marabu, Germany). Cells were incubated with a primary antibody followed by a secondary antibody. CD74 was labelled with FITC Alexa Fluor 488 (green) and CD44 was labelled with Alexa Flour 555 (red). Colocalization of CD74 with CD44 was assessed by Pearson's correlation coefficient was used to analyze the degree of colocalization. The scale lay between -1 and 1, where 1 stand for colocalization, -1 stands for negative colocalization and 0 stands for no colocalization. 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) counterstain (Vector Laboratories, Burlingame, CA, USA) was used at a 1:250 dilution.

Quantitative colocalization analysis of confocal fluorescence microscopy images

To investigate whether CD74 and MIF co-localize, a high-precision single-cell bioimaging protocol was employed, previously developed by our research group [31]. The Pearson correlation coefficient (PCC) was used for quantitative analysis of colocalization [31], [32]. PCC provides the overall association of two probes in an image, statistically. It also indirectly measures the quantity, i.e. the fraction of one protein that colocalises with another protein. A Nikon A1Si confocal microscope (Nikon Instruments Inc.) with a plan-apochromatic VC1.4 N.A. 60X magnifying oil-immersion objective was used for image acquisition. Images were acquired in three channels, using one-way sequential line scans. DAPI was excited at 398.7 nm with laser power 1.6 arbitrary units, and its emission was collected at 450 nm with a PMT gain of 86. Alexa Fluor 488 was excited at 488 nm with laser power 5.8; its emission was collected at 525 nm with a PMT gain of 117. Alexa Fluor 555 was excited at 560.5 nm with laser power 3.7, and its emission was collected at 595 nm with a PMT gain of 98. The scan speed was ¼ frames/s (Galvano scanner). The pinhole size was 35.76 µm, approximating 1.2 times the Airy disk size of the 1.4-NA objective at 525 nm. Scanner zoom was centred on the optical axis and set to a lateral magnification of 60 nm/pixel. Axial step size was 105 nm, with 80-100 image planes per z-stack.

Small interfering (si) RNA transfection

CAMA-1 and MDA-MB-231 cell lines were seeded in six-well plates at a density of 2×10^5 per well in 2 ml normal growth medium supplemented with 10% FCS. The cells were then allowed to grow until they reached 60-80% confluency. For each transfection, 4 µl of CD74 siRNA duplex at dose of 80 pmols (sc-35023) (Santa Cruz Biotechnology, USA) was diluted and 4 µl of siRNA transfection reagent was added at dose of 80 pmols (sc-29528) (Santa Cruz Biotechnology, USA) into 100 µl of siRNA transfection medium (sc-36868) (Santa Cruz Biotechnology, USA) separately without serum or antibiotics. Both diluents were mixed and incubated for 15-45 minutes at room temperature. Cells were then washed with 2 ml of siRNA transfection medium. The siRNA transfection reagent mixture was then added to each well, and the volume made up to 1 ml by adding 800 µl of siRNA transfection medium (Santa Cruz Biotechnology, USA). In the same manner, this was applied for negative control siRNA (sc-44230) (Santa Cruz Biotechnology, USA). The cells were then incubated overnight at 37ºC in a CO_2 incubator for 18-24 hr. Following incubation, the medium was aspirated and replaced with fresh 1X normal growth medium. Cells were assayed using the appropriate manufacturer's protocol 24-72 hours after the addition of fresh medium in the step above. Transfection efficiency was confirmed by western blot and microscopy. Once the transfection was confirmed, the effect of CD74 siRNA on the proliferation and apoptosis of CAMA-1 and MDA-MB-231 could then be studied.

Proliferation and Apoptosis assay

CAMA-1 and MDA-MB231 cell lines were cultured in 6 well plates at a density of (15×10^3 cell/well) or in 96 well plates at a density of (15×10^2 cell/well) at 37°C and then transfected with CD74 siRNA duplex as explained previously. The cells were then washed twice with 1X PBS and incubated with 2 µl of Annexin V-FITC (BioLegend, UK) at room temperature for 20 minutes in the dark. Cells were then thoroughly washed and then fixed with 4% PFA followed by washing steps in PBS. Finally, the samples were read using BD FACSAria and analysed by FlowJo 8.8.6. In the same manner, the MTT assay was then used to assess cell proliferation. Briefly, 20 µl of MTT solution (5 mg/ml in PBS) and 100 µl was added per well, and cells were incubated at 37°C with 5% CO_2 in a humidified chamber for 4 hr for colour development. The resultant Formazan crystals were dissolved in dimethyl sulfoxide (100 µl) and the absorbance intensity measured at 595 nm using a microplate reader (Versamax). The percentage of cell proliferation was calculated relative to the rate of proliferation in untreated cells.

Results

Cell-surface expression of CD74, MIF and CD44

The cell-surface expression of CD74, MIF and CD44 were analysed in CAMA-1, MDA-MB-231. Non-permeabilized were stained with an appropriate concentration of By2 (anti-CD74), ab55445 (anti-MIF) and 156-3C11 (anti-CD44) antibodies followed by 1 µl RAM-FITC secondary antibody. Cells without staining and isotype cells, stained with only secondary

antibody, were used as a negative control. CD74, MIF and CD44 expression were detected on the cell surface and cytoplasmic of CAMA-1 and MDA-MB-231. Monocytes, Raji cells, cervical cancer HeLa cells, and lymphocytes, (Jurkat) cells, were used as a positive control as they express high levels of CD74, CD44, and MIF respectively. This is displayed in Figure 1 in where empty histograms show CD74, MIF or CD74 protein grey filled the histogram.

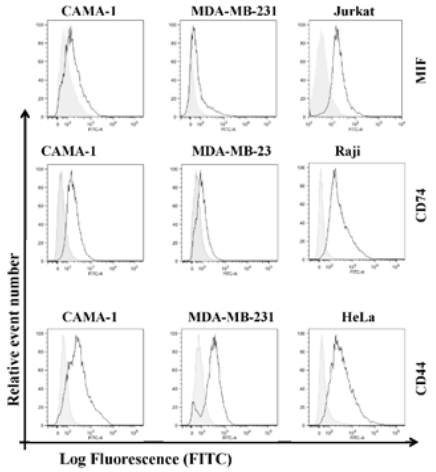

Figure 1: Flow cytometric analysis for cell surface expression of CD74, MIF and CD44 in the breast cancer cells displayed; Empty histograms represented the expression of CD74, MIF, and CD44; Expression in Raji, Jurkat, and HeLa cells are used positive controls. Whereas, grey-filled histograms were shown as a negative control obtained from isotype-matched with control antibody; The data are representative of three independent assays

Colocalization of MIF with CD74and CD44

To investigate whether MIF colocalised with CD74 or CD44 on CAMA-1 and MDA-MB-231 cells, all cell lines were immunostained with an appropriate primary antibody followed by a secondary antibody (Figure 2 and Figure 3).

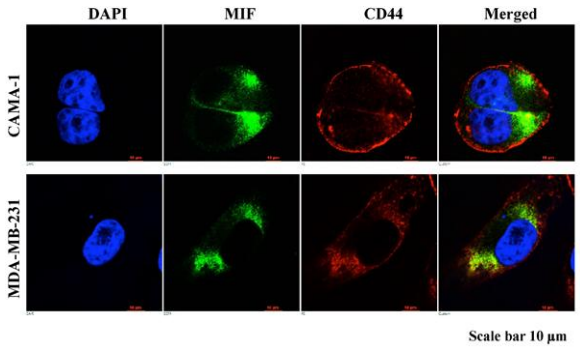

Figure 2: Figure 2: Colocalization of MIF and CD44 on the cell-surface of CAMA-1 and MDA-MB-231 cells, determined by confocal microscopy analysis; CAMA-1 and MDA-MB-231 and MDA-MB-435 cells were cultured in LabTek 8-well chambers at a density of 10 × 103 cells per well overnight; The cells were stained with MIF labelled with Alexa Fluor® 488 (green) or CD44 labelled with Alexa Fluor® 555 (red); Cell nuclei were stained with 4', 6-diamidino-2-phenylindole (blue); Fluorochromes were acquired separately to evaluate the expression of CD44 and MIF; Yellow/orange fluorescence reveals the potential colocalization of two antigens. The images represent three different experiments

CD74 or CD44 was labelled with Alexa Fluor® (red) and MIF Alexa Fluor® (green). CAMA-1 and MDA-MB-231 cells showed clear expression of MIF, CD74, and MIF. Merging green and red channels assessed the colocalization and Pearson's product-moment correlation coefficient (PCC) was used to analyse the degree of colocalization.

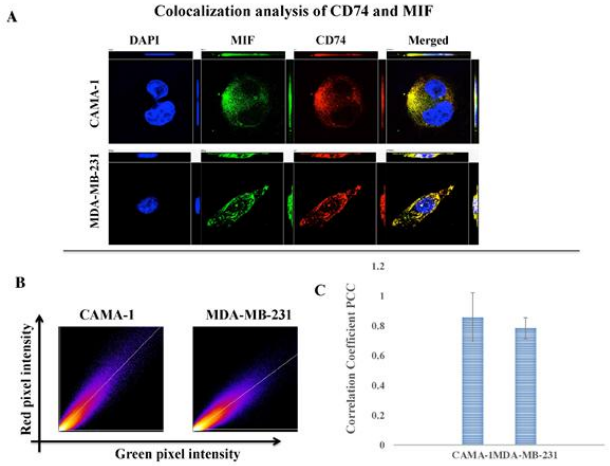

Figure 3: Figure 3: Colocalization of CD74 and MIF on the cell-surface of CAMA-1 and MDA-MB-231 cells, determined by confocal microscopy analysis; A) CAMA-1 and MDA-MB-231 cells were cultured in LabTek 8-well chambers at a density of 10 × 103 cells per well overnight; Cells were stained with MIF labeled with Alexa Fluor® 488 (green) or CD74 labeled with Alexa Fluor® 555 (red); Cell nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI) (blue); Fluorochromes were acquired separately to evaluate the expression of CD74 and MIF; Yellow / orange fluorescence reveals the potential colocalization of the two antigens; 3D images were acquired in the stack, with z-direction step size 0.14 µm using NIS element; Single-plane of z-stack is shown in three directions as xy, yz and zx; Data represent three different experiments; B) Each pixel in the image was plotted in the scatter diagram based on its intensity level in each channel; The color in the scatterplot represents the number of pixels plotted in that region; In this example, green is shown on the x-axis and red is shown on the y-axis; The scatterplot shows high colocalization and no bleed through either green or red channels; The scatter plot provides the rate of the area of association of two fluorochromes, calculated by linear regression; The scatter plot comprised of dots, appearing as cloud, indicates complete colocalization; B) Graphical representation of colocalization analysis based on the Pearson product-moment correlation coefficient (PCC) on each cell; The value for PCC ranges from +1 and −1 inclusive; A value of +1 would mean the total positive correlation, every pixel that contains Alexa Fluor® 488 (FITC) also contains Alexa Fluor® 555 (TRITC), while a value of −1 would mean the total negative correlation, every pixel that contains Alexa Fluor® 488 does not contain Alexa Fluor® 555 and vice versa; The PCC was calculated based on different images and indicates strong colocalization of CD74 and MIF on CAMA-1 and MDA-MB-231 Data represents three different experiments

Knockdown of CD74 expression in CAMA-1 and MDA-MB-231 cells by CD74 siRNA

Prior studies have reported that CD74 is over-expressed in human breast adenocarcinomas, and has a role in tumour progression along with MIF and CD44. The expression of CD74 in CAMA-1 and MDA-MB-231 cells was therefore evaluated. The expression of CD74 was found to be highest in CAMA-1 cells compared to MDA-MB-231 cells (Figure 4). In pilot experiments, it was found that a

concentration of 80 pmol/ml for 24 hr of specific CD74 siRNA was optimal for disrupted expression of CD74. Therefore, a dose of 80 pmol/ml was selected for optimal transfection of CAMA-1 and MDA-MB-231 cells with siRNA for all subsequent experiments.

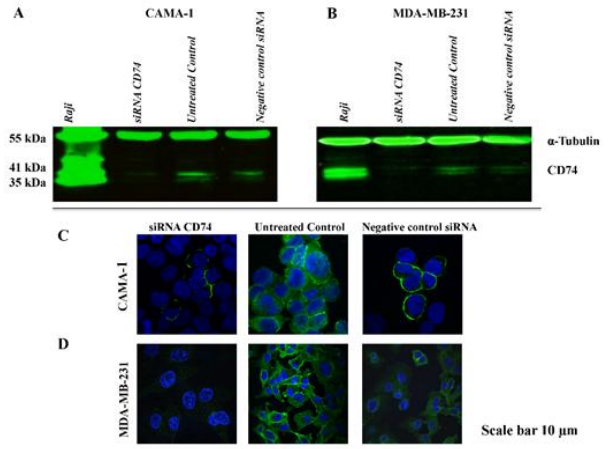

Figure 4: Figure 4: CD74 knockdown by CD74 siRNA transfection reagent in CAMA-1 and MDA-MB-231 cells; A) and B) siRNA-mediated knockdown of CD74 expression in CAMA-1 and MDA-MB-231 cells were detected by Western blot; An approximately two to five (siRNA) fold weaker signal of CD74 protein expression is apparent, as compared to the negative control siRNA group normalized to the expression of α-Tubulin; C) and D) Confocal images of CAMA-1 and MDA-MB-231 cells transfected with CD74 siRNA, untreated control and negative control siRNA; Data represent three different experiments

Knockdown of functional CD74 expression in CAMA-1 and MDA-MB-231 cells promotes apoptosis

In the light of the observations indicating apoptotic modes of cell death in CAMA-1 and MDA-MB-231 cells treated with CD74 siRNA next, multi-parameter flow cytometric analysis of siRNA-transfected CAMA-1 and MDA-MB-231 cells was pursued to obtain more sensitive and quantitative details of a possible apoptotic mode of cell death. Following 24 hr of a culture of CD74 siRNA-transfected CAMA-1 and MDA-MB-231 cells, flow cytometry was used to detect the expression of annexin V in the absence of PI staining.

Annexin V is a non-quantitative probe used to detect phosphatidylserine expressed on the cell surface, an indication of apoptosis. CAMA-1 and MDA-MB-231 cells treated with CD74 siRNA displayed significantly higher levels of annexin V staining (± 55% and 58% respectively) compared with negative control siRNA-treated counterparts (± 8% and ± 13% respectively) (Figure 5A). These observations indicate that CD74 might play important regulatory roles in apoptosis.

Effects of CD74 knockdown on CAMA-1 and MDA-MB-231 cell proliferation

CAMA-1 and MDA-MB-231 cell proliferation and viability were determined using the MTT metabolic and viability assay (Figure 5B). CAMA-1 and MDA-MB-231 cells treated with CD74 siRNA displayed significantly reduced proliferation compared to cells treated with the negative control siRNA control sequence.

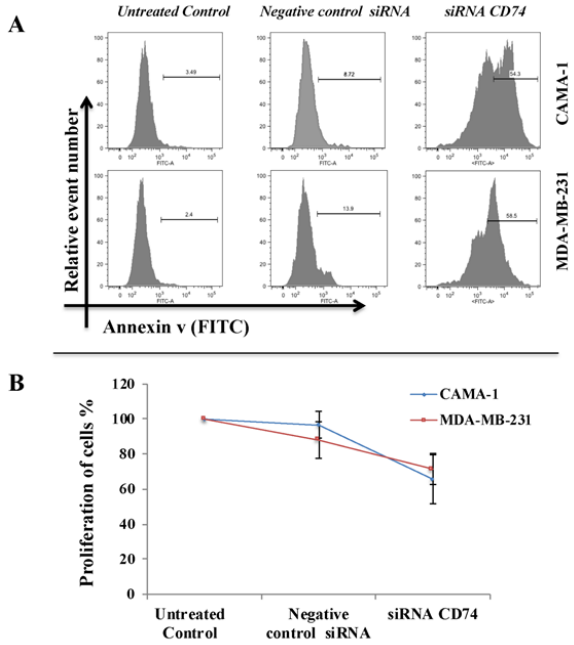

Figure 5: Figure 5: Effect of CD74 siRNA on the apoptosis and proliferation of CAMA-1 and MDA-MB-231 cells; A) Flow cytometric determination of the effect of CD74 siRNA on apoptosis of CAMA-1 and MDA-MB-231 cells; Cultured CAMA-1 and MDA-MB-231 cells were divided into three groups: nontransfected cells, cells transfected with negative control siRNA and cells transfected with CD74 siRNA; After a 24 hr treatment, the cells were harvested for quantitation of apoptosis by determining changes in the cell-surface expression of annexin V; Displayed is also a description of the observed frequency of cells undergoing apoptosis, which was found to be much higher in the CD74 siRNA treated cells than in the negative control-treated group of CAMA-1 and MDA-MB-231 cells, respectively; B) Effect of CD74 siRNA on the proliferation of CAMA-1 and MDA-MB-231 cells; MTT assay showed that treatment of CAMA-1 and MDA-MB-231 cells with CD74 siRNA inhibited their proliferation; Each point in the curve represents the arithmetic mean OD values ± SD from representative experiments that were performed in triplicate

Discussion

The present study aimed to investigate the role of CD74 and its interrelation to MIF in breast cancer cells. This was achieved by studying the expression and colocalization of MIF with CD74 and CD44 molecules in the breast cancer cell lines CAMA-1 and MDA-MB-231 cells. The results obtained from confocal microscopy demonstrated that CD74 and MIF are highly colocalized on the cell-surface of all

breast cancer cells. Pearson's correlation coefficient and scatter plot analysis (Figure 3) also gave rise to the colocalization of CD74 and MIF [33], which accurately depicts the percentage of colocalization of CD74 and MIF molecules. Several groups have studied the association of CD74 with MIF and CD44 in cancers since it was reported that CD74 and CD44 are involved in signalling with MIF [10], [15], [16]. We also showed that CD74 and CD44 colocalise in breast cancer cells using a non-invasive and validated bioimaging procedure [28]. Also, it was shown that the formation of a molecular complex between MIF, CD74 and CD44 in prostate carcinoma cells lines (DU-145) could mediate signal transduction including (gene regulation, apoptosis, and cell proliferation) in prostate cancer [34].

Previous studies by immunofluorescence have confirmed the colocalization of MIF and CD74 in non-small cell lung cancer [35]. Additionally, using correlation analysis, Zheng et al. identified a positive correlation between MIF and CD74 in gastric cancer cells [36]. Correspondingly, Starlets *et al*. showed that, in malignant B cells obtained from patients with chronic lymphocytic leukaemia (CLL), MIF binds to the extracellular domain of CD74 to initiate a signalling cascade leading to cell proliferation and survival [6]. The interaction of MIF with CD74 and CD44 has been reported, suggesting that MIF in association with CD74 and CD44, as a complex, plays a significant role in bladder cancer cell proliferation [37]. Similarly, Meyer-Siegler *et al*., reported that the interaction between MIF and CD74 activates the ERK1 and ERK2 signalling pathway, presumably through interaction with CD44, in the prostate cancer cell lines DU-145 and LNCaP, but not in normal human prostate epithelial cells (PrEC) or benign prostate epithelial cells (BPH-1) [34]. However, human benign prostate hyperplasia epithelial cells (BPH-1) and PrEC prostate cancer cells do not express CD74 on the cell surface, so for this reason, both cells do not interact with MIF and CD44 [34]. Correspondingly, Shi *et al*. showed that mammalian COS-7 cells do not bind MIF unless engineered to express the extracellular domain of CD74 [10].

To investigate the role of CD74 in apoptosis and proliferation, siRNA that targeted CD74 was used. Western blot results (Figure 4A and 4B) showed that CD74 expression was strongly knocked down in CAMA-1 and MDA-MB-231 cells in comparison with the control and CD74 siRNA. Microscopic results also confirmed that CD74 expression was strongly knocked down in both cell lines (Figure 4C and 4D). When CD74 expression was knocked down, apoptosis was observed in CAMA-1 and MDA-MB-231 cells. Both cell lines, when treated with CD74 siRNA, displayed significantly higher levels of annexin V staining (± 55% and ± 58% respectively) compared to negative control siRNA-treated counterparts (± 8% and ± 13% respectively). In the same manner, it was found that in CAMA-1 and MDA-MB-231 cells treated with CD74 siRNA, a significantly reduced proliferation was observed compared to cells treated with the negative control siRNA control sequence and untreated cells. Likewise, it has been reported that knockdown of MIF or CD74 expression by RNA interference inhibits DU-145 cell proliferation and downstream MIF signalling [34], [38]. It is also reported that knockdown of the functional expression of MIF markedly decreased H460 cell proliferation and induced apoptosis, as seen by augmented expression of annexin A5 following treatment of H40 cells by MIF siRNA [39]. In particular, Verjans *et al.* showed that anti-MIF and anti-CD74 antibodies potently blocked cell proliferation of non-invasive MDA-MB-468 and invasive MDA-MB-231 breast cancer cells; however, this was not observed in non-tumorous MCF-12A cells [40]. This could be explained by the absence of the cell-surface portion of CD74 in MCF-12A cells. It is also reported that CD74 regulates Fas death receptor signaling in lymphomas by decreasing the levels of Fas receptors on the cell surface [41]. In the same manner, Liu *et al*., have shown that CD74 promotes tumor growth, angiogenesis, and cancer cell metastasis *in vivo* [42]. The effect of CD74 in tumor growth and cell proliferation was studied by blocking the activity of MIF or CD74 in HEK / CD74 or a renal cell carcinoma (Caki-1) cells. The data showed that CD74-upregulated vascular endothelial growth factor D (VEGF-D) positively regulates the expression of cyclin D and E, which results in the promotion of cell cycle progression [42]. It was reported that G1 / S phase proteins cyclin D and cyclin E, were upregulated by CD74 and promoted cell cycle progression [6]. The recent finding also showed that the expression of CD74 was associated with MIBC / high grade of the UCB, while the knockdown of CD74 attenuated the proliferation, invasion, and angiogenesis of HT-1376 [18]. Figure 6 shows the proposed signal transduction pathway of MIF with CD74 and CD44.

In conclusion, it was observed that the interaction of MIF with CD74 CD44 could be a potential tumor marker for breast cancer cells. Moreover, level of co-expression of MIF and CD74 could be a surrogate marker for the efficacy of anti-angiogenic drugs, particularly in breast cancer tumors. Also, knockdown of CD74 by CD74 siRNA significantly reduced CAMA-1 and MDA-MB-231 cell proliferation and increased the level of apoptotic cells.

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The Role of VEGF and TNF-Alpha on Epithelialization of Diabetic Foot Ulcers after Hyperbaric Oxygen Therapy

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Abstract

BACKGROUND: Around 15-25% of diabetes mellitus (DM) patients will develop diabetic foot ulcers (DFUs) with high morbidity, many studies have been proposed to search the most effective healing techniques.

AIM: This study was conducted to demonstrate the ability of hyperbaric oxygen therapy (HBOT) as a complementary therapy in DFUs healing through raising vascular endothelial growth factor (VEGF) levels and suppressing tumour necrosis factor-alpha (TNF-α).

METHODS: All patients received the same treatment including wound debridement and wound care, but the patients in the HBOT group, breathed 100% oxygen at 2.4 ATA for 90 minutes in total of 20 sessions (four weeks).

RESULT: There were 32 diabetic patients with DFUs Wagner 3-4. VEGF levels after four weeks of HBOT was significantly elevated compared to the control group ($p = 0.013$). The effect size of VEGF levels was $p = 0.005$. TNF-α levels after four weeks of therapy were decreased ($p = 0.01$). Faster epithelialization is seen in the HBOT group ($p < 0.001$). We also performed path analysis, HBOT had a significant effect on the epithelialization ($p < 0.001$) and VEGF levels affected the epithelialization process ($p = 0.042$).

CONCLUSION: HBOT administration leads to increased VEGF levels, decreased TNF-α levels, and accelerated wound healing of DFUs patients. HBOT directly aids epithelialization and indirectly through VEGF upsurge and TNF-α downturn.

Introduction

Diabetes mellitus (DM) is a chronic disease caused by inadequate insulin production, or when insulin cannot be used effectively. This results in elevated blood sugar levels causing damage to the heart, blood vessels, eyes, kidneys, and nerves in longstanding uncontrolled DM [1], [2]. In patients with DM, the most common complications in blood vessels are macroangiopathy, neuropathy, immunosuppression that facilitate inflammation, ischemia, infection, and cell death [2], [3]. These complications may lead to foot abnormalities such as a chronic ulcer, called diabetic foot ulcers (DFUs).

An ulcer occurs because, at tissue damage or death, that associated with the degree of peripheral vascular disease in inferior limb and may be accompanied by infection [4], [5]. It is estimated 15-25% of DM patients will develop DFUs with high morbidity, 40-80% will have high infection risk, and 10-20% will require amputation [6].

The standard therapies of DFUs are to regulate normal blood sugar levels, antibiotics medication to prevent and treat infection, ulcer debridement, wound care, off-loading the affected limb, and to improve blood flow or revascularisation [2], [6], [7], [8], [9]. In addition to these standard therapies, there are many adjuvant therapies, such as hyperbaric oxygen therapy (HBOT), growth factor therapy, stem cells therapy [7], [10], autolytic debridement [4], and percutaneous transluminal angioplasty [11]. These modalities are also performed in the management of DFUs. Because of DFUs high morbidity risk, many studies have been proposed to search the most effective healing techniques.

According to Undersea and Hyperbaric

Medical Society (UHMS), HBOT is an intervention in which a person breathes 100% oxygen intermittently inside a hyperbaric chamber at a pressure greater than sea level pressure (1 atmosphere absolute or ATA). Increased 1 ATA pressure is equivalent to a depth of 10 meters underwater. The therapeutic condition is achieved with a minimum pressure of 1.4 ATA and breathes with 100% oxygen [12]. In general, DFUs therapy uses 100% oxygen and pressure 2-3 ATA inside the hyperbaric chamber for 90 minutes per day [10], [13], [14].

The HBOT's mechanisms increase the tissue oxygen levels, decreased oedema, and kills anaerobic bacteria, resulting in the acceleration of wound healing [14]. Based on these mechanisms, many researchers use HBOT as one of the method therapies to DFUs [2], [6], [9], [13], [15]. HBOT is also able to improve the angiogenesis process, characterised by an increased in vascular endothelial growth factor (VEGF) levels. It can boost epithelial and granulation processes [13], [16], [17].

The wound or ulcer healing theory with angiogenesis mechanisms through the role of platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β), and vascular endothelial growth factor (VEGF) have been extensively studied in animals. However, the use of HBOT in DFUs patients, in HBOT's role in increasing VEGF through the angiogenesis process has not been widely discussed [18]. Tumour necrosis factor-alpha (TNF-α) is certainly elevated in inflammation, but in DFUs patients who get HBOT has not been explained why HBOT can decrease TNF-α levels. Fundamentally, the role of HBOT heals wounds through oxidative stress and can suppress inflammatory reactions with decreased TNF-α [19].

It is explained that in the DFUs healing process requires the study of the biomolecular role. Based on these theories, we were challenged to learn, understand, and explore more about the HBOT, not only in accelerating wound healing as a clinical feature, but also the changes in biomolecular that may help DFUs patients.

This study aimed to demonstrate the HBOT role as a complementary therapy to provide more rapid clinical recovery through increased VEGF and decreased TNF-α in DFUs wound healing.

Material and Methods

This research is an experimental study using randomised pre and post-test control groups design, with permuted block to HBOT and control group. All patients received the same wound treatment. In the HBOT group, the patient got HBOT, but the control group did not. This research was conducted at Sanglah General Hospital, Denpasar, Bali, after obtaining the ethical clearance of research 1582/UN.14.2/Litbang/2015 from Research Unit of Medical Faculty of Udayana University and Sanglah General Hospital. All subjects of this research were willing to follow the research by signing the agreement after getting the explanation (informed consent).

Inclusion criteria were DM patients with DFU Wagner grade 3 and 4, aged 40-60 years, and $TcPO_2$ > 40 mmHg. TcPO2 measurement was performed at the proximal of the ulcerated foot. Exclusion criteria were DM patients with DFU Wagner 1, 2, and 5, patients with other organ abnormalities such as heart failure, pulmonary infections, pulmonary emphysema, pneumothorax, chronic obstructive pulmonary disease, liver disease or hepatitis, stroke, kidney failure, and sepsis or multiple organ failure.

Before the debridement, serum sampling was performed to get a baseline value of VEGF and TNF-α biomarkers. Another laboratory test was taken, such as random blood sugar and serum albumin. Blood centrifugation was done at the velocity of 3000 rpm for 10 minutes. Serum was inserted in the safe lock microcentrifuge tube and stored in a freezer at -20°C in the laboratory. Biomarker examination used quantitative sandwich enzyme immunoassay. VEGF test used the catalogue number PDVE00, and TNF-α used the catalogue number DTA00C. After debridement, the wounds were treated with normal saline, sterile gauze, and elastic bandage as standard management.

The total sample was 32 patients, divided into two groups (HBOT and control), with 16 patients, respectively. In HBOT group, patients breathed 100% oxygen at 2.4 ATA in a multiplace hyperbaric chamber for 90 minutes each session per day, and five days in a week until 20th session (four weeks). At the end of therapy after four weeks from the surgical debridement, second serum sampling was performed again to check VEGF and TNF-α biomarkers in the same way as serum sampling at the beginning of the study. Epithelialization was also performed at the end of therapy. We measured the average of epithelial growth of the entire edges of the wound, in a circle, every 1 cm, from healthy skin to the edge of the ulcer.

Data analysis was conducted by the IBM SPSS statistics version 23.0 for Windows (IBM Corporation). Descriptive analysis to described patient characteristics in both groups. We evaluated the normality of numerical data with the Shapiro-Wilk test. If the data were normally distributed, parametric test with paired T-test for pre and post-test data, and evaluation of value between groups with independent T-test. If numerical data were not normally distributed, data transformation with base-e logs was also known as natural logs (Ln), and it tested by the same test. If after transformation, the data was not normally distributed, then used the non-parametric test, such

as a paired T-test replaced with Wilcoxon test and independent T-test with Mann-Whitney U test. The further analysis used path analysis with Stata 12.0 (StataCorp). Significant test was p-value < 0.05.

Results

The subjects collected in this study were 32 DM patients with DFUs Wagner 3-4 (Table 1), with average age 52 years old, duration of DM 6 years, and body mass index (BMI) around 23 kg/m^2 (normal limit). In the control group, patients had foot ulcers longer, slightly higher $TcPO_2$, higher random blood sugar, and lower albumin serum than HBOT group. Both groups were comparable with p-value > 0.05.

Table 1: Characteristics of patients before treatment

	HBOT (n = 16)	Control (n = 16)	p value
Age (years)[a]	52.56 ± 5.81	52.75 ± 5.17	0.924[b]
DFUs duration (weeks)[a]	5.75 ± 4.19	7.53 ± 12.98	0.779[c]
DM duration (years)[a]	6.33 ± 6.81	6.75 ± 6.16	0.319[c]
BMI (kg/m^2)	23.43 ± 3.86	23.99 ± 4.23	0.687[c]
$TcPO_2$ (mmHg)[a]	58.11 ± 4.87	59.18 ± 12.94	0.759[b]
Random blood sugar (mg/dl)[a]	238.00 ± 106.55	266.50 ± 122.40	0.488[b]
Albumin serum (mg/dl)[a]	3.08 ± 0.75	2.92 ± 0.55	0.692[d]
Sex (%)			
Male	8 (50)	5 (31.25)	0.280[e]
Female	8 (50)	11 (68.75)	

[a]Mean ± standard deviation; [b]ndependent T-test; [c]Ln Independent T-test; [d]Mann-Whitney U test; [e]Chi-square test.

Table 2 showed average VEGF levels at baseline was similar 151 pg/ml in both groups, but after four weeks therapy there was significant escalation of VEGF in HBOT group than control group (277.42 ± 171.75 pg/ml, 95% CI 185.90-368.94 pg/ml vs 169.21 ± 78.92 pg/ml, 95% CI 127.16-211.26 pg/ml, respectively, p = 0.013). We showed pre and post-test VEGF levels in HBOT was different significant p < 0.001 with escalation value of 125.75 ± 116.76 pg/ml, but in control group was a slight escalation of 17.94 ± 81.26 pg/ml, p = 0.439. The escalation value (effect size) between HBOT and control groups was a significant difference, p = 0.005.

Table 2: VEGF levels between groups

VEGF (pg/ml)	HBOT	Control	p-value
Baseline	151.67 ± 96.46	151.27 ± 51.98	0.501[b]
After therapy	277.42 ± 171.75	169.21 ± 78.92	0.013[b]
p value	< 0.001[a]	0.439[a]	

All value in mean ± standard deviation; [a]Ln Dependent T test; [b]Ln Independent T test.

We analysed TNF-α levels between groups in Table 3. It showed similar value at baseline (p = 0.91), but TNF-α levels at four weeks therapy was significantly different between groups (p = 0.01), in HBOT group was 28.51 ± 4.25 pg/ml, 95%CI 26.24-30.77 pg/ml, and in control group was 35.33 ± 13.82 pg/ml, 95%CI 27.96-42.69 pg/ml. TNF-α levels in HBOT group was reduction 4.43 ± 5.03 pg/ml, but in control group was escalation 1.94 ± 10.72 pg/ml (p = 0.02). There was a significant reduction of TNF-α levels in the HBOT group (p = 0.005).

Table 3: TNF-α levels between groups

TNF-α (pg/ml)	HBOT	Control	p-value
Baseline	32.93 ± 4.43	33.38 ± 5.29	0.91[b]
After therapy	28.51 ± 4.25	35.33 ± 13.82	0.01[b]
p value	0.005[a]	0.814[a]	

All value in mean ± standard deviation; [a]Wilcoxon test; [b]Mann-Whitney U test.

Besides the biomolecular analysis, we measured the epithelialization of ulcers at the end of therapy. There was faster epithelialization in HBOT group than the control group (3.81 ± 1.38 mm, 95%CL 3.07-4.54 mm vs 1.27 ± 0.61 mm, 95%CI 0.95-1.60 mm, respectively, p < 0.001). We also performed path analysis to evaluate the relationship between HBOT, VEGF, and TNF-α to epithelialization (Figure 1). The relationship that occurs as a direct and indirect effect on epithelialization. Directly, the HBOT had a significant effect on the epithelialization (p < 0.001). But indirectly, HBOT affected epithelialization through the escalation of VEGF levels and reduction of TNF-α levels. However, the most important role of epithelialization was VEGF levels (p = 0.042).

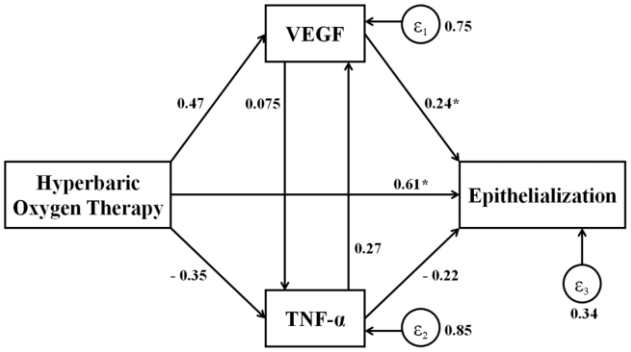

*Figure 1: Diagram of path analysis of HBOT to VEGF, TNF-α, and epithelialisation. Note: *p <0.05. - HBOT did not significantly escalate VEGF by 47.35%, p = 1.00; - HBOT did not significantly reduce TNF-α by 35.46%, p = 1.00; - HBOT significantly escalated epithelialization by 61.02%, p <0.001; - VEGF did not significantly escalate TNF-α by 7.52%, p = 1.00; - VEGF significantly escalated epithelialization by 24.13%, p = 0.042; - TNF-α did not significantly escalate VEGF by 26.96%, p = 1.00; - TNF-α did not significantly reduce epithelialization by 22.14%, p = 0.054*

Discussion

Hyperbaric oxygen therapy is tailored made for each disease. The frequency of HBOT varies from three to five sessions for acute cases [13], [20]. Ten to twenty HBOT sessions for DFU [2], [6], [9], [10], even fifty to sixty times for chronic cases and slow healing wound [13], [20]. The 5-10 HBOT sessions or less than 20 sessions on DFUs gives no clinical results maximum. The 30 HBOT sessions or more 20 sessions gives the same result with 20 HBOT sessions. Therefore, we use 20 sessions of 100%

oxygen and 2.4 ATA in a multiplaced hyperbaric room, one session for 90 minutes intermittent (every 30 minutes, patients resting 5 minutes), one session per day, and five sessions per week [2], [9], [10], [13], [14]. For this study indicated for type 2 DM with DFU Wagner 3-4.

In this study VEGF levels can be seen in Table 2, that the control group VEGF levels increased, but not statistically significant ($p = 0.439$), while the HBOT group experienced a very significant increase ($p < 0.001$). The increase in VEGF in the HBOT group was higher than the control group with significant difference ($p = 0.005$).

In the Asano et al., Study [21] using mice, ligated left femoral artery and given HBOT found VEGF mRNA increased, at 24 hours and 72 hours, but returned to normal after day 14. Increased VEGF mRNA is due to ischemia process. In contrast to bFGF (basic fibroblast growth factor) and HGF (hepatocyte growth factor) which at 24 and 72 hours also increased, but greatly improved on day 14 after administration of HBOT. This indicates that VEGF mRNA is elevated because it is induced by ischemia alone. Yuan et al., Study [22] obtaining VEGF did not increase in the administration of HBOT. The early increasing of VEGF, occurs due to lactate and increased NO (nitric oxide) and not due to hyperoxia in HBOT.

In the Al Waili et al., Study [23] found that VEGF levels increased significantly after HBOT administration, while PGE2 (prostaglandin E2) and Cox-2 (cyclooxygenase-2) mRNA values decreased. It was concluded that cytokines, prostaglandins, and NO probably induced by increased VEGF levels. VEGF plays an important role in liver regeneration, and the effects of VEGF are mediated through two tyrosine kinase receptors [24]. Different oxygen pressures will lead to activation of signalling pathway differences that stimulate VEGF expression to angiogenesis. Hyperoxia causes ROS (reactive oxygen species) production that affects HIF-1 (hypoxia-inducible factor-1) and causes VEGF expression [25].

The increase of VEGF levels in the HBOT group was greater and significantly higher than the control group; this represents that HBOT may activate angiogenesis through increasing VEGF levels. According to Thom [19], HBOT can increase the reactive oxygen compound, will synthesise more growth factors through increased SDF-1 (stromal-derived factor-1) ingredients, angiopoietin, FGF, TGF-β1, and VEGF through HIF-1. From these components, it is the improvement of neovascularisation. The ROS or RNS (reactive nitrogen species) compound affects the cell, in which the PKC (protein kinase C) is activated and various gene expression occurs such as endothelin-1, VEGF, TGF-β, PAI-1 (plasminogen activator inhibitor-1), NF-κB, NAD(P)H oxidase, and decreased eNOS (endothelial nitric oxide synthase) [26].

Wound healing factors in DFUs depend on growth factor, angiogenic response, macrophage function, collagen accumulation, the barrier function of the epidermal cell, keratocyte granulation quality, fibroblast migration, epidermal nerve proliferation, bone healing, ECM (extracellular matrix) accumulation, and remodelling of MMPs (matrix metalloproteinases) [27].

The most important growth factor in the angiogenesis process is VEGF. VEGF of 17-23 kDa can stimulate proliferation and endothelial cell migration. VEGF-A is believed to be responsible for fatty tissue angiogenesis [28]. VEGF-B (21 kDa) of 43% is identical to VEGF-A 165. It also stimulates angiogenesis and has implications of ECM degradation through plasminogen activation regulation [29]. VEGF-C (23 kDa) showed 35% homologous with VEGF-A 165, which play an important role in angiogenesis and lymphangiogenesis [30], [31]. VEGF-D (22 kDa) of 48% identical to VEGF-C also promotes the growth of lymphatic channels [32].

The TNF-α levels changes in our study, shown in Table 3. There was no significant increase in TNF-α levels ($p = 0.814$). In the HBOT group, there was a significant decrease in TNF-α levels ($p = 0.005$) after treatment. TNF-α levels after four weeks were significantly different between the control group and the HBOT group ($p = 0.01$). The difference of TNF-α levels between the initial treatment and after four weeks was significantly different ($p = 0.02$).

This condition was described by Thom [19], that hyperbaric oxygen therapy increases oxygen levels in the cell, resulting in the formation of reactive oxygen compounds or reactive nitrogen compounds (ROS or RNS). These reactive compounds will increase at hyperoxia state. Reactive oxygen compounds will suppress monocyte cells and reduce the synthesis of chemokines. The small number of monocytes and low level of chemokines will decrease the amount of cytokine production such as TNF-α. Thus, HBOT decreases the overall inflammatory response. In vivo, TNF-α is a major regulator of inflammatory immune responses, both locally and systemically. There are homologous genes from TNF, such as TNF-α and lymphotoxin (TNF-ß). These genes are present on the short arm of chromosome 6 [33]. A systemic inflammatory response will decrease the synthesis of various cytokines, including a decrease in TNF-α levels. Besides, the decrease of TNF-α may be induced by the HIF-1 effect mechanism [19]. TNF-α is a molecule formed by activated mononuclear phagocytes, including endothelial cells and fibroblast cells. In hyperglycaemia, increased ROS level may induce the release of TNF-α, IL-1, and IL-6 through the NF-κB pathway [34]. While on the administration of HBOT, this condition will not happen [19].

Excessive formation of ROS or RNS will be followed by the formation of an oxidant as a

scavenger, that will counter the overproduction of ROS. There are two kinds of antioxidants, such as enzymatic (superoxide dismutase, catalase, thioredoxin-glutathione dependent peroxidase, and reductase) and non-enzymatic (vitamin C, vitamin E, thioredoxin, glutathione, uric acid, β carotene, and carotene). These antioxidants are adequate to fight against oxidants. The decrease of TNF-α in the HBOT adjuvant therapy, depending on duration, frequency is given HBOT, and HBOT dose per session. Administration of HBOT 3 ATA for one-hour at massive bleeding decreased TNF-α compared to control. One-hour 100% oxygen delivery with 2.8 ATA also inhibits elevated TNF-α in ischemia-reperfusion injury of intestinal [35], [36]. Clinical judgement of DFUs was done by observation and measurement of epithelial width growth, from the margin of healthy skin into the wound, measured after four weeks of treatment. The control group, the epithelial growth of DFUs, was 1.27 ± 0.61 mm. The HBOT group, the epithelial growth of the patient, was 3.81 ± 1.38 mm. There was a significant difference in both groups' epithelial growth. Epithelial growth in the HBOT group was much better than the control group ($p < 0.001$).

In acute lesions, the epithelial growth of normal skin cells is 0.1 mm per day or 0.75 mm per week and 3 cm during the proliferative phase [37], whereas in chronic wounds, the epithelial growth slows down. In a chronic condition, the network healing process fails to achieve functional and anatomy integrity as in normal condition. Various factors that cause chronic wounds are still not fully understood, but one of the most important factors is the occurrence of oxygen deficiency resulting in prolonged tissue hypoxia. The ECM deposit also becomes less due to the production of fibroblasts and collagen remodelling highly dependent on the adequacy of tissue oxygen. In the development of chronic wounds, the healing process can be stopped at every phase, especially in the inflammatory or proliferative stage. Inhibition of the proliferative phase causes the build-up of neutrophil production in tissues that would otherwise destroy growth factors and degrade ECM components. This causes the tissue to become fragile [38]. In diabetes, the persistent inflammatory phase leads to prolonged maturation time of the granulation tissue and the parallel reduction of tensile strength [39]. In acute healing, there will be a gradual wound healing process based on the hemostasis phase, the inflammatory phase, the proliferation phase, and the remodelling phase. While in chronic wounds, the inflammatory phase persistently occurs [40].

Based on the description above, we can assemble the role of each molecule or compound in the cell or body to physiologically wound healing process and progression [41]. HBOT role as adjuvant therapy may activate the system to accelerate the DFUs healing process. According to Mendes et al., [41] several molecules and compounds take a big role in DFUs healing process, including genetic changes. It is an endless orchestra of harmony; there are many things that have not been revealed and fully understood. HBOT offers a variety of beneficial effects. In this study that HBOT can be used for the treatment of DFUs because HBOT causes a change in pathobiology, such as increased VEGF in the plasma will stimulate angiogenesis and neovascularisation. A decrease of TNF-α as a sign of reduced inflammatory reactions and improvement of immunity in plasma, followed by increased granulation as fibroblast work undergoes proliferation and migration. The clinical improvement of epithelial acceleration in adjuvant HBOT proved that HBOT improves ulcer healing through various growth factors, cell proliferation and migration, increased vascular permeability through vasoconstriction mechanisms, and improves vascular endothelial function.

The role of HBOT to VEGF, TNF-α and epithelial growth can be seen in the path analysis (Figure 1). By path analysis, the relationship was HBOT directly affecting epithelialization significantly ($p < 0.001$). HBOT indirectly affects epithelialization via VEGF and TNF-α. However, the most important role in the indirect relationship between HBOT and epithelialisation was VEGF ($p = 0.042$). According to Mendes et al., [41] in the wound healing process, many things are involved and influential. Molecules and compounds take their roles, genetic change of chain, interrelated, and dependent. But Yuan et al., Study [22] obtaining VEGF did not increase in the administration of HBOT.

In conclusion, HBOT administration leads to increased VEGF, decreased TNF-α, and accelerated wound healing of DFUs patients. Clinical efficacy in humans reported by previous investigators can be demonstrated through this biomolecular study. The biomolecular findings reinforce the theoretical basis, helping to explain human clinical findings, and adding biomolecular research to animals thus adding to the validity of previous studies. HBOT directly affects epithelialization, but also induces indirectly through VEGF enhancement mechanism and decreases TNF-α. However, the most important role in indirect epithelialization process is VEGF.

Data Availability

The data of this study is available by request.

Author contributions

I Nyoman Semadi contributed to study design,

conduct research, surgical debridement, data and statistical analysis, interpretation of findings, and drafting of the manuscript.

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Clinical Science

Live Birth Rates in Poor Responders' Group after Previous Treatment with Autologous Platelet-Rich Plasma and Low Dose Ovarian Stimulation Compared with Poor Responders Used Only Low Dose Ovarian Stimulation Before in Vitro Fertilization

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Keywords: Poor ovarian reserves; Platelet-rich plasma; Ovarian rejuvenation; IVF; Live birth rates

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Abstract

BACKGROUND: This prospective pilot study determined the efficacy of previous transvaginal intraovarian injection with autologous platelet-rich plasma (PRP) in poor ovarian responders (PORs) fulfilling the Bologna criteria before in vitro fertilisation (IVF)/intracytoplasmic sperm injection (ICSI) with low dose ovarian stimulation. Current knowledge of efficient treatment for PORs is limited and often contradictory; also, LBRs of IVF remains disappointingly low.

AIM: We assessed the live birth rates (LBRs) in PORs after previous ovarian treatment with PRP.

METHODS: Overall, 40 patients undergoing IVF/ICSI between June 2017 ending December 2018 were included. A transvaginal intraovarian injection of PRP was performed on 20 patients. Both compered groups were balanced for all basic characteristics, and multivariate analysis was performed to adjust for all known confounders.

RESULTS: Between the groups, a statistical significance in clinical pregnancies and LBR was not found. Clinical pregnancy and live birth rates were 33.33 ± 44.99 and 40.00 ± 50.71 in the PRP group and 10.71 ± 28.95 and 14.29 ± 36.31 in control group retrospectively. However, there is a trend towards higher implantation rates and LBRs in patients with previous treatment with PRP. Anyhow, the number of patients used in the research is insufficient to make a concrete conclusion, and more studies are needed in the future to confirm these results entirely.

CONCLUSION: Even though the treatment of POR responders remains as a therapeutical challenge, the usage of intraovarian injection of autologous PRP in PORs before the IVF performance brings a glimpse of new hope in increasing the success of IVF defined by clinical pregnancy and LBRs.

Introduction

The term poor ovarian responders (PORs) determines a subgroup of in-vitro patients who showcase a decreased response to classical ovary stimulation with gonadotropins, usually as a result of decreased ovary reserves. The IVF procedure results in a decreased number of received oocytes, a decreased clinical pregnancy and LBR [1]. The latest meta-analysis performed by the American Society for Reproductive Medicine (ASRM) in regards to the efficiency of different protocols of ovary stimulation demonstrates an increased cost-benefit with the usage of low dose stimulation using GnRH antagonists [2]. When taking into consideration the need for donating egg cells and different ethical and religious questions that impose themselves, it's clear that there's a need for an additional alternative option that will solve these issues. Therefore, the use of platelet-rich plasma (PRP) is considered as a justified and potentially successful opportunity with which the fertility outcome in PORs may be increased [3], [4].

PRP, as a method in many medical fields, has already demonstrated its beneficial effect on tissue regeneration, angiogenesis activation, inflammation control and anabolism [5]. Unfortunately, there are still insufficient clinical data in the field of ovarian infertility. PRP has been used for the first time as a medical

term in 2007 as: "a preparation consisting of concentrated platelets in a limited plasma volume. It is used in various regeneration procedures of surgical tissues, where growth factors from platelets can affect the speeding up of healing and regeneration of the wounds" [6]. Platelets are cytoplasmic fragments of megakaryocytes, which are formed from the bone marrow and are approximately 2 μm in diameter [7]. Activating the alfa granules from the platelets is one of the most crucial steps which affects the availability of released bioactive molecules, and Ergo, the quality of the PRP. Namely, they contain more than 800 proteins, such as cytokines, hormones, and chemo-attractants of stem cells, macrophages, and neutrophils; these molecules have a fundamental role in the hemostasis and the tissue regeneration [8].

The method of receiving PRP is simple, minimally invasive and at a low cost. The high concentration of factors of growth and cytokines present in the PRP affects the balance between the anabolic and katabolic process, optimising the tissue's surroundings and favouring the process of tissue regeneration [9].

Material and Methods

Patient selection

In this pilot study, 40 patients were included. A written consent form was received by all patients. Also, all patients were completely informed regarding the case study and the way of stimulation during our research. Patients were divided into two groups, group A and B. The group A consisted of 20 patients, who received a transvaginal intraovarian injection of autologous platelet-rich plasma, before the commencement of an IVF. The choice of this treatment was made after a clear and thorough discussion with the patient/couple. In both groups, there wasn't a statistically significant difference in regards to age, BMI, hormonal status, number of antral follicles, previous IVF attempts and duration of infertility. Both compered groups were balanced for all basic characteristics, and multivariate analysis was performed to adjust for all known confounders. The timeframe between the application of PRP until ovary stimulation for IVF was 61 ± 18 days.

The study was approved by the local Ethics Committee, and the Institutional Review Board and each patient included in the study signed an informed written consent. The study included PORs who meet at least two of the following three Bologna criteria, published by the European Society of Human Reproduction and Embryology (ESHRE) in 2011 [10]. Only women whose partners' have normal semen analyses were reviewed [11]. It is important to note that only patients where the IVF process was completed with an embryo transfer were included in the study. The exclusion criteria were ovarian insufficiency due to gonadal dysgenesis and chromosomal abnormalities, immunoglobulin A deficiency, large surgical repairs of pelvic floor leading to the creation of severe pelvic adhesions, the use of anticoagulants, psychotropic medicaments, psychiatric disorders, carcinomas or a history of chronic pelvic pain [12]. Women with present infection, haemoglobin lower than 11 g/L or platelets lower than 150 x 10^3/μL were excluded from the study. Patients who participated in the study were aged from 35 to 42.

Sample preparation

According to the classification proposed by Ehrenfest, 4 different types of PRP are defined, depending on the content of cells and the presence of fibrin [13]. In regards to the Classification of PRP in this case study, it is used as a commercial type of PRP with the lower concentration (2.5 x 3 times) system, Regen PRP, (Regen Laboratory, Mont-sur-Lausanne, Switzerland) [14]. The process was carried out under strict aseptic conditions as well as optimum temperature regulations, i.e., 21-24°C. PRP was prepared according to the manufacturer's guidelines. In the last step, the volume immediately above the erythrocyte layer was collected. Calcium gluconate was used as an activator. After activation, in a period less than 2 min, approximately 3-5 ml of the PRP was injected into the ovaries under transvaginal ultrasound guidance. The intervention was made under propofol intravenous anaesthesia following a protocol set by our IVF department. The whole intervention lasted about 15 to 20 minutes. We used a 30 cm single lumen 17G aspiration needles (COOK/Australia)

The changes in hormones FSH, estradiol, AMH was closely monitored, both before and after the application of PRP. We observed changes in the number of antral follicles before and after the application of PRP. In both groups, we used the same protocol for ovary stimulation, a low dose stimulation using GnRH antagonists.

Stimulation protocols and IVF procedure

The ovarian stimulation was performed according to the recommendations of the Practice Committee of the American Society for Reproductive Medicine [2]. A mild ovarian-stimulation protocol was performed in all patients. The protocol used 100-mg/day clomiphene citrate on days 2-6 of the cycle, adding the low-dose human menopausal gonadotropin (≤ 150 IU/d) and an antagonist (Cetrotide 0.25) when a lead follicle was ≥ 14 mm. During the controlled ovarian stimulation, we followed a protocol set by our IVF department. We evaluated several parameters such as estradiol (E_2), number and size of follicles, endometrium thickness and

gonadotropins administration with ultrasonography and laboratory tests. When one or more leading follicles reached a mean diameter ≥ 18 mm and the estradiol level was ≥ 200 pg/ml, human chorionic gonadotropin (hCG, 5 000 IU; Pregnyl, N.V. Organon, Os, The Netherlands) was administered for triggering the maturation of oocytes. Administration of FSH continued up until the application of HCG. Transvaginal ultrasound-guided oocyte retrieval was performed under short intravenous anaesthesia (Propofol-Lipuro 1%, Braun Melsungen AG, Melsungen, Germany). In both subgroups, transvaginal ovary punctuation followed after 35 to 36 hours of the application of HCG. The ICSI technique was used for all research patients. Embryo-transfers were applied 3 to 5 days later, depending on the condition and the work schedule of the IVF department. The same was performed under ultrasound control. Semen analysis was performed 30 min following liquefaction followed by semen sample preparation by density gradient centrifugation using 90% and 50% PureCeption (SAGE, Trumbull, CT, USA). Embryos were classified according to the scoring system of Hardarson and colleagues [15]. All transfers were performed under abdominal ultrasonography guidance using an embryo catheter (K-SOFT 5000, Cook Medical, Eight Mile Plains, Brisbane, Australia). Intravaginal administered progesterone (Crinone 8%, Merck Serono, Darmstadt, Germany) was used as the luteal phase support. The pregnancy test was considered positive when positive serum hCG levels (> 30 IU/ml) was detected 14 days after embryo transfer (ET). Clinical pregnancy was considered established when at least one visible sac with heart beating was detected by transvaginal ultrasonography.

Statistical analysis

Data analysis is performed in a Statistic program 7.1 for Windows and SPSS Statistics 23.0. For normal distributed data, mean and standard deviation were used. Comparisons across means were evaluated by paired two-tailed Students t-test. The factors with a *P*-value of < 0.1 in the univariate analysis were included in the logistic model. A *P*-value of < 0.05 was considered statistically significant.

Results

Mean patient's age was in the group A 37.47 ± 3.87 years, BMI was 22.63 ± 3.81 kg/m²; infertility duration was 4.0 ± 2.1 year. The mean partner age was 42.40 ± 5.34 years. All patients had multiple previous IVF attempts. Most of the patients had previous diagnostic hysteroscopy and laparoscopy. Differences in serum concentration of FSH, estradiol, AMH and total AFC in both groups were demonstrated in Table 1. Both compered groups were balanced for all basic characteristics, and multivariate analysis was performed to adjust for all known confounders. Between the two groups, there wasn't a statistical significance found in regards to the age, BMI, the basal value of FSH and AMH, length of infertility, previous attempts of IVF. The mean value of platelet concentration was 226.27 ± 82.80/10⁹L. Further analysis was performed to identify factors that might correlate the platelet count in the PRP with the values of FSH, AMH, estradiol and total AFC post-PRP. None of the tests presented statistical significance.

Table 1: Baseline characteristics of patients in both groups

Characteristics	Group A with previous treatment With autologous platelet-rich plasma	Group B	P
Age	37.47 ± 3.87	37.64 ± 3.20	P = 0.99
Body mass index kg/m²	22.63 ± 3.81	24.07 ± 5.01	P = 0.42
Infertility duration	4.0 ± 2.1	4.5 ± 1.2	P = 0.37
Partners age	42.40 ± 5.34	41.26 ± 4.38	P = 0.83
FSH	19.27 ± 2.29	19.22 ± 4.05	P = 0.97
Estradiol on day 3	71.06 ± 31.30	72.54 ± 28.64	P = 0.85
AMH	0.35 ± 0.19	0.72 ± 0.42	P = 0.03

E2, estradiol; FSH, follicle-stimulating hormone; AMH, anti-Müllerian hormone; antral follicle count; p Values pre- vs post-PRP calculated by Students t-test.

Ovarian stimulation parameters such a mean total gonadotropin dose consumed during IVF, mean serum estradiol before HCG trigger, the number of M II oocytes obtained in patients and duration of stimulation are demonstrated in Table 2.

Table 2: Compered ovarian stimulation parameters between groups

Characteristics	Group A with previous treatment with autologous platelet-rich plasma	Group B	P
Number of ampules 75IE	38 ± 13.9	42 ± 12.91	P = 0.08
Estradiol on day of HCG pmol/L	444.53 ± 331.87	528 ± 315.99	P = 0.57
Number of oocytes	1.87 ± 1.13	3.71 ± 2.40	P = 0.20
Duration of stimulation	10 (9.8-10.2)	10.2 (10-10.4)	P = 0.92

*Data presented as Breakdown & one-way ANOVA.

ICSI was used in all cases. All results were made based on IVF/. ET cycle. For all patient's fresh embryo transfers were performed. None of the patients experienced any complications from controlled ovarian stimulation or oocyte retrieval.

Our results of IVF outcome group A such a fertilisation rate 80.67 ± 25.42, implantation rate 33.33 ± 44.99, clinical pregnancy rate 33.33 ± 44.99 and live birth rate 40.00 ± 50.71 and in group B 65.60 ± 25.35, 10.71 ± 28.95, 10.71 ± 28.95, 14.29 ± 36.31 retrospectively were demonstrated in Table 3.

Table 3: Compared parameters of IVF treatment outcomes between groups

Characteristics	Group A with previous treatment with autologous platelet-rich plasma	Group B	P
Fertilization rate	80.67 ± 25.42	65.60 ± 25.35	P = 0.44
Implantation rate	33.33 ± 44.99	10.71 ± 28.95	P = 0.70
Clinical pregnancy rate	33.33 ± 44.99	10.71 ± 28.95	P = 0.69
Live birth rate	40.00 ± 50.71	14.29 ± 36.31	P = 0.71

*Data presented as Breakdown & one-way ANOVA.

Discussion

In this study, we can track a tendency of increasing the rate of clinical pregnancy and the live birth rates in Bologna poor responders, after the usage of the PRP method before the commencement of ovary stimulation during the IVF process.

Currently, there isn't a published randomised controlled study, which researched the effect of intraovarian injection of autologous PRP in poor responders. Available studies by the pioneer of PRP, professor Pantos and Sills [3], [4], showcasing the efficiency of using PRP on a larger indication scale.

The way PRP affects the patient is still not completely evaluated. With the use of platelet-derived growth factors (PDGFs), dysfunctional ovarian tissue is believed to be supplied with essential factors necessary for ovarian regeneration. In this context, it is necessary to mention angiogenesis and follicular vascularisation and their significant role in the ageing of the follicles. Receptors for growth factors are present on granulose cells confirming their association with the activation process of the primordial follicles [16]. Confirmation of all the above statements is also obtained from the Hosseini study [17]. On the other hand, the presence of ovarian stem cells on the surface of the ovarian tissue, under certain conditions, can produce de novo primordial follicles and thus the appearance of new antral follicles [18]. For this reason, it is considered that it can also be a result of the possibility of stimulation of germinative cells from the ovary cortex [19].

Besides, it is also not completely clear the exact reason to which we attribute the trend to increasing LBRs compared to the group b where a PRP was not performed before performing an IVF. Is it an improvement on the number of egg cells and/or their quality?

Anyhow, it is generally accepted that these patients need to be presented with all possible alternative treatments for a successful IVF with their genetic material. With a special emphasis on patients who fulfil the Bologna Criteria for POR and for patients who do not wish to enter a program for donating egg cells.

In conclusion, even though the treatment of POR responders remains as a therapeutical challenge, the usage of intraovarian injection of autologous PRP in PORs before the IVF performance brings a glimpse of new hope in increasing the success of IVF defined by clinical pregnancy and LBRs.

The conclusion in this study has to be reviewed extremely carefully because of the small number of patient participants in this pilot research study. For us to make a conclusion that would have significant statistical value, we would need a larger number of studies with a larger number of patients involved.

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Clinical Science

Risk Factors Analysis for Catheter-Associated Urinary Tract Infection in Medan, Indonesia

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Keywords: Urinary Tract Infection; Catheter; Risk Factor

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Abstract

BACKGROUND: Catheter-associated urinary tract infection (CAUTI) is one of the most common infections in health care caused by several risk factors.

AIM: This study aims at analysing the risky factors triggering CAUTI.

METHODS: This research was designed by applying prospective study. It was conducted from July to November 2018 by involving 82 patients attached to the catheter and treated in the General Hospital of Medan as the sample. The study instrument used observational sheets by measuring the occurrence of urinary tract infection using urine culture analysis ≥ 105 CFU/ml.

RESULTS: The results showed that there was a relationship ($p < 0.05$) amongs age ($p = 0.01$; RR = 0.51), diabetes mellitus ($p = 0.00$; RR = 7.61), duration of catheterization ($p = 0.00$; RR = 0.01), indications for catheter use ($p = 0.00$; RR = 0.34) with CAUTI, and there were not significant relationship ($p > 0.05$) amongs genre ($p = 0.06$; RR = 1.72), drainage system ($p = 0.43$; RR = 0.43) and catheter care ($p = 0.08$; RR = 0.50) with CAUTI. Diabetes mellitus ($p = 0.00$; OR = 8.92 95% CI = 1.02-11.83) and duration of catheterization ($p = 0, 00$; OR = 32.84 95% CI = 3.81-322.74) were the most significant factor related to CAUTI.

CONCLUSION: CAUTI is influenced by various factors, and it can be controlled by understanding those factors so that the right interventions to prevent the infections can be taken and the quality of nursing care can be increased as well.

Introduction

Urinary tract infection is the most common infectious disease in healthcare services worldwide. Urinary tract infection is mostly caused by catheter placement. About 40% of infections in healthcare are urinary tract infection in which 80% of it is triggered by catheter placement [1]. Approximately 12%-16% of the adult patients used indwelling catheters during a stay in the hospital, and 3%-7% of patients had catheter-associated urinary tract infection [2]. Urinary tract infection is the most common infection, which 560.000 per year and 387.550 were catheter-associated urinary tract infection [3]. The number of urinary tract infection estimated 222 million people worldwide. The number of urinary tract infection estimated 90-100 cases of 100.000 people or 180.000 new cases for one year in Indonesia [4].

Infection often occurs after placement of urine catheter, and infection increases 5% urine bacteria for catheter placement every day [5]. The number of infections 3%-5% and 3%-10% every day for indwelling catheter placement in short-term and long-term [6]. Urinary tract infection increases morbidity, mortality, length of stay and charge in the hospital. Catheter-associated urinary tract infection spends maintenance cost as much as 876 dollars for diagnosis and 1.764 dollars for treating patients in the intensive care unit [7]. A patient suffering from urinary tract infection got the harmful impact the presence of bacteria in the urine [8], [9], [10]. The impact can cause harm to patients. The nurse has a big role in care patients with catheter placement [11].

The nurses are very important for preventing the infection at patient by identifying potential risk factor that can affect urinary tract infection through a methodology approach to nursing care, namely

assessment, diagnoses, planning, implementation and evaluation [12]. Data about risk factor analysis catheter-associated urinary tract infection was very required judgment to do appropriate nursing care as a measurement tool for assessing the success of nursing care provided. To prevent the occurrence of urinary tract infections, it is necessary to know the risk factor catheter-associated urinary tract infection as basic to care for the patient. This study aims at analysing the risk factor catheter-associated urinary tract infection.

Methods

This research was designed by applying prospective study. It was conducted from July to November 2018 by involving 82 patients attached to the catheter and treated in the General Hospital of Medan as the sample. The sampling technique was a nonprobability sampling, namely; convenience sampling. The inclusion criteria of sample were: 1) patient placed in a catheter placement at General Hospital Medan showing signs and symptoms such as fever ≥ 38°C, suprapubic and costovertebral pain > 48 hours; 2) the culture result ≥ 10^5 CFU/ml or more than two bacteria identified; and 3) length of stay patient was a least two days. The exclusion criteria of the sample were: 1) patients were diagnosed with an infectious disease; 2) patient placed in a catheter placement showing signs and symptoms such as fever ≥ 38°C, suprapubic and costovertebral pain < 48 hours; 3) patients were treated in paediatric room; and 4) patient didn't want to be respondent. The study has been approved by the Ethics Commission of Nursing Faculty Universitas Sumatera Utara number 1503/VI/SP/2018 and the researcher also asked for respondent's agreement with informed consent.

Measurement to determine catheter-associated urinary tract infection used Center Disease Control (CDC) criteria such as temperature ≥ 38ºC, suprapubic and costovertebral pain > 48 hours, the culture result ≥ 10^5 CFU/ml or more than two bacteria identified. The study steps have been performed by researchers, namely: 1) the researcher filled the respondents' demographic data, such as age, genre, diabetes mellitus, an indication of catheter placement in the observation sheet. The researcher observed the duration of catheter placement, drainage system, catheter care, and signs and symptoms of catheter-associated urinary tract infection, such as temperature ≥ 38°C, suprapubic and costovertebral pain, the culture result ≥ 10^5 CFU/ml or more than two bacteria identified; 2) The patients were observed about signs and symptoms of catheter-associated urinary tract infection after catheter placement by the researchers; 3) The patients were observed for seven days long-time according to signs and symptoms of catheter-associated urinary tract infection by the researchers, and 4) The urine specimen of patients were collected for urine culture.

The authors made the fifth day of catheterisation was taken as the cut-off point for this study because according the research by Al-Hazmi showed that 7 patients from 46 patient who got catheterised placement have infections at third days and 30 patients from 44 patients at the seventh days. The author estimated the seventh day as the longest of the day — fifth days as the cut-off point in this study and median in this research. CAUTI could occur at after second days of catheter placement. The CAUTI began on the third day.

A urine catheter was collected for culture. Hand hygiene was performed before, and after the specimen's collection, gloves were used during the procedure. The drainage port was cleansed with an antiseptic before and after specimen's collection. The drainage system was clamped under the port drainage. The specimen was collected with a sterile syringe. A urine sample was aspirated 3 ml to 5 ml. The clamp was removed to prevent urine reflux. The urine sample was transferred from the sterile syringe to the specimen cup. The specimen cup was put into cooler-box and transferred to the laboratory.

The urine culture was performed by microbiologists. The urine specimens were inoculated on Blood Agar and Mac Conkey. The plates were incubated at 35°C for 24 hours and counted the total plate (CFU/ml). The identification of bacteria morphology and type of bacteria used conventional microbiology methods. It was found that urinary tract infection occurred if there was ≥10^5 CFU/ml or more than two bacteria identified.

Statistical analysis test was performed by using SPSS. Chi-square and Fisher's exact test was used for bivariate analysis because the variable had an ordinal scale, and Logistic regression test was used for multivariate analysis. The p-value significance level was set at $p < 0.05$. The multivariate quantified analysis was the most influential variable to catheter-associated urinary tract infection. The significance level to enter the logistic regression models was set at $p < 0.25$. The logistic regression models were performed for six times. The Two most influential variables were obtained finally.

Results

The study population: Characteristics of Patients

During the study period, there was 82 patient who attracted by urine catheter. Which, 36 patients were suffering from catheter-associated urinary tract

infection, and 46 patients did not suffer from catheter-associated urinary tract infection. The characteristic of patients is illustrated in Table 1.

Table 1: Characteristics of Patients in the Study (n = 82)

Characteristic	Absolut Frequency (n)	Relative Frequency (%)
Gender		
Male	44	53.66
Female	38	46.34
Age		
< 60 Years ± Mean	54 ± 45.66	65.85
> 60 Years ± Mean	28 ± 62.10	34.15
Diabetes mellitus (DM)		
Type II DM	42	51.22
No DM	40	48.78
Long History of Diabetes Mellitus		
> 10 Years ± Mean	20 ± 4.30	24.40
< 10 Years ± Mean	22 ± 12.63	26.80
Immobilisation		
Yes	71	86.59
No	11	13.41
Type of Immobilization		
Total	19	23.20
Partial immobilization	52	63.40
Duration of catheterisation		
< 5 days ± Mean	34 ± 3.42	41.46
> 5 days ± Mean	48 ± 6.42	58.53
Indication catheter used		
Surgery	37	45.12
Urology noninfection	14	17.07
Cardiology	7	8.53
Others (noninfectious disease)	24	29.28
Catheter care		
Yes	20	24.39
No	62	75.61
Catheter Size		
16 fr	50	60.98
18 fr	32	39.02

Based on Table 1. the proportion of males was higher than females 53.66%. The mean age was ± 45.66 to ± 62.10 years. The percentage of patients with diabetes mellitus 51.22% with long history was ± 4.30 to ± 12.63 years. Majority patient has immobilisation 86.59% with type partial immobilisation 63.4%. The mean duration of catheterisation was ± 3.42 to ± 6.42 days. The indication catheter used was surgery at 45.12%. Majority of urine catheter care was not done by nurses 75.61%. The catheter size was 16 as much as 60.98%.

Description of Research Result

Based on Table 2, Data was analysed by Chi-square. There was a significant relationship ($p < 0.05$) amongst age, diabetes mellitus, duration of catheterisation, indication catheter used and catheter size with catheter-associated urinary tract infection. There was not a significant relationship ($p > 0.05$) amongst gender and catheter care catheter-associated urinary tract infection. Diabetes mellitus and duration of catheterisation were the most influencing variable with catheter-associated urinary tract infection by logistic regression. It showed that the significant variable ($p < 0.05$) have a relationship with catheter-associated urinary tract infection, diabetes mellitus ($p = 0.001$) and duration of catheter used ($p =$ 0,001). If the strength relationship according to the value of Odds Ratio (OR), so the most influential variable on catheter-associated urinary tract infection were diabetes mellitus (OR = 8.92; 95%CI, 2.09–37.95) and duration of catheter used (OR = 32.84; 95%CI, 3.71–290.30).

Table 2: Analysis of Relationship between risk factor with Catheter-Associated Urinary Tract Infection (n = 82)

Variable	CAUTI		No CAUTI		Relative Risk	95% CI	*P*-value
	N	%	N	%			
A. Bivariate[a]							
Gender							
Female	12	31.58	26	68.42	0.57	0.33–0.99	0.06
Male	24	54.54	20	45.45			
Age							
< 60 Years	18	33.33	36	66.67	1.92	1.20–3.07	0.01
> 60 Years	18	64.29	10	35.71			
Diabetes mellitus (DM)							
Type II DM	32	76.91	10	23.81	7.61	2.96–19.60	0.001
No DM	4	10.00	36	90.00			
Immobilisation							
Yes	35	49.30	36	50.70	5.42	0.82–35.66	0.03
No	1	9.09	10	90.00			
Duration of catheterisation							
< 5 days	1	2.94	33	97.05	24.79	3.56–172.27	0.001
> 5 days	35	72.92	13	27.08			
Indication catheter used							
Medical	28	62.22	17	37.78	2.87	1.49–5.53	0.001
Surgery	8	21.62	29	78.38			
Catheter care							
No	3	50.00	31	50.00	2.00	0.90–4.46	0.08
Yes	5	25.00	15	75.00			
Catheter Size							
16 fr	16	32.00	34	68.00	1.95	1.02–3.17	0.01
18 fr	20	62.50	12	37.50			
B. Multivariate[b]							
Diabetes mellitus (DM)	-	-	-	-	8.92	2.09–37.95	0.001
Duration of catheterization	-	-	-	-	32.84	3.71–290.30	0.001

Note: [a]) *Chi-Square;* [b]) *Logistic Regression.*

Based on Table 3, the result of urine culture identified as various organisms caused by catheter-associated urinary tract infection. The majority organism was Escherichia coli 13 (36.11%).

Table 3: Organism (n = 36)

Organism	n	%
Escherichia coli	13	36.11
Enterococcus	1	2.78
Klebsiella pneumonia	1	2.78
Staphylococcus aureus	7	19.44
Staphylococcus epidermis	3	8.33
Pseudomonas aeruginosa	5	5.56
Enterobacter aerogenes	1	2.78
Proteus mirabilis	1	2.78
Acinetobacter baumannii	2	5.56
Enterococcus faecalis	2	5.56

Discussion

The catheter placement was a major common urinary tract infection in healthcare. Our finding shows that approximately 43.90% of patients had catheter-associated tract infection [13]. We used the *Center Disease Control* criteria to set as catheter-associated tract infection [14]. Bacteria type found was Escherichia coli 36.11%. Similarly, the study [8], [9], [15] that Escherichia coli was the most commons catheter-associated urinary tract infection. Type 1 Fimbriae was Escherichia coli gen causing infection [16]. These bacteria would locate and require the right antibiotic therapy. The risk of inappropriate antibiotic therapy could cause Multidrug Resistance (MDR) to the microorganism in the urinary tract [17].

We found that the genre has not a relationship with catheter-associated tract infection. Male and Female have the same opportunity to suffer from infection, but the numbers of a male suffering from infection were more than female in proportion. The study was contradictive with the theories found

that females had more risk to suffer from infection because females have a short urethra [18]. However, the study similarity with) [19] catheter-associated urinary tract infection. The increasing of catheter-associated urinary tract infection at male caused the difference of hormone and microorganism in urine [20]. The same opportunities to have catheter-associated urinary tract infection were caused by personal hygiene. The poor personal hygiene in the male and female, especially the genital area had risk suffer from disease-related infection [21].

Not surprisingly, we found that more than 60-year-old have more risk to suffer from catheter-associated urinary tract infection. It is consistent with theories of immunosenescence [22] and the previous study [21] with cohort study that high rates catheter-associated urinary tract infection in elderly patients with average age 64.6 years.

Diabetic Mellitus was as an independent factor for catheter-associated urinary tract infection and has been shown in another study [23]. We found that diabetes mellitus patient had 8.92 times risk have catheter-associated urinary tract infection. The increasing would continue with length to suffering from diabetes mellitus. The Diabetic Mellitus patient has a risk suffer from catheter-associated urinary tract infection because of autonomy neuropathy [24]. This problem can cause incomplete bladder emptying and cause microorganism colonisation. Furthermore, the diabetic Mellitus patient has pancreatic betta cells damage or do not produce enough insulin and cause hyperglycaemia. If there is a hyperglycaemia condition, the kidneys cannot reabsorb glucose. The glucose levels will be high in the urine. The glucosuria influences leukocyte function and performs as a growth medium of pathogenic microorganisms. The poor control and decreasing immune system become a risk factor of diabetic mellitus patients to suffering from catheter-associated urinary tract infection [24], [25].

A patient who has catheter placement continuously and bed rest can complicate to infection. We found the immobilisation has a relationship with catheter-associated tract infection. Immobilisation could cause urine flow to become static. Urine was flowing from renal pelvis to the bladder through ureter because of gravity in an upright position. When the patient was in the supine position, the peristaltic of the ureter is unable to produce gravity [26]. Urine would reflux from bladder to kidney [27].

Duration of catheterisation was the most influential independent factor with catheter-associated urinary tract infection. It has been shown in another study [21], [28], [29]. The odds of the duration of catheterisation 32.85 higher for a patient who inserted a catheter for five days or more. The length the catheter insertion, the more susceptible to infection [30]. Patients who insert the indwelling catheter have a risk to growth bacteriuria [31]. The catheter urine will form a biofilm. Bacteria can enter after catheter insertion or after three days [32]. Biofilm development occurs when cells (planktonic) contact with the surface of the catheter with the thin film [33].

Indication of catheter placement was indicated by medical and similar with previous studies [10], [28] that indication of catheter placement was indicated by medical, such as orthopaedic, urology and urine incontinence. The appropriate indication can decrease catheter-associated urinary tract infection. The inappropriate indication could cause 1.86-time to decrease catheter-associated urinary tract infection [34].

We found that catheter care did not have a relation with catheter-associated urinary tract infection. Because of all the number of the patient hadn't performed catheter care were more than the patient had performed. This finding is puzzling because it contradicts with theories of catheter urine care can decrease infection [26], [35]. Catheter urine care is a nurse's duty. In this study, the nurse is more likely to do the non-nursing job so it can increase nursing workload. The survey in 2017 conducted by AMN Healthcare in California America that approximately 35% of nurses performed a non-nursing job [36]. The relationship between catheter care and catheter-associated urinary tract infection warrants more research.

Catheter size affects the incidence of urinary tract infections in patients with catheters. Larger urine catheter sizes are used for the management of blockages or sediments. The nurses also believe that a larger catheter size prevents urine leakage. But on the other hand, a larger catheter size can irritate the bladder sphincter and aggravate leakage [37]. A smaller catheter size recommendation is recommended [38]. Small catheters can reduce trauma during insertion [39].

Our study had several limitations. First, patients may have been performed catheter-associated urinary tract infection. This is suspected that the use of diapers in patients. The organism enters the urinary tract from anal or area around the perineum. Second, the error location of urine catheter fixation caused urine catheter touch with perineal area. Third, clinical specimens may have been collected incorrectly and contaminated by the organism. It can be possible to caused bias in this study.

Besides our study have a limitation, our study has an implication too. Our finding is useful as information for health workers especially nurse and for hospital staff decided hospital's policies in infection prevention control by considering risk factor catheter-associated urinary tract infection, namely: maintaining hand hygiene, training infection prevention control for all hospital staff, socializing the catheter-associated

urinary tract infection bundle and removing catheter before seven days.

In conclusion, catheter-associated urinary tract infection can be influenced by various factors. This infection can be controlled by understanding the risk factor infection so that it can determine the right intervention to prevent infection the number of infection and increase the quality of nursing care as well. The specific nursing care is necessary from the nurse for a patient who has inserted a urine catheter with diabetes mellitus and duration of catheterisation for five days or more. Future research is needed to clarify the relationship between drainage system, catheter care, and catheter-associated urinary tract infection.

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Authors Contribution

Hariati conceived and carried out the research and wrote the manuscript. Dewi Elizadiani Suza and Rosina Tarigan reviewed the research process, design and results analysis of research.

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Clinical Science

Comparison of Ondansetron and Granisetron Effects for Prevention of Nausea and Vomiting Following Strabismus Surgery

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Abstract

BACKGROUND: Postoperative nausea and vomiting (PONV) is a common complaint after strabismus surgery that leads to unpleasantness, increased hospitalisation time and increased costs. In severe cases, it can lead to dehydration, electrolyte disturbances, aspiration, pneumonia, and even sutures opening.

AIM: This study was conducted to compare the effects of both ondansetron and granisetron on the reduction of PONV after strabismus surgery.

METHODS: This randomised, and the double-blind clinical study was conducted on patients with ASA I and II undergoing strabismus surgery with age over 3 years old in Shafa Hospital, Kerman University of Medical Sciences during 2017 under general anaesthesia. Patients with inclusion criteria were randomly assigned to one of three groups including Ondansetron (A), Granisetron (B) and control group (C). Matching cases and controls on drugs were fully completed. Furthermore, 100 µg/kg of Ondansetron was intravenously injected, followed by injection of 40 µg/kg Granisetron for another intervention group. All patients underwent the same anaesthetic procedure and intravenous injection of drugs during anaesthesia induction. The severity of nausea and vomiting in recovery, 6 and 18 hours after the operation were verified according to the Verbal Rating Scale (VRS). Our data were analysed by Chi-square, ANOVA and TUKEY tests via SPSS version 18.

RESULTS: There was no significant difference between the three groups in terms of age and sex. The incidence of postoperative nausea in recovery among three groups of A, B and C was determined to be 15, 7.5 and 37.5%, respectively. No significant difference was found between the two groups A and B ($P = 0.68$), although there was a significant difference between these two groups and group C ($P < 0.05$). The incidence of nausea at 6 hours after surgery in groups A, B and C was recorded as 40, 15 and 65% respectively, indicating that the incidence of nausea in group B was significantly lower than the other two groups, while showed a significant difference with group A ($P = 0.039$) and group C ($P < 0.05$). Also, the incident of nausea between groups was not statistically significant 18 hours after surgery ($P < 0.05$). Additionally, no significant difference was found in different groups in terms of vomiting incidence in recovery, 6 and 18 hours after surgery ($P < 0.05$).

CONCLUSION: Our study suggests that Granisetron is more effective in preventing PONV during 6 hours after the surgery in comparison with Ondansetron which makes it a favourable alternative for preventing PONV.

Introduction

Postoperative nausea and vomiting (PONV) are the second most adverse and complicated event following surgery [1], [2]. This usually occurs in the post-anaesthetic phase and ends within 24 hours, which its incidence ranged from 30 to 20% [3]. Risk factors for postoperative nausea and vomiting include females, duration of anaesthesia for more than half an hour, age above 3 years, type of surgery, personal or familial history of postoperative nausea and vomiting, or motion sickness [4], [5].

The type of surgery is not necessarily the most important cause of PONV. The occurrence of PONV following various surgeries is more due to the factors related to the patient and anaesthesia than the self-surgery issue [6], [7]. PONV is an unpleasant experience that patients often find worse than postoperative pain [7]. Prevention of PONV in patients at high risk of this complication improves postoperative satisfaction [8]. Although PONV is self-

limiting, its occurrence following surgery can lead to multiple complications such as aspiration of stomach contents, suture opening, oesophagal rupture, subcutaneous emphysema and pneumothorax [9], [10]. PONV delayed the patient's recovery and prolonged unexpected hospitalisation after an outpatient operation [11]. PONV prolongs the discharge time to 61-47 minutes. The annual impact of PONV on healthcare costs is estimated to be several hundred million dollars in the United States [12]. The reason for the lack of definitive prevention of PONV is the presence of multiple factors associated with this disorder, so the main solution to prevent this complication is still the use of anti-nausea drugs in clinical cases. Ondansetron is a serotonin receptor antagonist that is considered as the most effective anti-vomiting drug for preventing PONV [13], [14]. Granisetron is a serotonin 5-HT_3 receptor antagonist, with a half-life of about two times that of Ondansetron [13], [14].

Strabismus surgery is among the operations with a high prevalence of PONV (75-40%), which causes long recovery, high costs, increased bleeding, increased eye pressure and patient dissatisfaction due to the nature of the strabismus surgery caused by the muscle tightening [15]. Therefore, we compared the effect of two Ondansetron and Granisetron drugs on nausea and vomiting following strabismus surgery.

Material and methods

This randomised, and the double-blind clinical study was conducted on patients undergoing strabismus surgery with age over 3 years old in Shafa Hospital, Kerman University of Medical Sciences during 2017. Sampling was done based on purpose and field information was collected using a questionnaire.

Considering the maximum sample size, the standard deviation in similar studies, alpha coefficient of 5% and power of 80% [16], [17], 120 patients with strabismus surgery were randomly assigned to 3 groups of 40 subjects in the placebo, Ondansetron and Granisetron.

Alpha	=	*0.05*	*(two-sided)*	*Z (1-α/2)*	*1.959964*
power	=	0.8		Z (1-β)	0.841621
m1	=	2		D	1
m2	=	3		s^2	1.69
sd1	=	1.2		sd_1^2	1.44
sd2	=	1.4		sd_2^2	1.96
				n1	26.68619
				n2	26.68619

$$n=\frac{(Z_{(1-\alpha/2)}+Z_{(1-\beta)})^2(sd_1{}^2+sd_2{}^2)}{d^2}$$

Inclusion criteria: 1) Patients undergoing strabismus surgery over the age of 3 years And 2) Class 1 & 2 American Society of Anaesthesiologists' (ASA) classification of Physical Health. Exclusion criteria: 1) History of previous surgery and anaesthesia with postoperative nausea and vomiting; 2) History of Ménière's disease, motion sickness or migraine; 3) Dissatisfaction with surgery; 4) Use of drugs that affect nausea and vomiting within 24 hours of surgery; 5) Pregnancy or breastfeeding.

Patients with inclusion criteria were randomly assigned to one of three groups, including Ondansetron, Granisetron and placebo (normal saline administration). In a randomised clinical trial was blinded, in which neither the patient nor the evaluating person informed the type of drug used. Furthermore, matching cases and controls on drugs were fully completed.

Induction of all three groups was performed with midazolam 1 mg, fentanyl 100-50 micrograms, thiopental 5 mg/kg and atracurium 0.2 mg/kg. Maintenance of anaesthesia was carried out in all three groups with propofol infusion (100 µg/kg/min) and 50% N2O-O2 mixture.

After giving premed to the patient, 100 µg/kg of Ondansetron was intravenously injected, followed by injection of 40 µg/kg Granisetron for another intervention group.

All recovery personnel and section were unaware of the patient groups and the type of drug used. The severity of nausea and vomiting were evaluated by an anesthesiologist at the recovery and after transferring to the section during 6 hours following the operation and then after discharge from the hospital during the next 18 hours. A drug for the treatment of nausea and vomiting was not given to patients during anaesthesia. In case of severe nausea and vomiting, a dose of dimenhydrinate (mg) was administered to the patient. Additionally, patients who suffered from nausea and vomiting in recovery, received metoclopramide 0.1 mg/kg.

Because the two drugs used are both anti-nausea and -vomiting, and only their effect and potency are different, no additional drugs have been given to patients in recovery. Recording the age, height, weight and duration of anaesthesia, the severity of nausea and vomiting in recovery were done by anaesthesia technician. After transferring the patient to the section, the patients were evaluated by the anaesthesia resident within 6 hours after the operation and then after discharge from the hospital within the next 18 hours. The severity of nausea is measured according to the Verbal Rating Scale (VRS). By this method, an individual without nausea is assigned zero, in case of less than two nausea episode, mild nausea was considered, as followed twice or more (moderate nausea). The incidence of vomiting is considered to be the same as severe nausea.

Ethical considerations included: 1. Approval of

the plan by the Research Council of the School of Medicine; 2. Informed consent was obtained from the patient to participate in the study, of mention, obtained by the legal parents for the children; and 3. Both anti-nausea drugs are used routinely without any complications if any unwanted side effects were observed, each treatment was followed by a withdrawal from the study, but no complication was observed.

Data analysis

Information for each group was coded as 1, 2 and 3, and the recorded in the checklist for each drug or placebo, followed by statistical analysis through the SPSS version 18 software. Data analysis Information for each of the 3 groups, coded 1, 2 and 3, was recorded in the checklist for each drug or placebo, and then entered the SPSS version 18 software. Using the Kolmogorov-Smirnov test, the data were analysed for normal distribution. Depending on the distribution of data and quantitative or qualitative values, quantitative data such as age were analysed using one-way ANOVA test regarding their normalisation, while Non-parametric Kruskal-Wallis H test was used for data with skewed distribution. If parametric or parametric tests showed significant values, Dunn's multiple comparisons test and Tukey tests were employed to compare the groups. Chi-square test was applied to evaluate the qualitative variables. Regarding the lack of time assessment, repeated tests such as repeated measures ANOVA were not used. In all tests, a significant level of 0.05 was considered.

Results

Our study was conducted on 120 patients who referred to the operating room of the hospital for strabismus surgery and randomly divided into three groups: A (Ondansetron), B (Granisetron) and C (Placebo). Study population consisted of 76 male patients (63.3%) and 44 (36.7%) female. Among them, 26 patients received A, 21 drug B and 29 C, respectively. Among the female patients, 14 received A, 19 B and 11 C, respectively. Forty people were considered for each group A, B and C, without regarding gender.

The results of the chi-square test showed no significant relationship between nausea rate in all three groups and sex (P = 0.175). The mean age and standard deviation in each of groups A, B, C were determined as 43.68 ± 19.3, 22.78 ± 16.1 and 97.74 ± 13.1.

The mean age of patients in Ondansetron, Granisetron and control groups was 19.43, 16.22 and 13.97, respectively. The mean age of the subjects was 16.49 years. Based on the ANOVA test, no significant difference was found between the groups in terms of age (P = 0.332).

Table 1 shows the magnitude and severity of nausea and vomiting in recovery. Of the 120 participants, 15% had a feeling of nausea in group A, followed by group B (7.5%) and group C (37.5%). The best possible status for lack of nausea episode was in group B with 92.5%, followed by group A (85%) and group C (62.5%). The highest vomiting (3 cases) was recorded in group C, followed by group A (one person) and group B (none), (Table 1).

Table 1: The rate and severity of nausea and vomiting in recovery among three groups (A, B, C)

Variable		An (Endonestrone)		B (Granisetron)		C (control)		Total	
		Frequency	Percent	Frequency	Percent	Frequency	Percent	Frequency	Percent
Recovery	No nausea	34	85	37	92.5	25	62.5	96	80
	Mild nausea	4	10	3	7.5	7	17.5	14	11.7
	Moderate nausea	1	2.5	0	0	5	12.5	6	5
	Severity Nausea (Vomit)	1	2.5	0	0	3	7.5	4	3.3

Table 2 indicates the measurement of nausea episodes and severity in 6 hours after surgery. Of the 120 patients, 60% of the subjects did not feel nauseous, while 23.3% had mild nausea, followed by moderate nausea (7.5%) and severe nausea (vomiting: 9.2%).

The most common nausea episodes were observed in group C (placebo) with 65%, while episodes were 40% in group A and 15% in group B. In other words, the best possible situation was seen in Group B (85% without nausea). Vomiting in groups A, B and C was 7.5, 2.5 and 17.5%, with the lowest vomiting in group B and the highest in group C (Table 2).

Table 2: The rate and severity of nausea and vomiting in the first 6 hours after surgery in the three groups (A, B, C)

Variable		An (Endonestrone)		B (Granisetron)		C (control)		Total	
		Frequency	Percent	Frequency	Percent	Frequency	Percent	Frequency	Percent
6h after	No nausea	24	60	34	85	14	35	72	60
	Mild nausea	10	25	4	10	14	35	28	23.3
	Moderate nausea	3	7.5	1	2.5	5	12.5	9	7.5
	Severity Nausea (Vomit)	3	7.5	1	2.5	7	17.5	11	9.2

The incidence rate and severity of nausea and vomiting in 18 hours after surgery are listed in Table 2. After 18 hours of operation, 96.7% of the patients had no nausea episode One in group A, one in group B and two in group C patients, expressed the feeling, while vomiting was seen only in the group C (Table 3).

Table 3: Incidence and severity of nausea and vomiting 18 hours after operation in three groups (A, B, C)

Variable		An (Endonestrone)		B (Granisetron)		C (control)		Total	
		Frequency	Percent	Frequency	Percent	Frequency	Percent	Frequency	Percent
18h after	No nausea	39	97.5	39	97.5	38	95	116	96.7
	Mild nausea	1	2.5	1	2.5	1	2.5	3	2.5
	Moderate nausea	0	0	0	0	0	0	0	0
	Severity Nausea (Vomit)	0	0	0	0	1	2.5	1	0.8

The incidence of postoperative nausea in recovery in the three groups of Ondansetron, Granisetron and control was determined to be 15, 7.5 and 37.5%, respectively (Table 4). The result of the test demonstrated a significant general relationship between the groups. Based on the results of Tukey test, we found that despite the low nausea rate in the Granisetron group compared to the Ondansetron group, no significant difference was found between the two groups in terms of nausea (P = 0.658), although there was a significant difference between the two groups and the control group (P < 0.05) (Table 4).

Table 4: Comparison of the three groups in terms of postoperative nausea in recovery

Variable	An (Endonestrone)		B (Granisetron)		C (control)		Total		Results of the Chi-Square Test
	Frequency	Percent	Frequency	Percent	Frequency	Percent	Frequency	Percent	
Recovery Nausea	6	15	3	7.5	15	37.5	24	20	0.002

Regarding the incidence of vomiting in recovery, although it was relatively reduced in the Granisetron group in comparison with Ondansetron and placebo, but no statistically significant difference was observed between the groups (P = 0.164) (Table 5).

Table 5: Comparison of the three groups in terms of postoperative vomiting in recovery

Variable	An (Endonestrone)		B (Granisetron)		C (control)		Total		Results of the Chi-Square Test
	Frequency	Percent	Frequency	Percent	Frequency	Percent	Frequency	Percent	
Recovery Vomiting	1	2.5	0	0	3	7.5	4	3.3	0.164

The incidence of nausea in the 6 hours after strabismus surgery in the Ondansetron, Granisetron and control groups was 40, 15 and 65% respectively, indicating that the incidence of nausea was significantly lower in the Granisetron group when compared with the other two groups.

Table 6: Comparison of the three groups in terms of postoperative nausea in 6 hours after surgery

Variable	An (Endonestrone)		B (Granisetron)		C (control)		Total		Results of the Chi-Square Test
	Frequency	Percent	Frequency	Percent	Frequency	Percent	Frequency	Percent	
6H after Nausea	16	40	6	15	26	65	48	40	0.0001

Although the incidence of vomiting demonstrated a decreasing trend in the granitic group at 6 hours after the operation as compared to placebo and indomethacin, there was no statistically significant difference between the groups (P = 0.061; Table 6 and 7).

Table 7: Comparison of the three groups in terms of vomiting in 6 hours after surgery

Variable	An (Endonestrone)		B (Granisetron)		C (control)		Total		Results of the Chi-Square Test
	Frequency	Percent	Frequency	Percent	Frequency	Percent	Frequency	Percent	
6h after Vomiting	3	7.5	1	2.5	7	17.5	11	9.2	0.061

The incidence of nausea was not significantly different between the groups 18 hours after surgery (P = 0.772).

Table 8: Comparison of the three groups in terms of nausea 18 hours after surgery

Variable	An (Endonestrone)		B (Granisetron)		C (control)		Total		Results of the Chi-Square Test
	Frequency	Percent	Frequency	Percent	Frequency	Percent	Frequency	Percent	
18h after Nausea	1	2.5	1	2.5	2	5	4	3.3	0.772

Also, there was no significant difference between the groups in terms of vomiting incidence 18 hours after the operation (P = 0.336; P < 0.05; Table 8 and 9).

Table 9: Comparison of the three groups in terms of vomiting 18 hours after surgery

Variable	An (Endonestrone)		B (Granisetron)		C (control)		Total		Results of the Chi-Square Test
	Frequency	Percent	Frequency	Percent	Frequency	Percent	Frequency	Percent	
18 h after Vomiting	0	0	0	0	1	0.52	1	0.8	0.365

Discussion

Today, the increasing number of surgeries has increased the desire for early release of patients from recovery. Decreasing the incidence of PONV has accelerated the discharge of patients, reduces the length of hospitalisation or re-admission, and leads to patient satisfaction [18], [19]. In this regard, we aimed to compare the effect of both Ondansetron, and Granisetron on nausea and vomiting following strabismus surgery.

The findings of the statistical tests showed that there was no significant difference between the level of nausea in all three groups and gender (p = 0.175). Furthermore, the mean age of patients in the three groups revealed no significant difference between the groups in terms of age (P = 0.332). The best possible status for lack of nausea was observed in group B (92.5%), followed by group A (85%) and group C (62.5%). Despite the low nausea rate in the Granisetron group in comparison with Ondansetron, there was no significant difference between them (P = 0.658);

The highest vomiting (3 cases) was recorded in group C, followed by group A (one person) and group B (none), (Table 1). However, there was a significant difference between the two intervention groups and the control group (P < 0.05).

The highest vomiting (3 cases) was recorded in group C, followed by group A (one person) and group B (none). Although recovery vomiting was relatively reduced in the Granisetron group as compared to Ondansetron and placebo, but no

statistically significant difference was found between the groups (P = 0.164).

Based on the results presented herein, 60% of the subjects did not feel nauseous, while 23.3% had mild nausea, followed by moderate nausea (7.5%) and severe nausea (vomiting: 9.2%).

The most common nausea episode was observed in group C (placebo) with 65%, while the episode was 40% in group A and 15% in group B. The best possible situation was seen in Group B (85% without nausea); Statistically, the incidence of nausea in the Granisetron group was significantly less than the other two groups, and this difference was significant in comparison with the Ondansetron group (P = 0.039) and the control group (P < 0.05).

In addition, vomiting in groups A, B and C was determined as 7.5, 2.5 and 17.5%, where the lowest vomiting was observed in group B and the highest in group C. Although, relative reduction of vomiting was observed in the Granisetron group as compared to placebo and Ondansetron, but no statistically significant difference was found between the groups (P = 0.061).

The incidence rate and severity of nausea and vomiting in 18 hours after surgery are listed in Table 2. After 18 hours of operation, 96.7% of the patients had no nausea episode. One in group A, one in group B and two in group C expressed episodes while vomiting was seen only in the group C.

The incidence of nausea was not statistically significant among the groups 18 hours after surgery (P = 0.772). Moreover, there was no significant difference between the groups in terms of vomiting18 hours after surgery (P = 0.365), (P < 0.05).

Very limited studies have been conducted on the effects of both Granisetron and Ondansetron on reducing the incidence of PONV in strabismus surgery, although more studies are available for other surgeries such as laparoscopy. Most studies have shown that Granisetron is more effective than Ondansetron which is consistent with our study, although some studies have also shown contradictory results.

A study assessed the effect of Granisetron, Ondansetron, Midazolam combination with Dexamethasone for reduction of PONV after strabismus surgery among 100 children ASA class I and II, where findings did not show significant differences between different groups [20].

Another study evaluated antiemetic effects of Ondansetron and Granisetron in preventing postoperative nausea and vomiting in subjects undergoing daycare laparoscopic tubal ligation where minimal emetic episodes in patients receiving intravenous granisetron when comparing with those receiving ondansetron and placebo. The study mentioned above indicated that emetic episodes were attributed to 7% of patients receiving intravenous granisetron, followed by 20% in the ondansetron group and 50% in placebo group C [21].

In a randomized double-blind investigation, 100 female patients were assessed for effect of Ondansetron and Granisetron in preventing PONV (intervals of 6-0, 12-6, 18-12 and 24-18 after surgery) in subjects undergoing elective laparoscopic cholecystectomy, where a significant was found in the incidence of PONV among two groups after 6 hours to 24 hours [16].

Gauchan et al. evaluated the antiemetic efficacy of Ondansetron and Granisetron in patients undergoing laparoscopic cholecystectomy during the first 24 hours after anaesthesia. They indicated that Granisetron was effectively capable of increasing incidence of PONV as compared to Ondansetron in the first 24 hours [17].

In another study by Savant et al., the effects of ondansetron (4 mg, 2 mL) and granisetron (2 mg, 2 mL) was assessed in preventing PONV in subjects undergoing oral and maxillofacial surgery.

Except for the headache side effect, it has been shown that the incidence of nausea and vomiting in the Granisetron group was significantly lower than that of Ondansetron [22].

In conclusion, our study suggests that Granisetron is more effective in preventing PONV for 6 hours after the surgery in comparison with Ondansetron which makes it a favourable alternative.

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Prevalence and Risk Factors for Hyponatremia in Preterm Infants

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Abstract

BACKGROUND: Hyponatremia is the result of a negative sodium balance caused by inadequate salt intake or excessive salt loss due to immature renal or intestinal function in preterm infants.

AIM: The aim of our study was to define the incidence of and factors affecting its development in preterm newborns.

METHODS: This was a retrospective cohort analysis of 126 preterm infants born before 36 weeks of gestation between June 2016 and July 2018 at Neonatal Intensive Care Unit of Hue Central Hospital, Vietnam. Hyponatremia was defined as a sodium level ≤ 132 mEq/L or 133-135 mEq/L with oral sodium supplementation. We used the serum sodium level to define hyponatremia.

RESULTS: There were 37 infants who had hyponatremia, accounting for 29.4% of the infants enrolled in the study. A lower gestational age, the presence of respiratory distress syndrome, the use of furosemide, and feeding with breast milk were significant risk factors for hyponatremia in preterm newborns.

CONCLUSION: Hyponatremia occurred at a relatively high frequency. This result exemplifies the importance of serum sodium monitoring and supplementation for the correction of hyponatremia.

Introduction

Sodium, an element essential to growth, is contained in bone, cartilage and connective tissue and is indispensable for the development and operation of the central nervous system. Mild hyponatraemia is common in preterm babies and is not known to cause significant adverse effects [1]. On the contrary, extreme hyponatraemia is rarely seen and increases the risk of neurodisability [2], [3]. Many factors predispose preterm babies to hyponatraemia: impaired reabsorption of sodium at both proximal and distal tubules [4], inadequate salt provision, *e.g.*with donor breast milk [5], immaturity of endocrine mechanisms of water and sodium homeostasis [6]. Hypoxia, medications and respiratory distress can also aggravate hyponatremia associated with kidney tubular damage. However, few studies have addressed the incidence of and risk factors for hyponatremia in preterm newborns is achieved.

In this study, we aimed to define the incidence of and factors affecting its development in preterm newborns.

Material and Methods

Our retrospective cohort was composed of 126 preterm infants born before 37 weeks of gestation and admitted to the Neonatal Intensive Care Unit of Hue Central Hospital, Vietnam between June 2016 and July 2018. The data were collected via a retrospective chart review. The collected data included the infants' perinatal histories, clinical characteristics (including hyponatremia). This study was approved by the Hue Central Hospital institutional review board. Informed consent was waived by the board.

Hyponatremia was defined as a sodium level ≤ 132 mEq/L or between 133 and 135 mEq/L with oral sodium supplementation [7]. According to our nutritional policy, total parenteral nutrition was started from birth and also protein and lipid supplementation was started at day 1. Sodium supplementation was started after diuresis begins according to the capillary or serum sodium level. If there is a hyponatremia, we routinely checked sodium level every 1-3 days to assess whether hyponatremia is relieved. The duration of hyponatremia was defined as the length of

time when the sodium level was ≤ 132 mEq/L. We used the serum sodium level to define hyponatremia.

All the numerical data are expressed as the means±standard deviation. The *t*-test or the Mann-Whitney test was used for continuous variables. The chi-square test or Fisher's exact test was used for the analysis of categorical variables.

To evaluate the risk factors for hyponatremia, we compared the perinatal factors of the hyponatremia and non-hyponatremia groups during the study period. A multiple logistic regression using a stepwise selection was employed, and we included significant variables (those with *P* values below 0.05) in a univariate analysis. The statistical analysis was conducted using SPSS Statistics version 20.

Table 1: Univariate analyses of perinatal and neonatal factors between hyponatremia group and non-hyponatremia groups

Parameters	Non-hyponatremia group (n = 89)	Hyponatremia group (n = 37)	*P* value
GA at birth (weeks), mean ± SD	32.1 ± 2.7	29.3 ± 3.4	< 0.01
Birth weight (g), mean ± SD	1,550.2 ± 439.3	1001.2 ± 343.6	< 0.01
M:F ratio	1: 1.12	1: 1.08	0.65
5 min AS < 7, No. (%)	28 (31.4)	18 (48.6)	0.03
SGA, No. (%)	24 (26.7)	10 (27.0)	0.97
Oligohydramnious, No. (%)	11 (12.4)	6 (16.2)	0.58
Maternal hypertensive disorders, No. (%)	21 (23.6)	6 (16.2)	0.29
GDM, No. (%)	13 (14.6)	4 (10.8)	0.47
PROM > 18 hr, No. (%)	26 (29.2)	19 (51.3)	< 0.01
Prenatal antibiotics, No. (%)	32 (35.9)	21 (56.7)	< 0.01
Prenatal steroid, No. (%)	69 (77.5)	27 (73.0)	0.47
RDS, No. (%)	11 (12.3)	18 (48.6)	< 0.01
PDA, No. (%)	34 (38.2)	29 (78.4)	< 0.01
Sepsis, No. (%)	8 (9.0)	9 (24.3)	< 0.05
IVH ≥ Gr3, No. (%)	5 (5.6)	5 (13.5)	0.11
Antibiotics use, No. (%)	59 (66.3)	34 (91.9)	< 0.01
BM feeding, No. (%)	29 (32.6)	24 (64.9)	< 0.01
Metabolic acidosis, No. (%)	11 (12.4)	3 (8.1)	0.53
Furosemide use, No. (%)	11 (12.4)	19 (51.4)	< 0.01

GA, gestational age; SD, standard deviation; M: F, male: female; AS, apgar score; SGA, small for gestational age; GDM, gestational diabetes mellitus; PROM, premature rupture of membrane; RDS, respiratory distress syndrome; PDA, persistent ductus arteriosus; IVH, intraventricular hemorrhage; Gr, grade by Volpe; BM, breast milk.

Results

Patient characteristics

A total of 126 infants born at 24 through 36 weeks of gestation were included in this retrospective cohort study. More than one-half of the entire cohort was male (59.5%), and 81% of the births were a singleton.

There were 37 infants who had hyponatremia, accounting for 29.4% of the infants enrolled in the study. The mean gestational age of the hyponatremia group was 29.1 ± 3.2 weeks (minimum-maximum; 23-33 weeks), and their mean birth weight was 973.7 ± 315.9 g (820-2,250 g). The mean onset of hyponatremia was 3.4 ± 1.5 days after birth (1-12 days), and the mean duration of hyponatremia was 3.23 ± 2.18 days (1-6 days). Of the 37 infants with hyponatremia, 13 (35.1%) experienced a hyponatremia duration of at least 4 days. There were no infants with serious neurologic complications associated with hyponatremia.

Risk factors for hyponatremia

The hyponatremia group had a lower gestational age and a lower birth weight than did the non-hyponatremia group ($P < 0.01$). Premature rupture of the membranes occurring more than 18 hr before the delivery, the use of prenatal antibiotics, respiratory distress syndrome, patent ductus arteriosus requiring medical, postnatal culture-proven sepsis, and the use of postnatal antibiotics or furosemide within two weeks after birth occurred significantly more frequently in the hyponatremia group ($P < 0.05$). Feeding with breast milk was more common in the hyponatremia group ($P < 0.05$) (Table 1).

According to the multiple logistic regression analysis, a shorter gestation, a shorter duration of parenteral nutrition, the presence of respiratory distress syndrome, the use of furosemide, and feeding with breast milk were independently associated with the development of hyponatremia (Table 2).

Table 2: Multivariate logistic regression analysis of risk factors of hyponatremia

Variables	Odds ratio	*P* value	95% CI for estimate (OR)	
			Lower	Upper
GA (per week)	0.514	0.000	0.318	0.892
RDS	2.925	0.032	1.431	5.410
Furosemide use	3.081	0.009	2.043	5.804
BM feeding	2.416	0.044	1.522	4.394

GA, gestational age; RDS, respiratory distress syndrome; BM, breast milk.

Discussion

During intrauterine life, the foetus lives in a warm, watery environment where it receives a constant supply of water and electrolytes from the mother. On the contrary, following birth, the neonate must adapt to a relatively cold, dry environment with much wider fluctuations than those experienced in the uterus [8], [9]. Variations in the hydro-electrolytic balance in the preterm neonate of very low weight are extreme due to the immaturity of the different organs. The immature kidney in particular leads to high toxicity caused by fluids: in excess when the supply of water is abundant and scanty when the supply of water is insufficient [10]. An abundant supply of fluids is the cause of generalized oedemas and insufficient lung activity brought about by the increase in interstitial liquid directly or indirectly through maintenance of a patent Botallo duct [11]. On the contrary, the insufficient administration of fluids may lead to

hypovolemia, hyperosmolarity, metabolic anomalies and kidney insufficiency [12]. Since the concentration of sodium in the serum is the main indicator of the water balance in the preterm infant, this group of patients presents a particular frequency of hyponatremia and hypernatremia [13]. Recently, serious sequels associated with hyponatremia and variations in sodium concentration in preterm infants have been reported [13], [14]. Hyponatremia is most frequently caused by an excessive supply of water rather than by a reduced sodium intake, while hypernatremia is most frequently secondary to a reduced supply of water or increased losses of water rather than by a excessive supply of sodium [15]. The questions and controversies in neonatology concern the supply of liquids in the first days of life and the amount of the sodium supplement.

In our study, 29.4% of preterm infants born before 36 weeks of gestation were affected by hyponatremia. Despite the relatively small sample size and limitation of a retrospective study, this is, to our knowledge, the first description of the incidence of hyponatremia in Vietnam. The significant risk factors for hyponatremia were a lower gestational age at birth, the presence of respiratory distress syndrome, furosemide use, and feeding with breast milk. Preterm neonates are at high risk for the development of hyponatremia because of (1) lower glomerular filtration rate, (2) reduced proximal tubular reabsorption of sodium, and (3) increased arginine vasopressin levels in response to illness [16], [17].

Some of the suggested pathophysiological causes of hyponatremia in premature babies are inadequate sodium intake and increased natriuresis, which can cause increased vasopressin. Our risk factor analysis supports these pathophysiological factors as causes of hyponatremia. In preterm infants, the immaturity of the proximal renal tubule can cause the decreased reabsorption of sodium. Numerous factors affecting the proximal tubule can aggravate hyponatremia, including hypoxia, respiratory distress, and the administration of drugs with tubular toxicity. Our study showed that the infants' gestational age at birth and birth weight tended to be lower in the hyponatremia group and that respiratory distress syndrome was a significant risk factor for hyponatremia. Another significant risk factor was furosemide, a well-known diuretic that affects kidney tubules, causing massive natriuresis. Regarding inadequate sodium supplementation, feeding with breast milk were significant risk factors.

In the present study, the incidence of hyponatremia was as high as 29.4%. It will, therefore, be of great importance to clarify the clinical consequences of a low serum sodium level in premature babies. Some preliminary data have already indicated that neonatal sodium deficiency may have unfavorable consequences for later cognitive functions [14], [18]. Furthermore, hyponatremia has been documented to be a risk factor for cerebral palsy in extremely premature babies [19].

In preterm infants, increased natriuresis due to renal tubular immaturity can lead to protracted volume contraction, which can stimulate aldosterone and arginine vasopressin (AVP) release, allowing further water retention and the progression of hyponatremia [20], [21], [22]. The elevation of plasma AVP levels in bronchopulmonary dysplasia (BPD) infants both during the fourth week of life and as a chronic condition has also been reported [23], [24]. Although an impaired renal response to AVP in hyponatremic patients prevents the further worsening of hyponatremia [20], elevated AVP levels in BPD patients can cause pulmonary fluid to accumulate, increasing pulmonary edema; therefore, hyponatremia may be a significant risk factor for BPD.

Postnatal growth retardation related to hyponatremia has also been reported [25]. Sodium is a significant growth factor that stimulates cell proliferation and plays a significant role in protein turnover [26]. NaCl deprivation inhibits growth, which is manifested in reductions in body weight, brain weight, body length, muscle and brain protein and RNA content, and brain lipid content (compared with controls). Subsequent NaCl supplementation restores the growth velocity; however, it does not induce catch-up growth [26].

What does this mean for the practicing clinician? Neonatologists should make every effort to keep serum sodium concentrations in the normal range. Hyponatremia in preterm infants is an iatrogenic complication that should be preventable, because newborns start out life with normal serum sodium concentrations. One practice that needs to be reconsidered how parenteral fluids are being prescribed to the preterm neonate. Current recommendations are to prescribe 5% dextrose in water, only adding sodium after weight loss has been achieved [27]. The reason for this recommendation is the concern that sodium supplementation in the immediate postnatal period will lead to extracellular volume expansion with the development of hypernatremia, worsening respiratory distress, necrotizing enterocolitis, and patent ductus arteriosus [28], [29].

In summary, hyponatremia occurred at a relatively high frequency. This result exemplifies the importance of serum sodium monitoring and supplementation for the correction of hyponatremia, especially if the hyponatremia persists for long periods despite a lack of acute symptoms. Nonetheless, as this was a retrospective study, hyponatremia might be only a marker of disease severity rather than an etiologic factor. Thus, further large cohort studies are needed in this area.

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Clinical Science

Effect of Levosimendan Compared to Conventional Inotropic Agents on Hemodynamics and Outcome in Patient with Poor LV Function Undergoing Cardiac Surgery

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Abstract

BACKGROUND: Patients undergoing heart surgery involving cardiopulmonary bypass (CPB) experience global myocardial ischemia with subsequent reperfusion which, despite cardioplegic protection, may result in different degrees of transient ventricular dysfunction. Levosimendan is a "calcium sensitisers", it improves myocardial contractility by sensitising troponin C to calcium without increasing myocardial oxygen consumption and without impairing relaxation and diastolic function.

AIM: To evaluate the adding effect of a calcium sensitiser (levosimendan) compared to the conventional inotropic and vasoactive agent used in the patient with poor left ventricular function undergoing cardiac surgery on different measured hemodynamic variables and the effect on the outcome.

METHODS: It is prospective observational studies were patients were divided into 2 groups of 30 patients each. The first Group received conventional inotropic and vasoactive treatment at different doses, while the other group received levosimendan additionally at a loading dose of 6-12mic/kg according to mean arterial pressure over 0.5 hr followed by 24 hrs infusion at 0.05 to 0.2 mic/kg/min. Hemodynamic data were collected at the end and 30 minutes after CPB, after that at 6, 12, 24, and 36 hours post CPB. Mean arterial pressure (MAP), central venous pressure (CVP), heart rate (HR), mixed venous saturation (Svo2), and base deficit (BD) were measured.

RESULTS: Levosimendan had significantly improved postoperative hemodynamic values as in the mixed venous pressure at different times postoperative ($p < 0.05$), also the base deficit at different times postoperative ($p < 0.05$), while there was a significant reduction in systemic vascular resistance as decreased mean arterial pressure in levosimendan group compared to conventional group at 6hrs postoperative mean 77.50 ± 10.81 vs 83.73 ± 10.81 with ($p = 0.029$), and at 12 hrs postoperative mean 77.37 ± 10.10vs 84.23 ± 13.81 with ($p = 0.032$), and there was no significant difference in heart rate at different times postoperative between both groups ($p > 0.05$), while there was no significant effect on mortality between both groups ($p = 0.781$).

CONCLUSION: Levosimendan had improved hemodynamic parameters significantly with no effect on mortality compared to conventional inotropic agents in a patient with poor left ventricular function undergoing cardiac surgery.

Introduction

Patients undergoing heart surgery involving cardiopulmonary bypass (CPB) experience global myocardial ischemia with subsequent reperfusion which, despite cardioplegic protection, may result in different degrees of transient ventricular dysfunction, also known as myocardial stunning in the immediate postoperative period [1], [2]. If severe enough, this dysfunction can cause postoperative low cardiac output syndrome, a complication with an estimated prevalence of about10% and a mortality of 17% [3].

Pharmacological support, in the form of vasodilator and inotropic therapy, as well as mechanical support, such as intra-aortic balloon counterpulsation and ventricular assist devices, is often necessary to restore adequate tissue perfusion in the immediate postoperative period [2]. Perioperatively, the most frequently used inotropes are beta-adrenergic and phosphodiesterase III inhibitors [4], [5], [6].

Levosimendan is a recently introduced

indicator, it belongs to a novel group of agents called calcium sensitisers, which increase the sensitivity of contractile proteins to calcium. The use of this agent in the treatment of decompensated heart failure is based on its dual mechanism of action. It improves myocardial contractility by sensitising troponin C to calcium without increasing myocardial oxygen consumption [7] and without impairing relaxation and diastolic function [8]. Also, it causes the opening of ATP-dependent potassium channels on smooth muscle fibres, which induces systemic, pulmonary and coronary vasodilatation and may offer cardioprotective effects during myocardial ischaemia [9], [10], [11].

Furthermore, growing evidence from *in vitro* and *in vivo* studies indicates that, unlike other inotropes, levosimendan does not affect or even improves diastolic function in failing myocardium [12], [13], [14], [15].

We aimed to evaluate the adding effect of a calcium sensitiser (levosimendan) compared to the conventional inotropic and vasoactive agent used in the patient with poor left ventricular function undergoing cardiac surgery on different measured hemodynamic variables and the effect on the outcome.

Patients and Method

Our study was a prospective observational study, conducted from the period of May 2016 to May 2017, in which patients admitted to the intensive care units following cardiac surgery in different cardiac surgery centres (Cardiothoracic Department, Cairo University and other cardiothoracic centres, Cairo, Egypt).

A total of 60 patients were enrolled in our study and were subsequently divided into two groups 30 patients each, group received conventional inotropic and vasoactive treatment, while the other group received levosimendan additionally at loading dose 6-12 mic/kg according to mean arterial pressure over 0.5 hr followed by 24 hrs infusion at 0.05 to 0.2 mic/kg/min. Hemodynamic data were collected at the end and 30 minutes after CPB, after that at 6, 12, 24 and 36 hours post CPB. Mean arterial pressure (MAP), central venous pressure (CVP), heart rate (HR), mixed venous saturation (Svo_2) and base deficit (BD) were measured.

Inclusion criteria

All patients with poor preoperative left ventricle function ejection fraction < 35% undergoing cardiac surgery for valvular, coronary bypass or aortic aneurysm repair.

Exclusion criteria

Patients with congenital heart disease.

Patients with allergy to levosimendan.

All patients enrolled in our study were subjected to:

- Full history and clinical examination.

- Preoperative logistic EUROSCOREII evaluation.

- Laboratory investigation including:

- Complete blood count.

- Renal and liver function tests.

- Coagulation profile.

- Pre and postoperative echocardiography.

- Chest x-ray.

- ECG.

Statistical analysis

Data were collected, revised, coded and entered to the statistical package for social science (IBM SPSS) version 20. Qualitative data were presented as number and percentages, while quantitative data were presented as mean, standard deviations and ranges. The comparison between two groups with qualitative data was made by using *Chi-square test*, the comparison between two independent groups with quantitative data and parametric distribution was made by using *Independent t-test,* while the Mann Whitney test is done for two independent samples with nonparametric distribution and the comparison between more than two groups with quantitative data and parametric distribution was done by using One Way ANOVA test**.** The p-value was considered significant if < 0.05.

Results

Our study enrolled a total of 60 patients: 46 males (77%), 14 females (23%), who were admitted to ICU following cardiac surgery with preoperative echocardiography revealing impaired cardiac functions EF < 35%.

Demographic data were presented in Table 1. There was no significant statistical difference between both groups (p > 0.05) regarding age, gender, preoperative ejection fraction, while there was a significant statistical higher incidence of BMI, diabetes, hypertension in the levosimendan compared to conventional group (p = 0.001, 0.002, 0.005 respectively).

Table 1: Study group demographic data

Demographic data		Conventional group No. = 30	Levosimendan group No. = 30	P value
Age	Mean ± SD	55.20 ± 8.59	59.67 ± 9.84	0-066
	Range	34 – 72	30 – 74	
Gender	Male	20 (66.7%)	26 (86.7%)	0-067
	Female	10 (33.3%)	4 (13.3%)	
Body mass index	Mean ± SD	30.93 ± 2.56	33.50 ± 3.05	0-001
	Range	24 – 36	29 – 39	
Diabetic	No	19 (63.3%)	7 (23.3%)	0.002
	Yes	11 (36.7%)	23 (76.7%)	
		28 (93.3%)	19 (63.3%)	0-005
Hypertensive	No	2 (6.7%)	11 (36.7%)	
	Yes			
Preoperative ejection Fraction	Mean ± SD	33.20 ± 3.35	31.80 ± 4.44	0.173
	Range	20 – 36	20 – 37	

Heart Rate

There was no statistically significant difference regarding heart rate at any time postoperative between the two groups (p > 0.05) as presented in Table 2.

Table (2): Comparison of postoperative measured heart rate between both groups

Heart rate		Conventional group No. = 30	Levosimendan group No. = 30	P-value
Immediate postoperative	Mean ± SD	102.13 ± 14.00	101.57 ± 20.08	0.900
	Range	77 – 132	65 – 160	
0.5hr postoperative	Mean ± SD	101.90 ± 13.17	101.90 ± 19.71	1.000
	Range	71 – 124	76 – 170	
6hrs postoperative	Mean ± SD	101.73 ± 12.67	99.17 ± 18.94	0.540
	Range	71 – 125	62 – 146	
12hrs postoperative	Mean ± SD	102.13 ± 15.14	101.50 ± 18.99	0.887
	Range	70 – 139	73 – 163	
24hrs postoperative	Mean ± SD	102.90 ± 15.83	106.33 ± 20.72	0.474
	Range	75 – 142	63 – 150	
36hrs postoperative	Mean ± SD	97.33 ± 18.96	107.37 ± 23.67	0.075
	Range	60 – 140	54 – 170	

Mean arterial blood pressure

Regarding mean arterial pressure, there was a statistically significant difference between both groups at 6hrs postoperative wherein the conventional group mean arterial pressure was higher 83.73 ± 10.81mmHg compared to the levosimendan group mean arterial pressure was 77.50 ± 10.81 mmHg (p = 0.029).

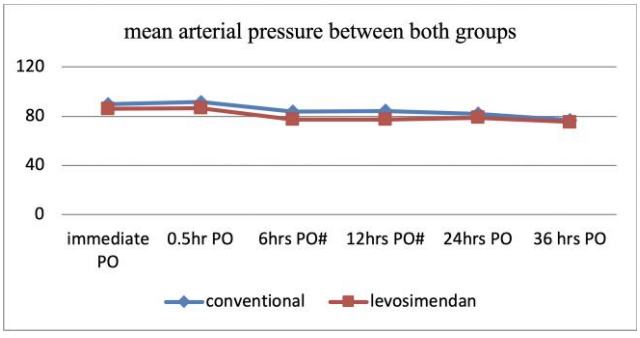

Figure 1: Mean arterial pressure between both groups (PO) postoperative; (#) significant P-value

Also, there was a statistically significant difference between both groups at 12 hrs postoperatively wherein the conventional group mean arterial pressure was higher 84.23 ± 13.81 mmHg compared to the levosimendan group mean arterial pressure was 77.37 ± 10.10 mmHg (p = 0.032). While there was no statistically significant difference at immediate, 0.5 hr, 24hrsand at 36hrs postoperatively (p > 0.05) as shown in Figure 1.

Central venous pressure (CVP)

Regarding central venous pressure, there was statistically significant lower CVP at 6hrs postoperative in the conventional group with median 6.00 (3.00 – 9.00) cm H2O compared to the levosimendan group median was10.00 (5.00 – 12.00) cmH2O (p = 0.004). Also, there was statistically significant lower CVP at 12hrs postoperative in the conventional group with median 7.50 (4.00 – 9.00) cmH2O compared to the levosimendan group median 10.00 (9.00 – 13.00) cmH2O (p = 0.006).

There was no statistically significant difference at immediate, 0.5 hr, 24 hrs and 36 hrs postoperatively (P > 0.05) as shown in Figure 2.

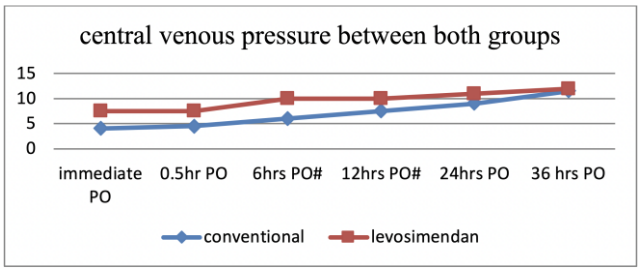

Figure 2: Central venous pressure between both groups (PO); postoperative (#) significant P-value

Base deficit

There was a highly statistically significant higher base deficit in the conventional group compared to the levosimendan group at immediate, 0.5 hr, 6 hrs, 24 hrs and 36 hrs postoperatively (p = 0.001, 0.001, 0.009, 0.021 and 0.001) respectively. While there was no statistically significant difference in the base deficit between both groups at 12 hrs postoperatively (p = 0.104) as shown in Figure 3.

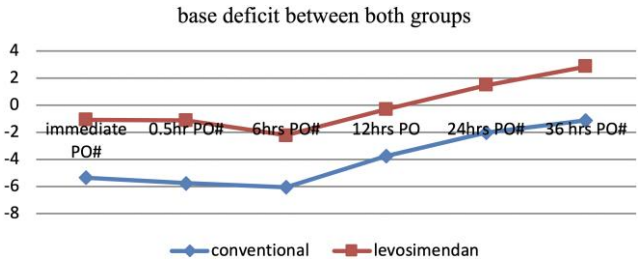

Figure 3: Base deficit between both groups (PO) postoperative; (#) significant P-value

Mixed venous oxygen saturation

There was a highly statistically significant higher mixed venous oxygen saturation in the

levosimendan group compared to the conventional group at immediate, 0.5 hr, 6 hrs, 12 hrs, 24 hrs and 36hrs postoperatively (p = 0.013, 0.015, 0.032, 0.024, 0.005 and 0.011) respectively as shown in **F**igure 4.

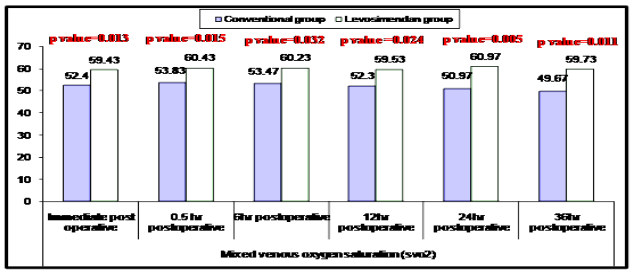

Figure 4: Mixed venous pressure between both groups

Outcome

Postoperative ejection fraction was significantly higher in the levosimendan group than the conventional group (p = 0.002), length of hospital stay was significantly lower in the levosimendan group than in conventional group (p = 0.028). Also, there was a statistically significant higher logistic EUROSCOREII in the levosimendan group than in the conventional group (p = 0.046) as presented in Table 3.

Table 3: Outcomes in the conventional and levosimendan group

Outcomes		Conventional group No. = 30	Levosimendan group No. = 30	P value
Postoperative ejection fraction	Mean ± SD Range	33.73 ± 2.96 25 – 39	36.90 ± 4.53 25 – 44	0.002
Mechanical ventilation duration in hours	Mean ± SD Range	51.43 ± 66.29 6 – 288	43.27 ± 36.48 6 – 120	0.557
Length of ICU stay in days	Mean ± SD Range	4.03 ±2.71 2 – 12	3.27 ± 1.68 2 – 8	0.193
Length of hospital stay in days	Mean ± SD Range	15.43 ± 9.70 5 – 46	10.87 ± 5.41 5 – 29	0.028
Logistic EUROSCOREII	Median (IQR) Range	1.88 (1.20 – 3.57) 0.76 – 21.06	3.19 (1.76 – 6.70) 0.68 – 21.72	0.046

There was no statistically significant difference between both groups regarding mortality with lower mortality in the levosimendan group 9 patients (30%) did not survive compared to conventional group were10 patients (33.3%) did not survive (p = 0.781) as presented in Table 4.

Table 4: Mortality in the two study groups

Mortality	Conventional group No. = 30	Levosimendan group No. = 30	P-value
Survival	20 (66.7%)	21 (70.0%)	0.781
Non survival	10 (33.3%)	9 (30.0%)	

Discussion

In our study using of levosimendan achieved statistically significant improvement in left ventricular function (EF%) postoperatively determined by echocardiography compared to conventional group (36.90 ± 4.53 VS 33.73 ± 2.96), (p = 0.002).

Supporting our results Husedzinovic et al., [16] in a double-blind, randomised trial evaluating the effect of levosimendan as a new strategy during off-pump coronary artery bypass grafting enrolling 24 patients received either placebo or levosimendan at a dose of 12 mic/kg as an infusion for 15 min before CABG. At 10 min and 60 min post-infusion, the cardiac index and the LVEF were significantly higher with levosimendan than with placebo (P = 0.018 each). The stroke volume index was significantly higher for levosimendan at 10 min (P = 0.018), but not at 60 min (P = 0.063).

In concordance to our study, Barisin S et al., [17] in his study levosimendan in off-pump coronary artery bypass: a Four-time masked, controlled study enrolling 31 patients: 10 patients received a high dose,10 patients received a low dose, and 11 patients received a placebo.

Two dose schedules: low dose(12 mic/kg), high dose (24 mic/kg) over 10min; treatment was given 20min before start of surgery result in significant increases in cardiac output and LVEF occurred after high-dose (P = 0.001; P = 0.006) and low-dose levosimendan (P = 0.001; P = 0.002). Both levosimendan doses produced significant increased stroke volume and decreased systemic vascular resistance.

In our study, the heart rate showed no significant statistical difference at all times postoperative between both groups (p > 0.05).

This went side by side with Stefan G. DeHert et al., [18] who conducted a study enrolling 30 patients evaluating the effects of levosimendan in cardiac surgery patients with poor left ventricular function had found that there was no statistically difference in the heart rate at all times postoperatively between both groups (p > 0.05).

Also, Polychronis Malliotakis et al., [19] who conducted a study enrolling12 patients evaluating the hemodynamic effects of levosimendan for low cardiac output after cardiac surgery had determined that there were no significant changes in heart rate postoperatively (p > 0.05).

In contrast, Ravikumar Gandham et al., [20] who conducted a study enrolling 60 patients evaluating a comparison of hemodynamic effects of levosimendan and dobutamine in patients undergoing mitral valve repair/replacement for severe mitral stenosis had found that there was a significant difference in heart rate being higher in the conventional group at mostly all times postoperatively (p < 0.05) this variance may be due to that he was mainly comparing dobutamine with levosimendan.

In our recent study, the mean arterial pressure was statistically significant at 6 and 12 hrs

postoperatively (p = 0.029 and 0.032 respectively) being higher in the conventional group than the levosimendan group.

This was concordance to Julian Alvarez et al., [21] who conducted a study enrolling 50 patient evaluating the hemodynamic effects of levosimendan compared with dobutamine in patients with low cardiac output after cardiac surgery had found that there was a significant mean arterial pressure difference between both groups at 6, 12, 24 and 48 hrs postoperatively (p < 0.05) being higher in the conventional group.

Polychronis Malliotakis et al., [19] showed significance mean arterial pressure difference between both groups at 6 and 24 hrs postoperatively (p < 0.05) being higher in the conventional group.

Ravikumar Gandham et al., [20] had found that significance means arterial pressure difference between both groups at immediate, 6 and 12 hrs postoperatively (p < 0.05) being higher in the conventional group.

Those agreements may be conducted to systemic and pulmonary vasodilator effect of levosimendan, leading to a reduction in blood pressure.

In our study, evaluating central venous pressure at 6 and 12 hrs postoperatively were statistically significant (p = 0.004 and 0.006 respectively) between both groups were lower in the conventional group.

In concordance to our results other studies like Julian Alvarez et al., [21] had found that significant difference in central venous pressure at 6, 12, 24 and 48 hrs postoperatively between both groups with (p < 0.05).

Polychronis Malliotakis et al. determined that there was a significant difference in central venous pressure at 6, 12 and 24 hrs postoperatively from baseline levosimendan infusion (p < 0.05).

Ravikumar Gandham et al., [20] found that there was a significant difference in central venous pressure at immediate, 6 and 12 hrs postoperatively from baseline levosimendan infusion with (p < 0.05).

They all found that there was a significant reduction in central venous pressure in levosimendan group as a result of a reduction in systemic and pulmonary vascular resistance this variation from our study may be due to there was no fixed postoperative IV fluid protocol among our patients.

In the current work, we used base deficit and mixed venous saturation being an indicator for adequate cardiac output and tissue perfusion they were strongly significant at almost all times postoperatively more base deficit in the conventional group than levosimendan group and higher mixed venous saturation in the levosimendan group than conventional group (p < 0.05).

In concordance with other studies Julian Alvarez et al., [21] showed that significant difference in mixed venous oxygen saturation at 6, 12, 24 and 48 hrs postoperatively between both groups with (p < 0.05).

Polychronis Malliotakis et al., [19] determined that there was a significant difference in mixed venous oxygen saturation at 6, 12 and 24 hrs postoperatively from baseline levosimendan infusion (p < 0.05).

Also, E. Al shawaf et al., [22] who conducted a study enrolling 30 cardiac surgery patients comparing levosimendan Vs milrinone in type2 diabetic patient with low LVEF undergoing elective coronary artery surgery found that there were significantly higher cardiac index and mixed venous oxygen saturation with levosimendan (p < 0.05), significantly lower pulmonary capillary wedge pressure, systemic vascular resistance and oxygen extraction ratios.

In our study, there was no statistically significant difference in mortality between the conventional and levosimendan groups (10 patients Vs 9 patients) with (p = 0.781).

Our results went hand by hand with Stefan G. DeHert et al., [18], who showed a non-significant effect on mortality (p = 0.224).

Also, Rajendrah Mehta et al., [23] in the mega trial levo.cts enrolled 880 patients evaluating the effect of levosimendan in patients with left ventricular systolic dysfunction undergoing cardiac surgery on cardiopulmonary bypass had found that levosimendan did not affect the primary outcome (mortality) (p = 0.45). Other studies like Julian Alvarez et al., [21], Polychronis Malliotakis et al., [19], and Ravikumar Gandham et al., [20] they were focusing mainly on the hemodynamic effect of levosimendan.

Limitation: The present study has the following limitations:

1. The small number of patients was a certainly serious drawback.

2. Presence of several confounding factors (e.g. mode and parameters of mechanical ventilation, amount of fluids or blood products infused during the observation period).

3. Lack of invasive cardiac function monitoring (cardiac index, stroke volume index, systemic and pulmonary vascular resistance) that might have affected our results cannot be ruled out.

4. The infusion of the drug beyond 36hours, particularly in the subgroup with low cardiac output syndrome, will not be addressed in the study. As we seek to evaluate the efficacy of levosimendan on the short and intermediate-term outcomes in patients undergoing high-risk cardiac surgery on CPB and will be unable to provide insights into the long-term efficacy of the drug.

In conclusion:

- Significant improvement in postoperative ejection fraction in the group receiving levosimendan ($p = 0.002$) and significant decrease in-hospital stay ($p = 0.028$).

- Significant improvement in hemodynamic parameters base deficit ($p < 0.05$) at almost all times postoperative, mixed venous Saturation ($p < 0.05$) at almost all times postoperative with no effect on heart rate ($p > 0.05$), with a mild reduction in mean arterial pressure due to inodilator effect of levosimendan.

- Levosimendan had no significant effect on mortality compared to the conventional group ($p = 0.781$).

- We recommend an increasing number of the study population, use fixed IV fluid protocol and more invasive cardiac function monitoring.

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Clinical Science

Are Histopathological Changes of *H. pylori* Infection in Young Dyspeptic Patients Necessitate Endoscopy?

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Abstract

BACKGROUND: *Helicobacter pylori* is an important gastrointestinal infective bacteria with many serious complications including gastric erosions and ulceration, duodenal ulcer, gastric carcinoma and MALT gastric lymphoma. The gastric biopsy is commonly performed in *H. pylori*-positive dyspeptic individuals, and many previous researchers studied the histopathological features of infected gastric biopsies however little previous studies focused on the histopathological findings in young population in comparison to the older one.

AIM: To make a focus on the histopathological effects of *H. pylori* infection in young patients compared with the older one and predicts the need for endoscopy in this population, also to estimates the prevalence of infection in Iraqi patients.

MATERIAL AND METHODS: the sample for this study is 180 patients in total, they attended Marjan medical city in Iraq for dyspepsia of more than 3 months and prepared for OGD. Patients asked for their permission to do immunological tests for *H. pylori*. Both serology for H. pylori antibodies and stool for antigen tests are used, and the case is included in the study only if both tests were positive, after OGD, the gastric biopsies are processed and examined histopathologically.

RESULTS: Normal gastric biopsy is the most common histopathological finding in young (< 25 years) patients (75%) while chronic atrophic gastritis is the most common one in patients > 25 years age (57%). The prevalence of *Helicobacter pylori* infection in dyspeptic patients was 73.3%, the correlation between infection and sex was insignificant (p-value 0.06), and no significant correlation between infection and age (p-value 0.07) was concluded.

CONCLUSION: *H. pylori*-related histopathological changes of gastric mucosa in young (< 25 years) are commonly mild and does not necessitate endoscopy at this age unless there are alarming signs.

Introduction

Dyspepsia is the most common gastrointestinal problem all over the world. Its meaning refers to a variety of unpleasant symptoms such as nausea, vomiting, epigastric pain or discomfort, heartburn, feeling of gastric fullness and early satiety [1]. In the recent Asian consensus, most of the members agreed to define dyspepsia as symptoms that related to the gastroduodenal region and that continues for three months or more [2].

Helicobacter pylori infection is one of the important treatable causes of dyspepsia. Its prevalence varies among different countries and regions in the same country, including Asian countries as a result of different social habits, geographical conditions, races and ethnicity [3], [4]. The prevalence of infection with this microorganism is 25-50 per cent in the developed countries and 70-90 percent in the developing countries in 2001 [5]. It had been found that individuals who lived in low social classes are more likely to harbour the organism as the chance for getting contaminated food and water is more. Overcrowding and bad hygiene are important predisposing factors for bacterial transmission [6]. The general prevalence of infection all over the world is about 50% [7]. *H. pylori* is a gram-negative actively motile bacteria. It is urease producing bacteria and its pathogenicity is mainly attributed to its production of urease enzyme, active motility and toxins production

[8]. The histopathological effect of *H*. *pylori* varies from just dyspepsia with normal endoscopic findings to chronic superficial gastritis in which inflammation is limited to the faveola, chronic atrophic gastritis with varying degree of glandular atrophy, peptic ulcer, gastric carcinoma, and even lymphoma. So, it is a risky organism that if it is given suitable attention and treated properly, this will prevent a problematic squally [8], [9]. Many previous studies had been made on the prevalence of *H*. *pylori* and found that there is no significant sex predilection [10], [11]. Age predilection for infection varies among studies with multiple studies refer to increased prevalence with age [10], [11]. However, it is common for this bacterial infection to be acquired during childhood and continue to adulthood [12], [13].

The common route for infection transmission is through oral-oral route, also feco-oral and less commonly incidentally through an endoscopy procedure. The infection with this microorganism is most common in developing Asian countries than in developed countries. This may be belonging to less hygienic water use, insufficient diet and overcrowding in these regions [14], [15]. The pathogenesis of *Helicobacter pylori* includes multiple steps. After entering the gastrointestinal tract, the organism utilises its ability to produce urease to neutralise gastric acidity and survive in the macrophages [16], then it uses its flagella to move toward the epithelial cell surface and attach to surface epithelial cells by adhesins- receptor interaction [17]. Finally, *H*. *pylori* has the capacity to produce cytotoxins: cytotoxin associated gene A (Cag A) and vacuolating cytotoxin A (VacA), these toxins together with the cytokines produced by the inflammatory cells that recruited by immunological response result in tissue damage and ulceration and it had been found that some types of these toxins may have a role in carcinogenesis [18], [19].

Many methodologies for the diagnosis of *Helicobacter pylori* are existing. Invasive and non-invasive methods may differ in their sensitivity and accuracy. Invasive antral biopsy with the histopathological examination, Giemsa stain and rapid urease test (RUT) prove its efficacy as gold standards method, however not all patients prefer this invasive procedure unless they are severely suffering [20]. The RUT also required a bacterial load of about 10^5 bacteria to be adequately sensitive, so it is not advisable for follow up after treatment [21], [22]. Non-invasive procedures including serology with IgG and IgM detection against microorganism in the sera of the patients and stool examination for bacterial antigen are rapid tests, of low cost and more acceptable to the patients. Blood examination with serological detection of anti-bacterial antibodies was less specific as it continues to give positive results for several months after eradication of microorganism [23].

On the other hand, detection of bacterial antigen in the stool proved to be more specific in the diagnosis as well as in patients follow up after treatment and eradication. A study of Mohammad Khalifehgholi et al., found that the sensitivity and specificity of stool antigen test is 74% and 78 % respectively [24]. The same study also found that the sensitivity and specificity of serology is 91.3% and 55.6% respectively [24]. The histopathological changes of gastric mucosa, which are the most important axis in this paper, are variable among most studies. In a study of Mohamed Hasan et al., gastritis had been identified histologically in 100% of H. pylori-positive population [25]. Non-atrophic (superficial) gastritis was detected in the majority of H. pylori-infected individual with no risk of transformation to peptic ulcer or adenocarcinoma [26] while chronic atrophic gastritis is often associated with metaplasia with increased risk of adenocarcinoma [27]. Multiple previous papers and articles talked about histopathological changes of H. pylori in the gastric biopsy of infected patients but little previous studies focused on the histological changes in dyspeptic young people harbouring the organism and if these changes are serious enough to recommend endoscopy in this age group. Our study will target this age group and comparing them with the older population to show if there is an urgent need for endoscopy or not.

Patients and Methods

In total, 180 dyspeptic patients were investigated for *H*. *pylori* infection over ten months. The cases were collected from Marjan medical teaching city for internal medicine/ laboratory unit and endoscopy unit between March 2018 and December 2018.

Exclusion criteria are 1. Patients with a known history of the previous infection with *Helicobacter pylori* and those who took a course of antibiotics for eradication recently (in the last month); 2. Those with dyspepsia for less than three months were excluded from the study because the histopathological changes might not be evident yet, and 3. patients with positive stool antigen but negative serology also excluded because of the possibility of recent infection and inadequate histological findings.

Ethical issues: the agreement of the hospital where the samples are collected had been taken. The permission for serum and stool antigen test had been provided by our patients. Gastric biopsies had been already planned for our patients as part of their management, and we only utilised the slides and paraffin blocks.

The complaints of the sample individuals vary, including nausea, epigastric pain, heartburn, flatulence, and bloating sensation. Patients with these

symptoms for three months or more were included in the study. Methods that used for diagnosis were both serology for IgG and IgM, stool for H. pylori antigen detection and histopathological examination of Giemsa stained sections of gastric biopsy. Patients who prepared for OGD attended the lab for routine investigations, and at that time, they asked for their permission for both blood and stool sample test of *Helicobacter pylori*. For the serological test, 5 ml of blood was drawn from the patients; the sera were separated and examined immediately for both IgG and IgM against organism using commercial *H*. *pylori* ELISA kit (Genedia). A stool sample was examined for *H*. *pylori* antigen using antibody of commercial ELISA kit (ARG80556). The case was regarded as positive if both serology and stool test were positive. Of 180 patients who prepared for the endoscopic examination, 5 patients did not attend the hospital at the time of endoscope (the remaining 175 samples). Of the 175 cases of endoscopic samples, 48 cases were excluded from histopathological assessment because they were negative for H. pylori by both serology and stool for antigen tests. After routine processing of the gastric endoscopic biopsies, histopathological examination was performed using formalin-fixed paraffin-embedded sections and hematoxylin & eosin-stained slides. The remaining 127 biopsies are reviewed by examination of H&E stained sections by two pathologists and independently from previous reports and sections of paraffin blocks are stained with Giemsa for H. pylori clarification in cases that were negative by H&E stained slides.

Statistical analysis

Chi-square test is used for data analysis and p-value of ≤ 0.05 is regarded as significant. The data were analysed with SPSS software version 20.

The descriptive analysis also used and percentages are calculated.

Results

Of the total 180 cases, 132 (73.3%) cases were positive for infection, 60 (33.33%) were male, and 42 (70%) of them was positive, while 120 (66.66%) were female with 90 (75%) cases were positive. Of the total 132 positive cases, 31.8% were males and 68.18% were females (Table 1). There is no significant correlation between the sex and *H*. *pylori* infection with p-value 0.06; however, about two-thirds of *H*. *pylori*-positive cases were female. The mean age for total positive cases was 33.4 year. Regardless of the infection with *H*. *pylori*, the number of females who are suffering from dyspepsia were more than males. The age distribution of infected individuals showed that the number (and percentage) of positive cases was more in the age group > 25 years (54.54% vs 45.45%).

There is a mild increase in the prevalence of infection in patients with age > 25 years. The histopathological examination showed that of 60 cases whom < 25 years old, 45 cases were normal gastric biopsy (75%) apart from a few scattered inflammatory cells infiltrate.

Table 1: prevalence of Helicobacter pylori infection and pattern of distribution among gender and ages

Variable	Patients	H.pylori positive No.(%)
Gender		
Male	60 (33.33 %)	42 (31.8%)
Female	120 (66.66 %)	90 (68.18%)
Total	180	132 (73.3%)
Age (year)		
≤ 25	72 (40%)	60 (45.45%)
> 25	108 (59.99%)	72 (54.54%)

While 12 biopsy (20%) showed mild superficial gastritis with chronic inflammatory cells restriction to the faveolar region and no atrophy, and only 3 cases (5%) showed moderate chronic superficial gastritis. So that the chance of serious histopathological findings (more than mild superficial gastritis) in young, infection positive people is low significantly (with p-value 0.035). In patients over 25 years age, mild superficial gastritis was found in 3 cases (4%), moderate superficial gastritis in 17 cases (23%), mild to moderate chronic atrophic gastritis in 20 cases (27%), severe atrophic gastritis in 22 cases (30%), chronic gastric ulcer in 8 cases (11%) and 2 cases (2.7%) of gastric adenocarcinoma (both of them were over 60 years age). In age group > 25 years, there was a predominance of chronic atrophic gastritis histologically over other pathological findings but there was no significant difference (p-value > 0.05), Table 2.

Examination of Giemsa stained sections and H&E stained sections showed positivity for H. pylori organism in gastric mucosa in 102 cases with only 30 cases where negative (23 negative cases were in young patients with near-normal biopsy). This might be attributed to the patchy colonisation of the mucosa or low-density bacteria. Giemsa stain was required only in 50 cases with 52 cases were positive by H&E stained sections. So that only 77% of H. pylori-positive patients by immunology proved to be positive by histopathological examination.

Table 2: distribution of histopathological findings among two population of H. pylori-positive patients (< 25 years age and > 25 years age group)

Histopathological findings	No. of patients < 25 yr. (%)	No. of patients > 25 yr. (%)
Normal gastric biopsy	45 (75)	0 (0)
Mild superficial gastritis	12 (20)	3 (4)
Moderate superficial gastritis	3 (5)	17 (23)
Mild to Moderate chronic atrophic gastritis	0 (0)	20 (27)
Sever chronic atrophic gastrits	0 (0)	22 (30)
Chronic gastric ulcer	0 (0)	8 (11)
Gastric adenocarcinoma	0 (0)	2 (2.7)
total	60 (100)	72 (100)

Discussion

The American Gastroenterological Association (AGA) recommended endoscopy in patients older than 55 years and those with alarming signs (weight loss, bleeding, persistent vomiting and family history of gastric carcinoma) [28]. In the current work, the histopathological finding matched the AGA recommendation about the importance of age as a limiting factor in endoscopy but with 25 years age rather than 55. In a study of Uemura, only atrophic gastritis can progress to metaplasia and adenocarcinoma [27]. So it is not horrible to find superficial gastritis which is the most serious finding that is revealed in this study, in young. Hong Koh et al., the study revealed mild atrophy in 55.2% and moderate atrophy in 3.4% [29]. While Kamada T et al., found that atrophy is present in 27.7% in the antrum and 28.6% in the corpus of Japanese young [30]. Both previously mentioned results were away from the findings of the current study, putting in mind the differences in the populations of the studies and their health and diet habit. The histopathological findings of Carvalho MA et al., research was very close to this study regarding the absence of gastric atrophy in young age group [31].

Histopathological examination proved the presence of H. pylori in gastric mucosa of only 77% of infection positive patients, somewhat similar to Mohamed Hasan et al., a study which discovered it histologically in 83.8% of stool antigen-positive [25]. This may be attributed to patchy colonisation of the microorganism.

The study showed that the prevalence of *Helicobacter pylori* infection in Iraq is 73.3%; the value is similar to that conducted by other studies from developing countries in Asia and the middle eastern [32]. Two studies performed in India showed that about 80% of the population was infected with Helicobacter pylori [33], [34]. Several studies that performed in other Asian countries such as Kuwait and Iran reported a decrease in the prevalence of infection in these regions with a prevalence of 49.7% and 47.9% respectively [35], [36]. In Iran, another two old studies reported a prevalence of 85% and 89.2%, so there is a decreased prevalence in this area as later study showed a prevalence of only 31% [35]. In this study, the prevalence is higher than other values that reported in nearby countries and this may reflect a low health care services in Iraqi community after a series of wars that follow in Iraq and the resultant low socioeconomic status. As in other similar studies, the correlation between the sex and the infection with *Helicobacter pylori* was insignificant. However, some of these researches, as in this study, showed a frank predominance of infection in dyspeptic females patients [3], [10], [35].

It had been found that there is a slight predominance of *Helicobacter pylori* infection in the age group of more than 25 years (54.54% in > 25 years vs 45.45% in ≤ 25 years). However, there was no significant correlation of infection with age. Studies of Ramin Niknam et al. and other researchers showed a significant correlation and increased prevalence with age [1], [3], [10].

In conclusion, the *H. pylori*-related histopathological changes of the gastric mucosa are less significant in young individual (< 25 years). The prevalence of *Helicobacter pylori* infection is still high in Iraq, in contrast, to declining prevalence in many Asian countries.

Recommendations: 1. there is no urgent need to do gastric endoscopy in H. pylori-positive people whom less than 25 years age (unless there are alarming signs) and its complications can be avoided; 2. studies about the causes of the high prevalence of helicobacter pylori are recommended to achieve a good preventive measure.

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Correlation between Breast Self-Examination Practices and Demographic Characteristics, Risk Factors and Clinical Stage of Breast Cancer among Iraqi Patients

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Keywords: Breast self-examination; Demographics; Risk-factors; Clinical stage; Iraqi patients

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Abstract

BACKGROUND: Breast Cancer (BC) is the most common cancer and the leading cause of cancer death among women globally. The disease can be cured with limited resources if detected early. Breast self-examination (BSE) is considered a cost-effective feasible approach for early detection of that cancer in developing countries.

AIM: To determine the correlation between BSE performance and demographic characteristics, risk factors and clinical stage of BC among Iraqi patients.

METHODS: This retrospective study included a total of 409 female patients diagnosed with BC at the Referral Training Center for Early Detection of Breast Cancer and the National Cancer Research Center in Baghdad. The studied variables included the age of the patient, occupation, marital and educational status, parity, history of lactation, contraceptive pill intake, family history of cancer and the clinical stage of the disease.

RESULTS: Our findings revealed that the most important predictors for practicing BSE was family history of BC or any other cancers (OR = 3.87, P = 0.018) followed by being a governmental employee (OR = 1.87, P = 0.024), history of contraceptive use (OR = 1.80, P = 0.011) and the high level of education (OR = 1.73, P = 0.004). On the other hand, there was no significant correlation between the practice of BSE and the BC stage at the time of presentation.

CONCLUSION: There is a relatively poor practice of BSE among Iraqi patients diagnosed with BC. It is mandatory to foster the national cancer control strategies that focus on raising the level of awareness among the community through public education as a major approach to the early detection of cancer in Iraq.

Introduction

Breast cancer (BC) is the most common cancer and the leading cause of cancer death for women which accounts for 23% of all female cancers globally [1]. The global cancer burden is estimated to have risen to 18.1 million new cases and 9.6 million deaths in 2018. One in five men and one in six women worldwide develop cancer during their lifetime, and one in eight men and one in eleven women die from the disease. Worldwide the 5-year prevalence is estimated to be 43.8 million [2]. In general, BC is a leading cause of cancer death in the less developed countries of the world [2]. For women aged 15 – 49 years, twice as many BC cases are diagnosed in developing countries than in developed countries [1]. In low-resource settings, seven out of ten people newly diagnosed with BC die versus two out of ten in high-resource settings [3]. Although BC tends to attack women in their most productive years of life, however, it could be cured with limited resources if detected early while treating advanced-stage disease is expensive, and the outcome is often poor [4].

Focusing on Iraqi Cancer Statistics, BC has become a major public health problem; its burden is rising with the increase in population size [2], [5]. It is the leading cause of death among the Iraqi population following cardiovascular diseases. The latest Iraqi Cancer Registry (ICR) reveals that a total of 25,556 new cases of cancer were registered among an estimated 37,883,543 Iraqi population in 2016; with a male to female ratio equivalent to 0.7: 1. Overall, breast cancer remains the most commonly diagnosed,

forming 19.6% of total and 34.3% of female cancers [5]. Iraqi studies reveal that a considerable proportion of BC patients are still diagnosed at relatively advanced stages [6], [7], [8], [9]; regrettably about 90% discover the disease accidentally by themselves [8], [9].

It has been demonstrated clearly that early detection of BC improves its outcome and thus remains the cornerstone for its control. Apart from the known screening tools for early detection of BC that include mammography and physical breast examination, breast self-examination (BSE) has been considered a cost-effective feasible strategy in low- and middle-income settings [9], [10], [11]. The procedure of BSE urges women to learn the topography of their breasts to seek advice when any abnormal changes occur later [9]. Previous reports indicated that among educated Iraqi female population only less than 50% of those who have heard about BSE did practice the manoeuvre for reasons attributed to lack of knowledge on how to perform BSE correctly [12], [13].

The current study aimed to assess the correlation between BSE practices and demographic characteristics, risk factors and clinical cancer stage among a sample of Iraqi patients diagnosed with BC.

Patients and Methods

This retrospective study was performed at the Referral Training Center for Early Detection of Breast Cancer/Medical City Teaching Hospital and the National Cancer Research Center/Baghdad University. Only cases with complete, valid data were included in this study which highlighted the clinical and pathological characteristics of 409 female patients diagnosed with BC during the period between 2016 and 2018.

The study tool was designed to collect information regarding the age of the patient, occupation, marital and educational status, parity, history of breast lactation, contraceptive pill intake, family history of breast or any other cancer and the clinical stage of the disease. The BC Stage was defined following the UICC TNM Classification System [14].

Each questionnaire was assigned with an identifying serial number when the data were entered and analysed by the researchers using the Statistical Package for Social Sciences (SPSS v. 25). The data were presented as frequency tables and bar charts. The chi-square test was applied to test the association between the categorical data; the level of significance was set at a P-value of ≤ 0.05.

A written consent was signed by each patient at the National Cancer Research Center of the University of Baghdad ethically approving the utilisation of the recorded data for research; keeping all information anonymous.

Results

Overall, 54.6% of the study sample was aged ≥ 50 years. The majority of the patients were married (87.5%), less than half were a governmental employee (47.4%), and nearly one-third was highly educated (31.4%). In general, 52.8% have never performed BSE; No statistical differences were noted regarding the practice of BSE among the categorised age groups. Likewise, no significant variations were observed between single and married women. On the other hand, the employed and more highly educated patients had a highly better experience with BSE than those who were housewives and illiterate (62.4% versus 33.5% and 65.9% versus 27.9% respectively) at $p < 0.001$ (Table 1).

Table 1: Association of Demographic Characteristics with the Practice of BSE

Variable	Practice BSE n. (%)	Total (%)	X^2 value	P-value
Age group (years)				
20-34	8(42.1)	19(4.6)		0.83
35-49	81(48.5)	167(40.8)	0.340	
≥ 50	104(50.5)	223(54.6)		
Occupation				
G. employee	121(62.4)	194(47.4)	34.13	0.001
Housewife	72(33.5)	215(52.6)		
Education				
Illiterate/Primary	39(27.9)	140(34.2)		0.001
Intermediate/Secondary	69(49.3)	140(34.2)	39.34	
Graduate/Postgraduate	85(65.9)	129(31.6)		
Marital status				
Single	15(42.9)	35(8.6)		0.41
Married	168(46.9)	358(87.5)	1.778	
Divorced/Widow	10(62.5)	16(3.9)		
Total	193 (47.2)	409 (100.0)		

Table 2 illustrates that the history of breast lactation was recorded in 70.7% of patients, contraceptive pills use in 32.5% and family history of BC or any other cancer in 41.3%. About 19% of the patients were nulliparous. Significant correlations were observed between BSE practices and history of contraceptive pills use, family history of BC and smoking ($P = 0.01$, $P = 0.01$ and $P = 0.03$ respectively).

Table 2: Correlation between BC Risk Factors and BSE Practices

Variable	Practice BSE n. (%)	Total (%)	X^2 value	P-value
History of breast lactation				
Yes	142(49.1)	289(70.7)	1.498	0.23
No	51(42.5)	120(29.3)		
History of contraceptive pills or hormonal use				
Yes	75(56.4)	133(32.5)	6.698	0.01
No	118(42.8)	276(67.5)		
Family history of Cancer				
Yes	92(54.4)	169(41.3)	6.074	0.01
No	101(42.1)	240(58.7)		
Parity				
Nulliparous	39(50.0)	78(19.1)	0.306	0.61
Gravida	154(46.5)	331(80.9)		
Total	193	409 (100.0)		

Overall, 61.6% of the studied population was diagnosed in Stages I and II. Statistically, there was no significant correlation between the practice of BSE and BC stage (Table 3).

Table 3: Relationship between the Stage of Breast Cancer and the Practices of BSE

Variable	Practice BSE n. (%)	Total (%)	X^2 value	P-value
BC staging				
Stage I	24(38.7)	62(15.2)	3.412	0.34
Stage II	97(51.1)	190(46.4)		
Stage III	68(45.3)	150(36.7)		
Stage VI	4(57.1)	7(1.7)		
Total	193	409 (100.0)		

As displayed in Table 4, on multiple logistic regression analysis, the only significantly associated variables with the practice of BSE were entered into the model. Our findings revealed that the most important predictors for practicing BSE was family history of BC or any other cancers (OR = 3.87, P = 0.018) followed by being a governmental employee (OR = 1.87, P = 0.024), history of contraceptive use (OR = 1.80, P = 0.011) and the high level of education (OR = 1.73, P = 0.004) (Table 4).

Table 4: Determinants of Practicing BSE by Logistic Regression Analysis

Factors	Odds ratio	95% C.I.		P-value
		Lower	Upper	
Family history of cancer	3.87	1.26	11.9	0.018
Governmental employee	1.87	1.08	3.24	0.024
History of contraceptive use	1.80	1.14	2.83	0.011
High education level	1.73	1.59	1.90	0.004

Discussion

BC is the most common female cancer in the Eastern Mediterranean region, where the witnessed demographic and socioeconomic transitions have increased the cancer burden within the last decades [15], [16]. Early-stage at cancer detection has been regarded as a key determinant of breast cancer outcome specifically in low- and middle-income settings; because of the limited resources required to provide adequate therapy [4], [7], [8], [9], [10]. Whereas no evidence-based data was reported to support the efficacy of BSE as a unique screening tool for BC [4], [10], nevertheless, in countries where breast cancer is diagnosed at an advanced stage it has been emphasised that screening by clinical breast examination with the teaching of BSE, as an integral component, will probably be effective in reducing breast cancer mortality [17].

The current study shows that 45.4% of the patients were diagnosed with breast cancer under the age of 50 years; 69% were aged 40-49 years. That was following the findings displayed in earlier studies from Iraq [5], [6], [7], [8], [9] which emphasises the high prevalence of that disease among the middle-aged female population. No statistical differences were noted regarding the practice of BSE among the categorised age groups. In a recent survey that aimed to highlight the main demographic and clinical profiles of 1172 female patients registered with breast cancer in Iraq the mean age at presentation was 51 years, 9.8% were not married, 19.2% graduated from universities, and 11% were nulliparous. History of lactation and hormonal therapy was noted in 57.6% and 19.4% respectively. Family history of cancer was positive in 28.8% and of breast cancer, specifically in 18.7% [18].

Our results demonstrate the relatively poor practice of BSE among Iraqi patients affected by BC; more than half of those (52.8%) have never experienced the manoeuvre. Variations in BSE performance rates ranging between 6 and 83% have been reported in different studies from developing countries [6], [8], [9], [12], [13], [19], [20], [21], [22]. When applying the logistic regression analysis, it was found that practising BSE by the affected patients was significantly associated with a family history of cancer, working as a governmental employee, history of using contraceptive pills and high level of education. These findings were by those recorded in previous studies from Iraq which displayed that the greater proportion of those who practised BSE was observed among the highly educated and employed sector of the community [12], [19], [20]. In general, it has been concluded that promoting the level of knowledge, attitudes and practices regarding BSE could play a positive impact on breast cancer screening [19], [20], [21], [22], [23], [24], [25], [26]. Fostering health education among females yields a better perception of breast-related symptoms and consequently raise their awareness of the significance of BSE and early medical consultation.

It was previously reported in the literature that history of contraceptive use and family history of cancer significantly influence the practice of the BSE among the BC affected patients [21], [24], [27]. In this study, family history of cancer among breast cancer patients was relatively high and constituted a significant predictor for practising BSE; reflecting a higher level of awareness towards the disease in the affected families. A previous study from Iraq revealed that 19.1% of breast cancer patients with a positive family history had two affected relatives while a first-degree relative was involved in 43.7% [28]. In that report the characteristics of the patients with a positive family history of cancer did not reveal any distinct clinical markers for their identification; recommending careful screening, regular follow and promoting public education to identify the high-risk groups.

Focusing on the BC stage in the present study, 38.4% of the patients were diagnosed in advanced stages III and IV. Compared to another study that was published in 2017, carried out on 603 Iraqi patients, it was observed that 9.5%, 47.1%, 33.2% and 9.1% of the patients presented at stages I,

II and III and IV respectively [7]. No statistical correlation was noted between the practice of BSE and BC stage in both studies. Nevertheless, earlier reports determined the favorable association between BSE performance and the clinical and pathological stage of breast cancer at first diagnosis [29], [30]. The authors concluded that although the specificity of BSE as a screening modality for BC is low and the costs in terms of false-positive results, anxiety and medical costs might be high, yet when adequately taught BSE could lead to earlier diagnosis.

In conclusion, there is a relatively poor practice of BSE among Iraqi patients diagnosed with BC. While no significant correlation was noted with the BC stage at presentation, the main predictor for practising BSE among the studied population was a positive family history of cancer, followed by being a governmental employee, history of contraceptive use and high level of education. These findings justify endorsing national cancer control strategies that focus on raising the level of awareness on BC among the Iraqi community through public education as a major approach to early detection [16], [31], [32].

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Clinical Science

Short Term Effectiveness of Gamma Knife Radiosurgery in the Management of Brain Arteriovenous Malformation

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Abstract

AIM: To evaluate the short-term effectiveness of Gamma knife radiosurgery as a modality of treatment of brain arteriovenous malformation.

METHODS: Sixty-three patients with arteriovenous brain malformations underwent Gamma knife radiosurgery included in this prospective study between April 2017 and September 2018 with clinical and radiological with MRI follow up was done at three months and six months post-Gamma knife radiosurgery. By the end of the 12th-month post-Gamma knife radiosurgery, the patients were re-evaluated using digital subtraction angiography co-registered with M.R.I. During the 12 months follow up, CT scan or MRI was done at any time if any one of the patients' condition deteriorated or developed signs and symptoms of complications. The mean volume of the arteriovenous malformations treated was 26.0 ± 5 cm^3 (range 12.5–39.5 cm^3) in The Neurosciences Hospital, Baghdad/Iraq.

RESULTS: By the end of the 12th month of follow up, the overall obliteration of the arteriovenous malformations was seen in six patients only (9.5%), while shrinkage was noticed in 57 patients (90.5%). Improvement or clinical stability was found in 24 out of 39 patients (61.5%) presented with epilepsy as a chief complaint before Gamma knife radiosurgery and 21 out of 24 patients (87.0%) complained of a headache before Gamma knife radiosurgery. Post-Gamma knife radiosurgery bleeding was found in only three patients (5.0%).

CONCLUSION: Even with the short term follow up, Gamma knife radiosurgery has an excellent clinical outcome in most patients with arteriovenous brain malformations. The clinical symptoms like headache and seizure were either diminished or controlled with the same medical treatment dose before Gamma knife radiosurgery. Long term clinical and radiological follow up is recommended.

Introduction

Brain arteriovenous malformation (AVM), is a complex of abnormal arteries and veins that directly communicate without an intervening capillary bed, with an incidence of 1.12 – 1.42 cases per 100,000 people each year [1], [2]. Haemorrhage is the most common presentation, occurring in 40 – 50% patients at initial diagnosis. The annual risk of haemorrhage ranges from 1.3 to 4%. The second most common presentation is a seizure, occurring in 20 – 30% of patients, followed by headache (5%–14%) and focal neurological deficits (around 5%) [3], [4]. AVM is the second most common cause of intracerebral haemorrhage in people < 35 years of age, following trauma. Treatment options for cerebral AVM include surgery, endovascular treatments, and radiosurgery. Among these, stereotactic radiosurgery (SRS) showed great benefit in the treatment of small- to medium-sized AVMs, by minimising the risk of future intracranial haemorrhage with a reduction in treatment-related morbidity. The benefit in appropriately selected patients who undergo SRS reaches approximately 80 – 85% [4].

We aimed to evaluate the short-term effectiveness of Gamma knife radiosurgery (GKR) is a modality of treatment of brain AVM.

Patients and Methods

This prospective clinical study which included

sixty-three outpatients, referred for radio-surgical treatment of an AVM using GKR in The Neurosciences Hospital, Baghdad/Iraq for the period between April 2017 and September 2018.

The inclusion criteria were any patient diagnosed (clinically and radiologically) to have an AVM. All the patients were older than 18, as shown in Table 1. All sizes of AVMs included.

The exclusion criteria were any previous AVM intervention, including vascular intervention, microsurgery, or previous GKR.

The average volume of the AVMs was 26.0 cm^3 (range, 12.5 – 39.5 cm^3). The G.K.R. dose was 16-25 Gy (mean = 20 Gy) — the dose of the radiation delivered as a single dose in one session and the target was the nidus. The model of the GKR was Leksell Gamma Knife, Elekta, Perfexion model.

All procedures performed without general anaesthesia. Instead, intravenous sedative agents (i.e., midazolam, thiopental, and/or pentazocine) used before frame fixation and local anaesthesia (lidocaine 2%) applied at the sites of frame fixation. The procedure was performed with the patient either fully conscious or under sedation, as considered appropriate.

The study protocol approved by our institutional review board and conformed to the principles of the Declaration of Helsinki. All patients provided written informed consent for the use of their data.

Follow-up

Clinical and radiological (MRI) follow up was done at three months and six months post-GKR. By the end of the 12th-month post-GKR, the patients were re-evaluated using digital subtraction angiography co-registered with MRI. During the 12 months follow up, CT scan or MRI was done at any time if any one of the patients' condition deteriorated or developed signs and symptoms of complications.

The policy of our institution is to re-evaluate the patients three years post-GKR using DSA and MRI then decide accordingly if the AVM is obliterated or further intervention is required.

Statistical analysis

Descriptive analysis in the form of percentage was calculated using Excel and presented in the relevant tables shown below. Chi-Square test was used for statistical analysis by utilising the Statistical Package for Social Sciences (S.P.S.S.) version17 (*p*-value < 0.05 was considered significant).

Results

The patients' characteristics included in this study were summarised in Table 1.

Table 1: Characteristics of the patients included in the study

Characteristics	Number of patients (%)
Male	39 (62%)
Female	24 (38%)
	Total 63 (100%)
Mean age (years)	47 (range 21-72)
Mean AVM volume (cm^3)	26.0 ± 5 (range, 12.50-39.50)
Presentation:	
Haemorrhage	36 (57%)
Epilepsy	39 (61%)
Headache	24 (38%)
Other (Dizziness, vertigo, focal symptoms)	9 (14%)
Location	
-Hemispheric	18 (85%)
-frontal	3 (14%)
-parietal	10 (47%)
-temporal	2 (9.5%)
-occipital	2 (9.5%)
-cerebellar	1 (5%)
-Deep (brain stem, thalamus)	3 (15%)

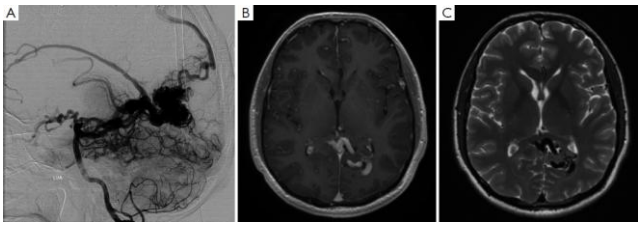
The results were concluded using the follow-up radiological studies (DSA, MRI, and CT Scans) and patients' objective presentation of symptoms.

Table 2: AVM size post-GKR at 12 months follow-up

AVM size follow up radiologically	Total cases 63	%	*P*-value
Obliteration	6	9.5%	*P* = 0.1
Shrinkage	57	90.5%	*P* = 0.01
No change	0	0.0%	

By the end of the 12th month of follow up, the overall obliteration of the A.V.M. was seen in six patients only (9.5%, *P* = 0.1), while shrinkage was noticed in 57 patients (90.5%, *P* = 0.01) (the shrinkage ratio was 32.2-78.4%, mean = 68.8%) (Table 2, Figures).

Pre-GKR imaging



One-year follow-up post-GKR

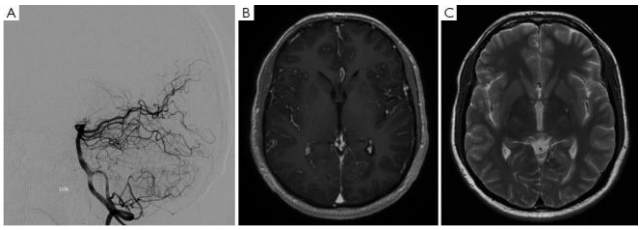


Figure 1: Pre-GKR imaging of left occipital AVM; A) DSA; B) MRI T1 weighted image with contrast; C) MRI T2 weighted image without contrast (top); One-year follow up post-GKR; A) DSA; B) MRI T1 weighted image with contrast; C) MRI T2 weighted imaging without contrast. Showing resolution of left occipital AVM (bottom)

Improvement or clinical stability at 12 months, without changing the dose of anti-epileptic drugs, was found in 24 out of 39 patients presented with epilepsy (free of attacks 15.5%, *P* = 0.01 with decreased amplitude time and frequency 46.0%, *P* = 0.02) as a chief complaint before GKR (Table 3).

Table 3: Seizure attacks post-GKR at 12 months follow-up

Seizure attacks post-GKR	Total of 39 cases	%	*P*-value
Free of attacks	6	15.5%	*P* = 0.01
Decreased amplitude-time-frequency	18	46.0%	*P* = 0.02
No change	15	38.5%	

Improvement or clinical stability at 12 months, without changing the dose of anti-epileptic drugs in 21 out of 24 patients who complained of headache (free of headache 62.5%, *P* = 0.001 with decreased frequency and severity 25.0%, *P* = 0.01) before GKR (Table 4).

Table 4: Headache attacks post-GKR at 12 months follow-up

Headache attacks post-GKR	Total of 24 cases	%	*P*-value
Free of headache	15	62.5%	*P* = 0.001
Decreased frequency and severity	6	25.0%	*P* = 0.01
No change	3	12.5%	

Post-GKR bleeding found in only three patients (5.0%). These three cases had complained of a sudden deterioration in the level of consciousness and severe headache after six months of the GKR. They were admitted to the hospital and managed conservatively (Table 5).

Table 5: Post-GKR haemorrhage

Post-GKS haemorrhage	Total of 36 cases	*P*-value
In 3 months	0	
In 6 months	0	
In 12 months	3	*P* = 0.1
No haemorrhage	60	

The complications encountered during the 12 months post-GKR follow up were hemianesthesia in 7 cases (11.1%), hemiparesis in 5 cases (7.9%), diplopia in 4 cases (6.3%), hemianopsia in 2 cases (3.1%), and ataxia in 1 case (1.5%). CT scan or MRI applied for each case. The duration of the symptoms was 2-10 days (mean = seven days). All were transient and responded well to steroid (dexamethasone).

Discussion

The most controversial issue on brain AVM treatment is the choice of interventional therapy modes. G.K.R. treatment could increase the safety of interventional surgeries for ruptured or unruptured brain AVMs, which are difficult to access by micro-neurosurgery, high Spetzler Martin (SM) grade, and eloquent in location [5].

The reported obliteration rate of the nidus in the brain AVMs after radiosurgery varies between 43 and 92%, [5], [6], [7], [8]. In our study, complete obliteration was achieved in six of the 63 patients (9.5%). This is explained by the short period of follow up (12 months).

Pollock et al., reported that serial MRI was predictive of total obliteration in 84% of patients in a period of two to four years [9]. Because of its low risk and high reliability, it is reasonable to use MRI to evaluate obliteration after radiosurgery[10].

Kano et al. found that factors associated with a higher rate of total obliteration on angiography included smaller target volume, smaller maximum diameter [8].

Friedman et al. reported that complete obliteration was obtained in 81% of AVMs between 1 ml and 4 ml in volume, in 89% of AVMs between 4 ml and 10 ml, and 69% of AVMs > 10 ml [11]. Murray et al. published increased volume was significantly associated with non-obliteration. Volume was a more critical factor than eloquent location, patient age, or gender [12]. Morphological features of the AVM and its density influence the success of AVM obliteration. Diffuse AVM structure is associated with a higher risk of radiosurgery failure [12], [13]. In our study, the size of the lesions was an essential factor as most AVMs were large (12.5-39.5 cm^3 with mean = 26 ± 5 cm^3).

The risk of bleeding remains in 1.8-5% of patients per year until obliteration can be confirmed [9], [11], [14]. The exact mechanisms through which radiosurgery reduces the chance of bleeding not entirely elucidated. However, the histopathological studies of AVM after radiosurgery have suggested some theories: A) progressive thickening of the intimal layer, which begins as early as three months after radiosurgery, appears to decrease the stress to the vessel walls, and B) partial or complete thrombosis of the irradiated vessels may decrease the number of patent vessels in the malformation [5], [15].

Der-JenYen et al. reported that concerning seizure control, significant seizure reduction found following the first 6-months period after GKR. A 28–66.7% seizure reduction rate compared with that before G.K.R. was achieved and no initial worsening of seizures in any of the patients after GKS [16]. In our study, the post-GKR seizure rate was somehow similar to those results, whereas 15.5% were seizure-free, and about 46.0% were with fewer symptoms. Also, no worsening was reported.

In conclusion, even with the short term follow up, GKR has an excellent clinical outcome in most patients with brain AVM. The clinical symptoms like headache and seizure were either diminished or controlled with the same medical treatment dose before the GKR. Long term clinical and radiological follow up is recommended.

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Traumatic Dural Venous Sinuses Injury

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Keywords: Traumatic dural; Dural border cell; Injury severity score

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Abstract

The traumatic dural venous sinus injury is one of the most dangerous complications of TBI, either due to fatal intracranial compressing venous bleeding, or disturbing the intracranial pressure which could be caused by injury to the SSS

On the other hand, post traumatic dural sinus thrombosis is considered a rare complication which may lead to hemorrhagic infarction with its serious consequences including epilepsy, neurological deficits, or death. Therefore, knowledge of the appropriate treatment of this kind of head injury is essential.

Introduction

The study was done prospectively in the Neurosurgery Department, Trauma Casualty Unit, Cairo University on patient admitted in the period from August 2013 to March 2014, suffering from traumatic brain injuries associated with dural venous sinus injury.

Forty patients with traumatic brain injuries associated with dural venous sinus injury requiring surgical intervention then followed up clinically and radiologically.

Inclusion criteria were: - All patients with traumatic brain injuries associated with dural venous sinus injury requiring surgical intervention; - No sex predilection and - No age predilection.

Exclusion criteria were: - All patients with traumatic brain injuries not associated with dural venous sinus injury; - Shock (the systolic blood pressure less than 90 mm Hg) for longer than 30 minutes; - Hypoxia [pulse oxygen saturation (SPO2) less than 90%] for longer than 30 minutes; - Serious extracranial injuries; - Bleeding tendency and - Clinical brain death on admission.

Prehospital Report

The patients were all treated with similar prehospital emergency treatment, and routine brain CT scan was performed (Table 1).

Table 1: Prehospital emergency treatment and routine brain CT scan

Mechanism	How did injury occur? Presence of drugs or alcohol. Deaths at the scene. Confounding issues.
Injury	Primary survey. Glasgow Coma Scale.
Vital data	Heart rate. Blood pressure. Respiratory rate. Oxygen saturation. Temperature (if applicable).
Treatment	Airway (airway management). Breathing (oxygen administration, needle or tube thoracostomy). Circulation (intravenous access established and fluids administered) Disability—neurologic (spine precautions). Extra information (medications administered, procedures performed).

History

Taken from the patient, or his relatives for; age, mechanism of injury, time of trauma, Time of loss of consciousness, presence of lucid interval and pre-hospital post-traumatic fits.

Clinical Findings

Conscious level (preoperative GCS), presenting symptom, scalp injuries, bleeding orifices, pupils and associated injuries (spine, cardiothoracic, orthopaedic …etc).

Radiological Findings

A) All patients underwent CT imaging with bone window to determine the type of intracranial injury (EDH, ICH, ASDH or compound depressed fracture) before a decision was made about the surgical procedure.

B) Imaging for associated injuries, (spine, cardiothoracic, orthopaedic, …etc).

Surgical procedures

In our study 10 cases (50%) the bleeding is controlled by direct compression by gel foam (gelatin compressed sponge), which is absorbable haemostatic material for few minutes, 5 cases (25%) the bleeding is controlled by direct application of gel foam on the sinus injury followed by stitching the dura up to the adjacent bone, 4 cases (20%) the bleeding is controlled by direct stitching the dural tear followed by gel foam compression and 1 case (5%) the bleeding is controlled by free muscle duroplasty. In some cases (not faced in the study) there is severe sinus injury with hemorrhage is out of control, ligation can be performed in non-critical areas (in the first quarter of the superior sagittal sinus). A temporary sinus- sinus shunt may be necessary to repair the sinus tear without compromising sinus blood flow properly, but we did not face that in our study. Blood transfusion is required in such cases.

Postoperative care

The conscious level will be assessed frequently using the Glasgow coma scale.

&follow up CT brain within 24 hours after surgery.MRV may be needed if there are manifestations of increased ICP, or venous infarction in follow up CT.

The following will be assessed

A) Clinical improvement of symptoms.

Table 2: Master table

No.	Sex	Age (year)	Mode of trauma	GCS	Associated	Image Preoperative	Sinus injury	Operative technique	Image Postoperative	Complications	Other surgery
1	f	2	Falling from height	15	-	LT frontal CD# crossing midline	Anterior part of SSS	Direct gel foam compression	Good	No	No
2	M	25	Blunt trauma	15	-	LT frontal CD# reaching mid line	Anterior part of SSS	Direct gel foam compression	Good	No	No
3	M	42	Blunt trauma	15	-	RT frontal CD# reaching mid line	Anterior part of SSS	Direct gel foam compression	Good	No	No
4	F	55	Road traffic accident	5	# ribs and haemothorax	LT parieto occipital CD#, massive brain edema, massive brain hypo perfusion	Left TS	Direct stitching of the tear with gel foam compression	Good control of sinus bleeding + Multiple brain infarctions	Multiple brain infarctions	No
5	M	29	Blunt trauma	9	-		Middle part of SSS	Stitching the dura up after application of gel foam on the tear	Good evacuation of the hematoma, Collection of EDH on contra lateral side	Collection of EDH on contra lateral side	Evacuation of hematoma
6	M	14	Trauma by sharp object	14	-	Occipital D#, bilateral occipital contusions	Posterior part of SSS, TS	Stitching the dura up after application of gel foam on the tear	Good control of sinus bleeding	Intra operative bleeding → 3 unites blood transfusion	No
7	M	80	Falling from height	7	# long bones	ASDH	Middle part of SSS	Direct gel foam compression	Massive ICH	ICH	Yes
8	M	19	Road traffic accident	15	-	RT parieto occipital CD# reaching sub occipital region	RT sigmoid sinus	Direct gel foam compression	Good control of sinus bleeding	No	No
9	M	25	Blunt trauma	7	-	LT fronto-temporo-parietal EDH	Middle part of SSS	Direct stitching of the tear with gel foam compression	Good evacuation of the hematoma, Collection of EDH on contra lateral side + Small RT parietal hemorrhagic infarction	=Collection of EDH on contra lateral side =Intra operative bleeding → 3 unites blood transfusion =Post traumatic transverse sinus thrombosis	Evacuation of hematoma
10	F	3	Blunt trauma	15	-	RT frontal CD# crossing mid line, underlying EDH	Middle part of SSS	Direct gel foam compression	Good evacuation of the hematoma	No	No
11	M	23	Road traffic accident	14	-	LT temporal EDH, frontal air sinus #	Anterior part of SSS	Direct gel foam compression + frontal air sinus repair	Good evacuation of the hematoma	NO	NO
12	M	35	Trauma by sharp object	5	-	LT high parietal CD#, underlying ICH	Middle part of SSS	Direct stitching of the tear with gel foam compression	Good evacuation of the hematoma	NO	NO
13	M	25	Road traffic accident	9	-	LT prieto-occipital EDH reaching sub occipital region	LT TS	Free muscle duroplasty	=Good evacuation of the hematoma =Massive brain edema, =Hemorrhagic infarctions	=Complete obstruction of LT TS→ Hemorrhagic infarctions → RT hemi paresis grade 3, Intra operative bleeding → 3 unites blood transfusion	NO
14	M	25	Blunt trauma	15	-	LT high parietal EDH	Middle part of SSS	Direct stitching of the tear with gel foam compression	ICH	=ICH = Intra operative bleeding → 4 unites blood transfusion	NO
15	M	15	Blunt trauma	15	-	RT high parietal CD#	Middle part of SSS	Direct gel foam compression	Good control of sinus bleeding	NO	NO
16	F	20	Road traffic accident	14	Mild pelvic collection	RT frontal EDH	Anterior part of SSS	Stitching the dura up after application of gel foam on the tear	Good evacuation of the hematoma	NO	NO
17	M	42	Blunt trauma	14	-	LT high parietal CD#+underlying EDH	Middle part of SSS	Direct gel foam compression	Good control of sinus bleeding	NO	NO
18	M	8	Blunt trauma	15		Mid line frontal CD#	Anterior part of SSS	Direct gel foam compression	Good control of sinus bleeding	NO	NO
19	M	47	Falling from height	5	# long bones	ASDH	Middle part of SSS	Stitching the dura up after application of gel foam on the tear	Good control of sinus bleeding	Intra operative bleeding → 4 unites blood transfusion	NO
20	M	4	Falling from height	15	-	LT parieto-occipital CD#	LT TS	Stitching the dura up after application of gel foam on the tear	Good control of sinus bleeding	NO	NO

M: Male; F: Female; LT: Left; RT: Right; CD#: Compound depressed fracture; EDH: Extra dural hematoma; ASDH: Acute sub dural hematoma; ICH: Intra cerebral hematoma; SSS: Superior sagittal sinus; TS: Transverse sinus.

B) The rate of complications.

C) The need for subsequent lines of management.

D) Wound healing.

E) The outcome was graded using the Glasgow Outcome Score (GOS), which defines.

1. Grade I as death,

2. Grade II as persistent vegetative state,

3. Grade III as severe disability (being conscious but disabled),

4. Grade IV as moderate disability (being disabled but independent), and

5. Grade V as good recovery.

Case Presentation

Case 1

The patient was operated upon by elevation of the depressed bone, carefully evacuation of EDH. There was bleeding from 2 small lacerations of the middle part of SSS, which was controlled by the direct application of gel foam on the lacerations with gentle compression for a few minutes (Figure 1).

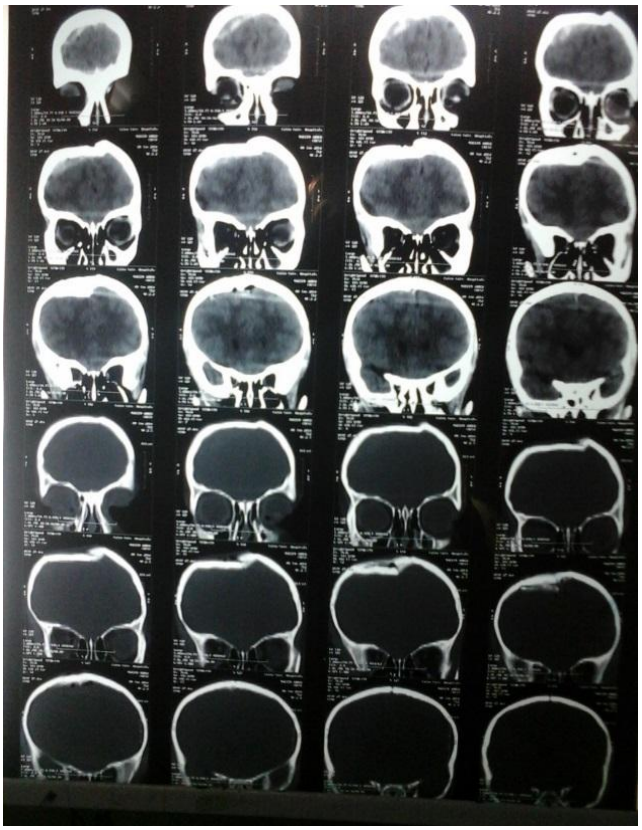

Figure 1: Pre-operative CT of Case 1

Postoperatively patient had the same GCS15/15, and there is no neurological deficit (Figure 2).

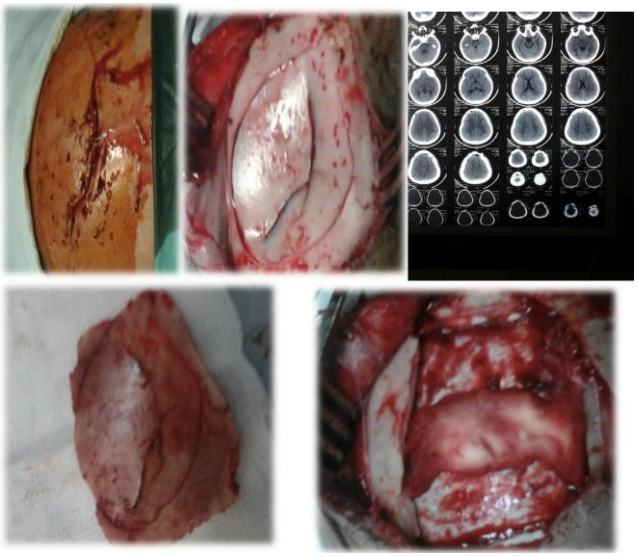

Figure 2: Postoperative CT of Case 1

Case 2

The patient was operated upon by careful evacuation of EDH. There was severe intraoperative bleeding from large injury of the left transverse sinus, which was controlled by free muscle duroplasty followed by application of gel foam and compression for a few minutes. During the operation, we need a massive blood transfusion, and there was severe brain oedema and the elevated bone cannot be replaced again (Figure 3).

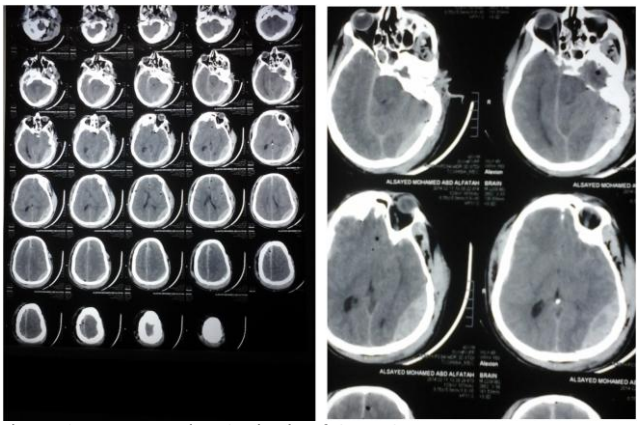

Figure 3: Pre-operative CT brain of Case 2

Postoperatively patient had the same GCS9/15, and there was contralateral hemiparesis motor power and was grade 2.

Postoperative CT scan showed good evacuation of the hematoma, but there were severe brain oedema and multiple hemorrhagic infarctions of left cerebral hemisphere (Figure 4).

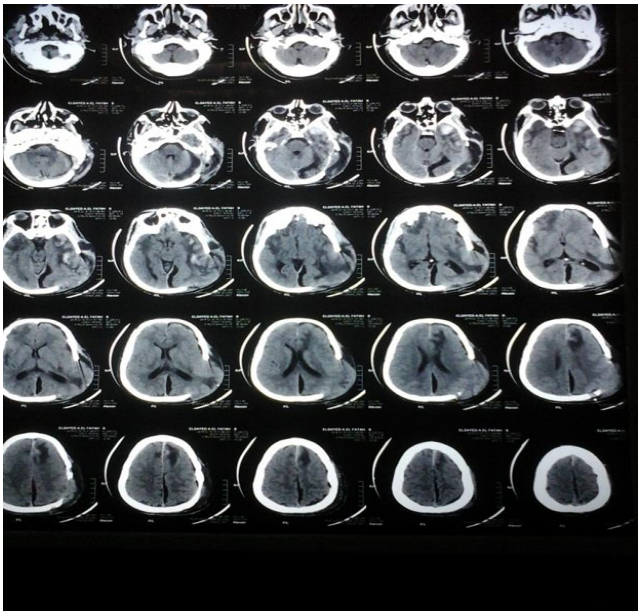

Figure 4: Post-operative CT brain of Case 2

MRV showed complete obstruction of the left transverse sinus (Figure 5).

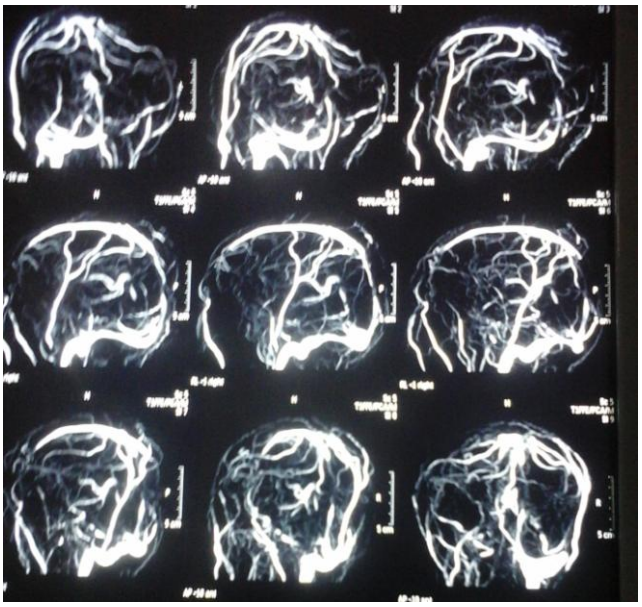

Figure 5: Post-operative MRV of Case 2

The patient gradually improved on dehydrating measures (mannitol 20% and Lasix), clexan (60 unites subcutaneous every 12 hours) and physiotherapy.

After one-week GCS became 14/15 and motor power improved in the right side up to grade 4.

Case 3

The patient was operated upon by elevation of the depressed bone, and duroplasty using ipsilateral pericranium. There was bleeding from small lacerations of the middle part of SSS, which was controlled by putting on the direct haemostatic sheath of gel foam on the lacerations and stitching the dura up (Figure 6).

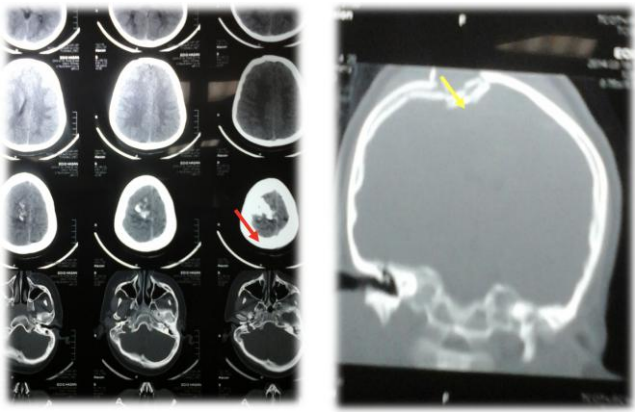

Figure 6: Pre-operative CT of Case 3

Postoperatively patient had the same GCS15/15, and there was an improvement in the motor power (Figure 7).

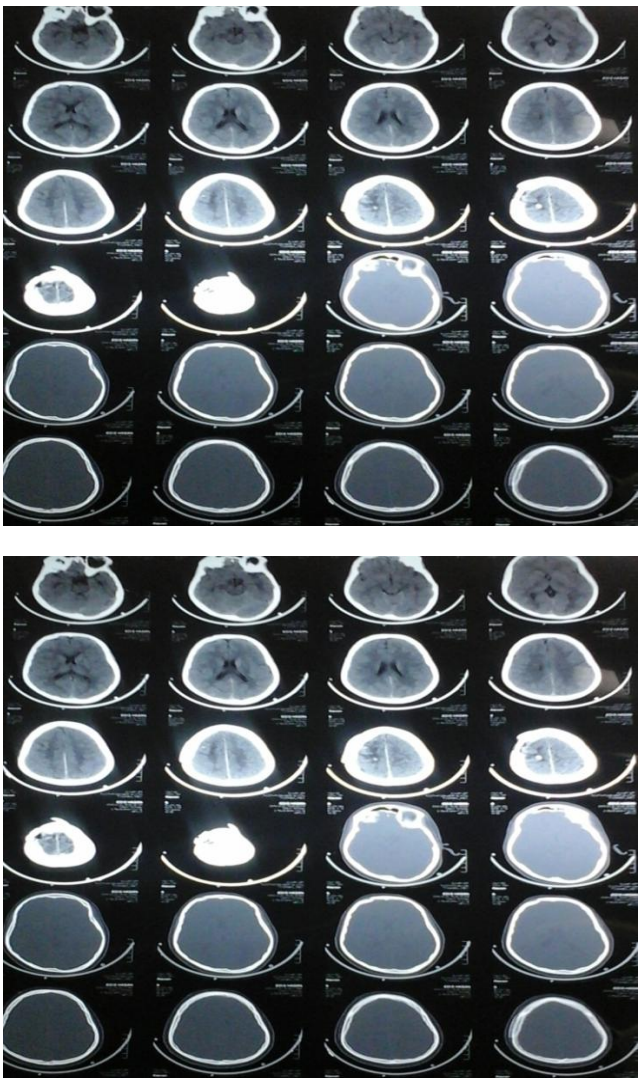

Figure 7: The depressed bone before its elevation (top); Post-operative CT brain of Case 3 (bottom)

Case 4

The patient was operated upon by careful evacuation of EDH. There was severe intraoperative bleeding from large injury of the middle part of SSS, which was controlled by direct stitching the tear followed by application of gel foam and gentle pressure for a few minutes. During the operation, we need a blood transfusion (Figure 8).

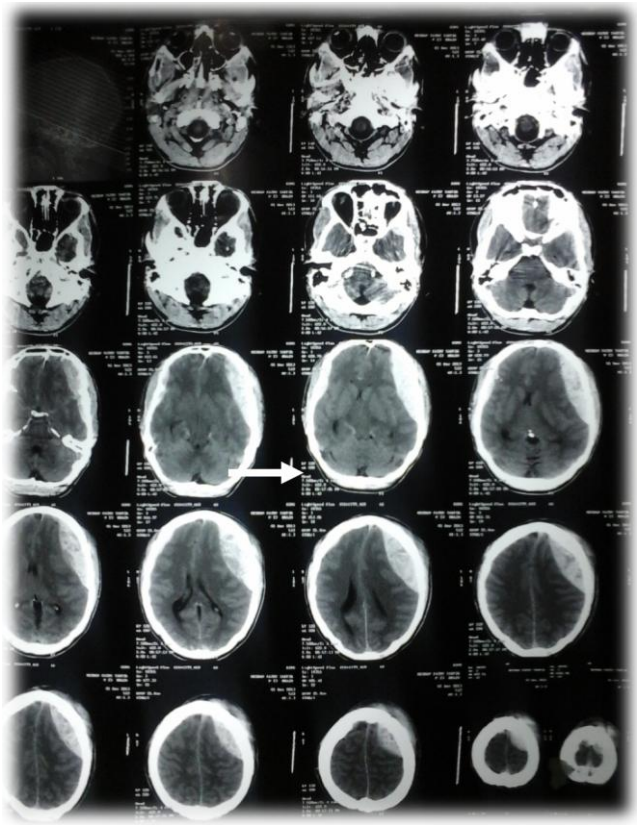

Figure 8: Pre-operative CT brain of Case 4

Postoperatively patient had the same GCS7/15.

Postoperative CT scan showed good evacuation of the hematoma, but there was a collection of EDH on the contralateral side which was evacuated, and there was small right parietal hemorrhagic infarction (Figure 9).

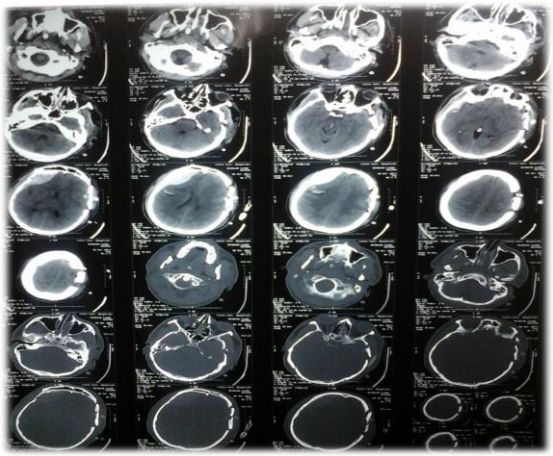

Figure 9: Post-operative CT brain of Case 4 showing RT frontal EDH and hemorrhagic infarction

MRV showed thrombosis of the medial half of the right transverse sinus and attenuated calibre of the middle part of SSS with no evidence of complete occlusion. Anticoagulation was used clexan (60 unites subcutaneous every 12 hours) (Figure 10).

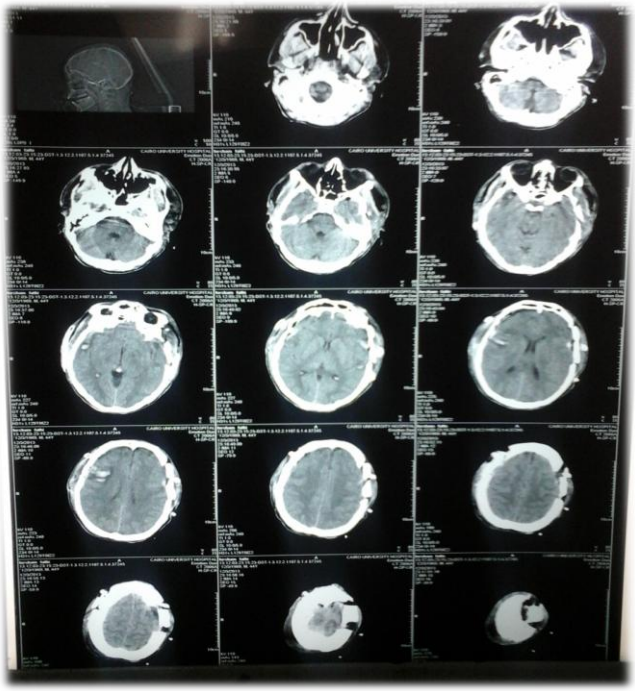

Figure 10: CT brain of case no (4) after the second operation

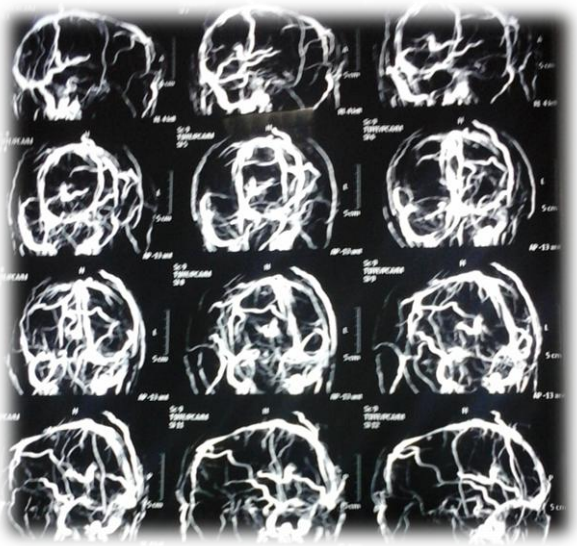

Figure 10: Post-operative MRV

Results

The data collected from 20 cases of traumatic dural venous sinus injuries in this study were evaluated. The study included 20 patients, 14 males and 6 females. Their age ranges between 2 and 55 years with a mean age of 28.5 years.

Sex

There were 6 female and 14 males. Male to female ratio was 3:1. Mode of trauma is shown in Fig. 11.

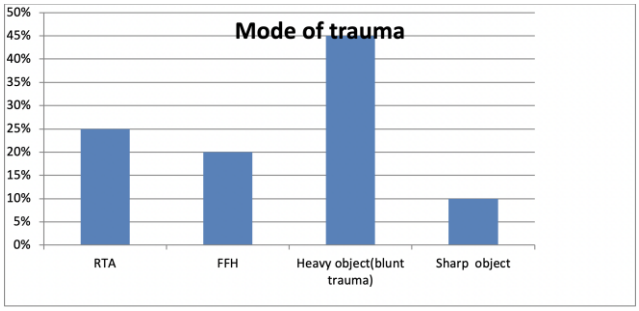

Figure 11: The mode of trauma

The GCS

Stated in Table 2 are GCS.

Table 2: GCS in the patients

No of patients	GCS	Percent
3	5/15	15%
2	7/15	10%
2	9/15	10%
4	14/15	20%
9	15/15	45%

The site of sinus injury

Table 3 shows the various dural sinuses injuries according to their localisation.

Table 3: The various dural sinuses injuries according to their localisation

The localisation of sinus injury	No of patients	Percent
Superior sagittal sinus	15	75%
Anterior part		
Middle part	6	
Posterior part	9	
	1(in combination with TS)	
Transverse sinus	3	15%
Sigmoid sinus	1	5%
Multiple sinuses	1(TS with the posterior part of SSS)	5%

The lesions associated with the sinus injury

The associated injuries were present in 20% (4 out of 20 cases) mainly orthopedic fractures of long bones (2 cases) 10%, hemothorax with fracture ribs (1 case) 5% and mild abdominal collection (1 case) 5%. The case with hemothorax and the two cases with fractures of long bones died indicating more severe trauma to the patients.

Table 4 shows different intracranial lesions associated with dural sinus injuries.

Table 4: Different intracranial lesions associated with dural sinus injury

The lesion	No of patients	Percent
CD#	7	35%
EDH	5	25%
ASDH	2	10%
Multiple lesions	6	30%

The operative techniques used in the treatment of sinus injury

Figure 12 shows different operative techniques used in the treatment of dural sinus injury.

Figure 12: Different operative techniques used in the treatment of dural sinus injury

In this study, 12 cases passed without any complications, either intra-operative or post-operative. Five cases had severe intra-operative bleeding and take blood transfusion, two cases of our study were complicated with extradural collection in the contralateral side which were managed by surgical evacuation, two cases were complicated by intracerebral hematoma one of them was managed conservatively and the other surgically, one case was

complicated with post-traumatic sinus thrombosis and hemorrhagic venous infarction, and one case was complicated with complete transverse sinus occlusion after free muscle duroplasty leading to hemorrhagic venous infarction and contralateral side weakness. As stated in (Table 5 and Table 6).

Table 5: Complications in the studied group

Complication	No of patients
No	12
Massive intraoperative bleeding	5
EDH collection on the other side	2
ICH collection	2
Post-traumatic sinus thrombosis	1
Complete transverse sinus occlusion after free muscle duroplasty	1

Glasgow outcome scale

The overall mortality (Grade I) was 15%, (Grades II vegetative) and (grade III severely disabled) were 0%, (Grades IV moderate disability) was 5% and (grade V functional recovery) was 80%.In our study, 3 patients died due to associated intra cranial lesions and other coexisting injuries in polytraumatized patients as haemothorax and bone fractures. The remaining 17 patients returned to daily living activity in the follow up period. In all patients the outcome was dependant on the primary and secondary brain injury and coexisting injuries in polytraumatized patients.

Table 6: Need for further surgical intervention

Further surgical intervention	No of patients
No	17
Yes	3

Discussion

Traumatic brain injury (TBI) is frequently encountered in the emergency department, and one of the most common causes of morbidity and mortality in developing countries. The traumatic dural venous sinus injury is one of the most dangerous complications of TBI, with its incidence to be raised to 4-12% of all TBI, with a reported mortality rate of 41% [1].

To understand the leading causes of the dangerous complications of traumatic dural venous sinus injury, we reviewed the literature and published papers which were concerned with the previous. So, we realised that either fatal intracranial compressing venous bleeding, or disturbing the intracranial pressure, which could be caused by injury to the SSS [2]. It is believed that cerebrospinal fluid (CSF) drains through the arachnoid granulations into the superior sagittal sinus [3].

The hematoma due to sinus bleeding may compress the SSS leading to impairment of cerebral venous drainage, so the absorption of cerebrospinal fluid is impaired, which may lead to the presence of venous blood hypertension and hydrocephalus leading to increasing intracranial pressure with restricted cerebral blood supply then occur, upon that, patients may be presented with clinical picture of increased intracranial pressure. If intracranial hypertension develops, it can induce uncal herniation and reflex brady-cardia, which may then result in sudden death. On the other hand, post traumatic dural sinus thrombosis is considered a rare complication which may lead to hemorrhagic infarction with its serious consequences including epilepsy, neurological deficits, or death [4].

The common causes of intracranial dural venous sinus thrombosis include head and neck infections, pregnancy and puerperium, use of oral contraceptives, and dehydration [5]. Following head injury, skull fractures or intracranial hematomas can cause thrombosis either by direct compression of the sinus [6] or by damaging endothelial lining of the sinus wall which will be followed by activation of the coagulation system resulting in sinus occlusion. Uncommonly, sinus thrombosis can occur after mild closed head injury with suture diastasis [5]. Early detection is important as early management with anticoagulation of this potentially treatable condition will result in good outcome, and thrombi in the sinuses frequently recanalize with time due to fibrinolysis [5].

Treating traumatic dural sinus injuries puts a high demand on every neurosurgeon as this kind of injury cannot be diagnosed in all cases pre operatively. Therefore, knowledge of appropriate treatment of this kind of head injury is essential. Recent development in computed tomography (CT) scan in the form of 3D reconstruction is helpful in this scenario as the preoperative knowledge of the anatomical site is essential for proper planning of surgical management of dural sinus injury. Sinus injury should be suspected if preoperative CT shows hematoma overlying venous sinuses, or fractures which crossed the sinus [7].

Follow up MRV should be a good definitive diagnostic tool for those suspected cases especially if the patient starting to have manifestations of increased intra cranial pressure or follow up CT showed evidence of venous infarction in the form of hyperdense petechial hemorrhages and hypo dense edema may be seen in the cortical grey matter and sub cortical white matter due to sinus obstruction. The other challenging issue is how to deal properly with the massive bleeding coming from the injured dural venous sinuses during surgically treated head injured patients. So we reviewed The literature that was concerned with the proper dealing with that issue which stated that Small holes or tears of the venous sinus can be managed with either by using absorbable haemostatic materials as Gel foam (gelatin compressed sponge) and Surgicel or fibrillar (oxidized regenerated cellulose), or by gentle pressure with baking with cottonoid bads [8], [9].

Direct closure of tears can be performed if it does not result in sinus stenosis. For larger ruptures, patch repair using a vein graft, per cranium or the fascia lata is required [10], [8].

If the hemorrhage is out of control, ligation can be performed in non-critical areas as in the first quarter of the SSS. A temporary sinus- sinus shunt may be necessary to properly repair the sinus tear without compromising sinus blood flow [11].

There are some respectable technical aspects, as follows, that may greatly facilitate proper management. Before the elevation of the bone fragments, preparations for rapid haemorrhage and air embolism should be in place and an assistant should be ready to manoeuvre the operating — table at a moment's notice [12]. Continuous generous irrigation over the sinus during the elevation of the bone fragments reduces the chance of embolism. Wet swabs should be at hand to immediately cover the sinus. The semi-sitting (lounging) position allows a good venous return without increased intracranial pressure. The operative exposure should be as extensive as possible. The skin flap and craniotomy should extend across the midline to permit visualisation of both sides of the sinus [13].

The bridging veins, especially in the rolandic outflow area, should be preserved [14]. To facilitate venous sinus patency after surgery, blood pressure, volume and viscosity must be carefully monitored.

To supplement knowledge in this field, we describe here the results of 20 patients with traumatic dural venous sinus injury were managed in Cairo University hospitals, in the emergency department.

In our study, male: female ratio was almost (2:1) [14 males and 6 females]. Similarly, other studies reported more incidence of trauma in males. e.g. [15], [16] found that 61.9% of people with TBI were males. Also, Andersson et al., at 2003 [17] concluded that TBI in Sweden males had 1.46 higher rate than females.

In our study we found that 85% of patients were in this age group (15-45 years), similarly, Gan et al., (2004) [18] concluded that the incidence of TBI peak in the younger patients aged 20-40 years.

In our study, the mortality increases with age, the incidence of mortality in patients > 40 years was 50%, while in patients < 40 years was 0%. Similarly, other studies reported that morbidity and mortality increase as age increase, with a mortality rate of 70% in patients older than 40 years [19], [20], [21].

The mechanism of injury had no significant difference in mortality rate. There was no significant difference in the prognosis between males and females in spite of a 3:1 male predominance. Similarly, other studies had the same results [22].

Mohanty et al., 1995 [23] and Kuday, et al., 1994 [24] have reported that lower GCS correlated with a more unfavourable outcome. In our study, 9 cases have GCS 15/15 45%, 4 cases with GCS 14/15 20%, 2cases with GCS 9/15 10%, 2 cases with GCS 7/15 10%, and 3 cases with GCS 5/15 15%, and there is mortality rate 75% (3 cases) in patients with GCS under 8/15.

In our study, we found that the SSS is the most common dural sinus injury, the anterior and central parts of the SSS 75% of cases, the transverse sinus injury 15% of cases, the sigmoid sinus injury 5% and combined injuries of different dural sinuses 5%. Similarly, other studies reported that SSS is the most injured sinus and the central part is the most affected e.g. [1], [15], [25].

Meier U et al., 1992 found that 69% of the cases with traumatic dural sinus injury had a closed head injury together with intracranial hematoma as EDH and ASDH, 31% of cases had an open head injury, but the results of our study didn't match with this result, as 65% of the cases with traumatic dural sinus injury had an open head injury while 35% of cases had a closed head injury together with intracranial hematoma as EDH and ASDH. As in our study, we had 7 cases (35%) with compound depressed fracture, 5 cases (25%) with EDH, 2 cases (10%) with ASDH and 6 cases (30%) have multiple cranial lesions, mainly compound depressed fracture associated with other intracranial lesions as, EDH, ICH and brain contusions, and this result correlates with [15], [25]

In our study, 10 cases (50%) the bleeding is controlled by direct compression by gel foam for few minutes, 5 cases (25%) the bleeding is controlled by direct application of gel foam on the sinus injury followed by stitching the dura up. In 4 cases (20%) the bleeding cannot be controlled by direct compression by gel foam only due to larger tears and needed direct closure of tears by sutures followed by gel foam compression. One case (5%) the bleeding is controlled by free muscle flap duroplasty, but during operation after control of sinus bleeding, there was sudden severe brain oedema with herniation of brain through the craniotomy, so we could not replace the bone flap again during the operation. The case was complicated by contralateral hemiparesis. Post-operative CT brain was done revealed hemorrhagic infarction. MRV was done that revealed complete obstruction of the transverse sinus. Anticoagulation and dehydrating measures were used with regular physiotherapy, and there was a gradual improvement. In the study of Ozer FD, et al., 2005 [15] massive blood loss occurred intra operatively could be controlled by digital pressure with gel foam or with a free muscle flap.

In this study, 12 cases passed without any complications, either intraoperative or postoperative. Five cases had severe intraoperative bleeding and take blood transfusion from (2-3 L). Two cases of our study were complicated with delayed extradural

collection in the contralateral side which was managed by surgical evacuation. The cause of this contralateral delayed EDH in our two cases was bleeding from the superior sagittal sinus in one case, and multiple fissure fractures in the other case. We reviewed the literature and published papers which were concerned with delayed EDH; we found that delayed EDH is one which either is not present or is in insignificant amount on initial CT scan but is found in significant quantity on subsequent CT scan. It comprises 9-10% of all EDH [26]. Almost half of them occur after a craniotomy to relieve another hematoma, possibly caused by loss of tamponade effect on the bleeding vessel [27], and are often related to a skull fracture of the overlying bone [28]. Low ICP, high BP, rapid correction of hypotension favours development of delayed EDH. Low ICP can bring about intracranial bleeding by itself without trauma as in cases of extracerebral haemorrhage complicating shunt surgery, ventricular and subarachnoid drainage, spinal anaesthesia, posterior fossa and spinal intradural operations [29].

Two cases were complicated by intracerebral hematoma; one of them was managed conservatively and the other surgically with no any apparent source of bleeding during the operation. One case was complicated with complete transverse sinus occlusion after free muscle duroplasty leading to hemorrhagic venous infarction and contralateral side weakness. One case had post-traumatic sinus thrombosis and hemorrhagic venous infarction without any neurological deficit just headache. Anticoagulation was used, and there was a gradual improvement.

In our study, 3 patients died due to associated intracranial lesions and other coexisting injuries in polytraumatised patients as haemothorax and bone fractures. The remaining 17 patients returned to daily living activity in the follow-up period. In all patients the outcome was dependant on the primary and secondary brain injury and coexisting injuries in polytraumatised patients, and this results nearly similar to the results of the study of Ozer FD, et al., 2005 [15] in which 2 cases died due to associated intracranial lesions, the remaining 15 patients returned to daily living activity in the follow-up period and the clinical success was 88%. In our study, clinical success was 85%.

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Indonesian Version of Addiction Rating Scale of Smartphone Usage Adapted from Smartphone Addiction Scale-Short Version (SAS-SV) In Junior High School

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Abstract

BACKGROUND: The increase of smartphone user among Indonesian junior high school students, particularly for teenagers, indicates the addiction factor of the smartphone.

AIM: This research is designed to adapt the Smartphone Addiction Scale-Short Version (SASS-SV) to Indonesian version based on cultural adaptation of the rating scale.

METHODS: This study involves 300 participants consisted of 151 of male children and 149 female children with an average age is 13.27 years-of-old. The validity of concurrent was used to obtain the validity, while the internal consistency and Receiver Operating Characteristic (ROC) were conducted to confirm the reliability of the rating scale.

RESULTS: In purpose to measure internal consistency, the Cronbach alpha has been applied. The Cronbach alpha value was 0.740, and Concurrent validity was checked to NMP the Nomophobia Questionnaire (NMP-Q) based on Indonesian version. The analysis of ROC showed that the value of Area under the Curve (AUC) was 0.997 (0.990-1.000), with cut-off value accounted for ≥ 32, a sensitivity value of 0.91 and specificity value was 0.973 for the male children. On the hand of female children, the results showed similarity with the AUC was 0.996 (0.998-1.000), and the cut-off, sensitivity and specificity values were accounted for ≥ 34, 0.91, and 0.974 respectively.

CONCLUSION: The Indonesian version of SAS-SV provided acceptable validation results as well as the reliability, and this version can be used to evaluate the smartphone addiction in Indonesia.

Introduction

Since the release of the smartphone, the anomaly usage of the smartphone has emerged a question which is whether the usage of a smartphone can cause addict [1]. A smartphone is a device which is consisted of a combination of state-of-the-art features so that people could send messages, make a phone call, listen to music and play games. However, a few experts have reported that several users become highly dependent on their smartphones without realising the condition itself [2].

In these days, addiction is not only indicated by the consumption of drugs and matter but also it implies to gambling, the Internet, games and even smartphones [3]. The term of addiction of smartphones, mobile phone dependence, and compulsive mobile phone use, has been coined for the same phenomenon, which shows the happiness during using their smartphones and abandon their daily activities [2].

According to the survey conducted by the National Information Society Agency (NIA) in 2012, the percentage of Korean teenagers which were categorised addicted to their smartphones had been reported to be increasing annually [4]. In Indonesia, the statistical data from the Information and Communication Technology in the past three years, the use of the Internet had elevated significantly started from 15% in 2014 to 51% in the year of 2017, placing Indonesia as a nation with high growth of internet users, five times more than the global average between the year of 2016 and 2017. The number of connected users to smartphones in

Indonesia had surpassed the total population in 2014, around 112%. This number was highly significant with the average users of the smartphone to Indonesia population [5].

It is unclear about which instrument is valid to measure the potential causes made by the usage of smartphones [6]. Several self-report questionnaires have been developed to measure the addiction scale caused by smartphones since recent years. Generally, the smartphone addictions were consisted by four main aspects, which were compulsive behaviours, tolerance, withdrawal and functional misbehaviour, which was identical to the internet addiction features [3], [7]. In South Korea, the NIA has developed the Korean Scale for the Internet Addiction (K-Scale) and Smartphone Scale for Smartphone Addiction (S-Scale), and this scale has been improved into a scale to measure the addiction level to the smartphones, and it was called the Smartphone Addiction Scale (SAS) [8]. This scale also has a short version, which is called a Smartphone Addiction Scale-Short Version (SAS-SV) [3], the Cronbach's alpha coefficient was 0.91. This short version scale had been considered as one of the instruments that had been developed and validated [6]. However, in Indonesia, with the increasing number of smartphones, no addiction instruments have been validated to measure the smartphone addictions.

This research is designed to adapt the Smartphone Addiction Scale-Short Version (SAS-SV) into Indonesian version. In this study, we performed the validity of concurrent to obtain the validity, whereas the internal consistency and Receiver Operating Characteristic (ROC) were conducted to confirm the reliability of the rating scale. This scale is adapted to be able to evaluate the presence of smartphone addictions in Indonesia. From our knowledge by literature search, this study is the first that validates the rating scale of smartphone addiction in the province of Sumatera Utara, Indonesia.

Material and Methods

Participants

In this study, the participants are 300 junior high school students from two schools that are located in the city of Medan, North Sumatra Province, Indonesia. One hundred fifty-nine are boy students and 149 girl students with an average age are 13.27 years-old. This study was conducted after the informed consent written and signed by both of the participants and the schools. The ethical clearance that was followed was based on the Nuremberg Code and the 1964 Helsinki Declaration, and each participant received informed consent before participating in this study. This research was approved by the Health Research Ethical Committee, Medical Faculty of Universitas Sumatera Utara, with approval No: 685 / TGL / KEPK / FK USU-RSUP HAM / 2018.

Measurements

All the statistical analysis was performed by using the IBM SPSS statistics 22 with as following parameters: 1. Socio-demographic Characteristics. Socio-demographic characteristics were presented descriptively; 2. Concurrent Validity of SAS-SV Indonesian Version. The analysis of concurrent validity of SAS-SV Indonesia version was conducted by performing the Pearson's correlation with NMP-Q, to determine the smartphone addiction of the participants. The NMP-Q Cronbach alpha value 0.931 and had been validated to Indonesian version [9]; 3. Internal Consistency Reliability for the SAS-SV Indonesian Version. To verify the reliability of the instruments, the Cronbach's alpha correlation coefficient was used; and 4. Receiver Operating Characteristic (ROC) of SAS-SV Indonesian Version. To determine the ability of the SAS-SV Indonesia Version, we performed the ROC analysis to predict the addiction based on the scale. Also, to measure the diagnostic ability with sensitive and specified results, the Area Under the Curve (AUC) of the ROC was analysed by selecting every alternative intersection within the graph resulted from the ROC.

Results

The processes of validation and reliability tests were conducted in two junior high schools in Medan, during the study hours at both schools. The research subjects were recruited by performing the non-probability sampling, i.e. purposive sampling was performed due to the restriction regulated by schools' policies, in which to be the research subjects, they must be junior high school students with an average age between 12 and 15 years-of-old who have smartphones. Before the survey, all the informed consent and ethical clearance have been obtained from the schools.

Table 1: Socio-Demographic Characteristics of Subjects

Variables		n	%
Sex	Boy	151	50.3
	Girl	149	49.7
Age	12 YoA*	52	17.3
	13 YoA*	121	40.3
	14 YoA*	120	40.0
	15 YoA*	7	2.3
Purposes in using smartphones	Social Media:	156	52.0
	* Facebook	5	1.7
	* Instagram	41	13.7
	* Line	22	7.3
	* Twitter	31	10.3
	* WhatsApp	57	19
	Entertainment (Mp3, Watching Movies, Playing Games)	127	42.3
	Internet Browsing (Google, Yahoo.)	17	5.7

*YoA = Years of Age.

The number of 300 students was obtained to be classified as the research subjects, and the following Table 1 is the socio-demographical characteristics of the subjects.

According to the Table 1, from 300 participants, it was obtained that 151 (50.3%) students were boy, while the 149 (49.7%) students were girl, with an average age was 13.27 years old. The social media factors were the main reason from the samples in using the smartphones, and the over the half of the population chose these reasons accounted for 156 subjects (52.0%) followed by 127 subjects (42.3%) with entertainment reasons such as listening to music, watching movies or playing games. Then, 17 subjects (5.7%) used their smartphones for browsing the Internet. The following Figure 1 shows the smartphone addiction based on the gender using SAS-SV Indonesian version.

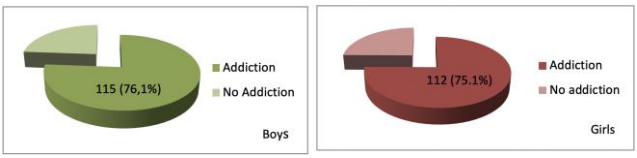

Figure 1: The percentage of smartphone addiction based on sex

Figure 1 present that the proportion of addiction in boy students was 76.1% and in a girl, students were 75.1%. Table 2, to the boy students, the highest percentage in using the smartphone is for entertainment which accounted for 94 people (62.3%), while for the girl students; almost a three-quarter of the samples chose social media for the purposes, accounted for 109 students (73.2%). Meanwhile, the least percentage of the purposes for both sexes when they use the smartphones is for internet browsing which accounted for 10 (6.6%) for boys and 7 (4.7%) for girls.

Table 2: Purpose in using smartphones based on sex

Purposes in using smartphones	Sex	
	Boy students	Girl students
Social media (Facebook, Line, Instagram, Twitter and WhatsApp)	47 (31.1%)	109 (73.2%)
Entertainment (Mp3, watching movies, playing games)	94 (62.3%)	33 (22.1%)
Internet browsing (Google, Yahoo)	10 (6.6%)	7 (4.7%)

Concurrent Validity

The concurrent validity was performed to confirm the validity of SAS-SV Indonesian version, which the scores resulted from the SAS-SV Indonesian version and NMP-Q are compared. The following Table 3 shows the correlation between SAS-SV and NMP-Q.

Table 3: The correlation between SAS-SV and NMP-Q

	NMP-Q
SAS-SV	r = 0.558 p < 0.001 n = 300

From Table 3, we use the Spearman test to analyse the data because the data was not normally distributed. In this validation test, the SAS-SV of Indonesian version is adapted from the original score which was constructed in South Korea in the form of English [3], [8], while the internal consistency shows the value of 0.931. In accordance with Indonesian culture, it has been classified as the value of r which in interval 0.5 to < 0.6 is classified as Medium [10].

Internal Consistency Reliability for SAS-SV Indonesian Version

The internal consistency reliability analyses were performed due to the adaptation process of SAS-SV into Indonesian version. The following Table 4 shows the internal consistency reliability of SAS-SV Indonesian version [11].

Table 4: The Internal Consistency Reliability of SAS-SV Indonesian version

No	Items	*Corrected Item / Total*	*Alpha if Item Deleted*
1	Kehilangan rencana kerja disebabkan oleh penggunaan smartphone *Missing planned work due to smartphone use**	0.308	0.723
2	Kesulitan Konsentrasi di kelas, sedang melakukan tugas atau sedang bekerja disebabkan penggunaan *smartphone.* *Having a hard time concentrating in class, while doing assignments, or while working due to smartphone use.**	0.381	0.722
3	Merasa nyeri di pergelangan tangan atau bagian belakan leher selama menggunakan *smartphone.* *Feeling pain in the wrists or at the back of the neck while using a smartphone.**	0.301	0.734
4	Tidak bisa bertahan karena tidak memiliki *smartphone.* *Won't be able to stand not having a smartphone.**	0.428	0.715
5	Merasa tidak sabaran dan resah saat saya tidak memegang *smartphone* saya. *Feeling impatient and fretful when I am not holding my smartphone**	0.514	0.702
6	Memikirkan *smartphone* saya walau saya sedang tidak menggunakannya. *Having my smartphone in my mind even when I am not using it**	0.432	0.715
7	Saya tidak akan berhenti menggunakan *smartphone* saya walapun kehidupan harian saya telah terpengaruh karenanya *I will never give up using my smartphone even when my daily life is already greatly affected by it.**	0.397	0.721
8	Mengecek secara konstan *smartphone* saya sehingga tidak ketinggalan percakapan di Twitter atau Facebook *Constantly checking my smartphone so as not to miss conversations between other people on Twitter or Facebook.**	0.320	0.732
9	Menggunakan *smartphone* lebih lama dari yang saya inginkan *Using my smartphone longer than I had intended.**	0.499	0.706
10	Orang-orang disekitar saya mengatakan bahwa saya menggunakan *smartphone* terlalu sering *The people around me tell me that I use my smartphone too much.**	0.432	0.715

*Adapted with permission from [3].

From Table 4 above, the result that was analysed from the internal consistency reliability instruments of SAS-SV Indonesia version had the reliability value; with the Cronbach's alpha value is 0.740. This value is acceptable. Based on Table 4, the values of the corrected item and the total item correlation are over 0.3, which indicate ten validated statements [10].

The Receiver Operating Characteristic (ROC)

In this study, we performed the analyses of Receiver Operating Characteristic (ROC) as well as the Area Under The Curve (AUC) for determining the addiction scale. Table 5 below shows the analysis of ROC with the parameters required.

Table 5: The Results of Analysis Receiver Operating Characteristic (ROC) Based on Sex

Sex	AUC	CI 95%	Cut-off	Sensitivity	Specificity
Boys	0.997	0.990 – 1.000	31.50	0.974	0.973
Girls	0.996	0.998 – 1.000	33.50	0.910	0.974

AUC, the area under the curve; CI, confidence interval.

The cut-off value of SAS-SV Indonesia version obtained for the boys was ≥ 31.5 with sensitivity and specificity percentages accounted for 97.4% and 97.3% respectively. The girl's students showed similarly, with the cut-off value was 33.5, followed by sensitivity and specificity, respectively 91% and 97.4%. Based on the scores, the subjects that are diagnosed to in experiencing the smartphone addiction produce a score for boys is ≥ 32, while for the girls is ≥ 34.

Discussion

To obtain a good measurement, the instruments are properly translated from the original language (English version) to the Indonesian version which is based on the targeted areas and cultures. Therefore, forward and backward translations were conducted in this study so that the subjects can understand the questionnaires. It means that the instruments must have to be the same, acceptable, conducted within the same guidance [12]. The forward translation which is considered to be valid requires at least two translators. Bilingual translators must be able to translate into their mothers' languages because of the awareness factors, and it is recommended to employ aware and unaware translators in determining the concepts of the questionnaires [13], [14]. Unaware translators are expected not to understand or inform the concepts of the questionnaires, and they have no medical or clinical educational backgrounds. It is exactly the same as the forward translation; the backward translation requires two translators.

In this study, the subjects are teenagers with interval ages from 12 to 15 years-old. All the subjects in this study own and use smartphones. In the original version of SAS-SV, the study has chosen the second grade of junior high school students in South Korea, so that in this study no limitation of educational levels due to the purpose in determining the smartphone addiction among the teenagers [3]. Over three-quarter of the junior high school students is addicted to their smartphones, which accounted for 76.1% for boys and 75.1% for girls. Kim et al. have argued that teenagers these days are easy to accept a new media of communication, such as smartphone [15]. As the first generation within the family, teenagers are the first members of the family that grows surrounded by sophisticated media. This also means that teenagers are more vulnerable to be affected by the bad effects of smart than those experienced by adults. For teenagers, communications based on the telephone are important factors in maintaining their social relationships [15]. On the other hand, they also depend on their parents due to the change in their physical and psychological attributes. However, they must also be independent during the growth, and smartphones are highly necessary for their life [16].

From Table 1, the main purpose of using the smartphones done by the teenagers is for social media, with over half of the samples population (52.0%). The most common social media applications used by the teenagers are WhatsApp (19%), Instagram (13.7), Twitter (10.3%), Line (7.3%) and Facebook (1.7%) because these types of social media are built-in to texted message services. These results are also concordance to the study conducted in South Korea [8] which Social Networking Sites (SNS) or Messenger, such as Facebook, Twitter, Kakao Story and Kakao Game due to their integrated systems to text message services [3]. In this study, the gender categories were analysed to determine the smartphone addiction. Based on Table 3, the entertainment purpose was the highest reasons chosen by the boy students for using their smartphones with the percentage of 62.3%, while the girl students used their smartphones for social media reasons, which accounted for 73.2%. Over half of the population, both boy and girls' students have spent their times for social interaction, such as the SNS. Overused of SNS while using their smartphones implies significantly to their academic performances, whereas a gaming smartphone that is connected to other user provides easy interaction throughout the others social networks [15].

The concurrent validity test of SAS-SV Indonesia version and NMP-Q shows r = 0.558, which contributes medium correlation result. This shows that the SAS-SV and NMP-Q are not completely identical, although it has a medium correlation. The internal concurrent reliability test obtained displays a good result (0.740), with Cronbach's alpha value is >0.6 based on the analysis. The average score of SAS-SV Indonesia version for the male students was 35.09 while the female student's score was 36.11, and the cut-off value which is analysed from the ROC test of both genders could be determined. The cut-off value of SAS-SV Bahasa Indonesia version for the boys was ≥ 31.5 with sensitivity and specificity values were 97.4% and 97.3% respectively, and these values were insignificantly different to the girls had ≥ 33.5 with 91%

of sensitivity and 97.4% of specificity.

To the best of our knowledge, this Indonesian version of SAS-SV provided acceptable validation results as well as the reliability, and this version can be used to evaluate the smartphone addiction in Indonesia. In this study, the limitation is on the uncontrollable demographical factors as the demographic results showed the ratio of the samples almost 1:1. Thus, this Indonesia version was considered to be used for identifying the smartphone addiction for teenagers aged between 12 to 15 years old. Further study needs to be conducted regarding the characteristics of the subjects.

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Medical and Social Factors of Pediculosis

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Abstract

Pediculosis is a global problem in public health. An important factor in the efficient eradication of lice is ensuring adequate recognition and treatment of the disease by the population. In the present study, awareness of the population about the physiological properties of head lice, the ways of infestation with head lice, and the methods of treatment and prevention were studied. Perception of the disease by the people who had had head pediculosis and other people around them was identified.

Introduction

Pediculosis is the most wide-spread parasitic disease in the world, regardless of the level of the economic development of the country [1]. According to the World Health Organization, several billion people around the world are susceptible to the permanent risk of infestation with pediculosis. The highest pediculosis incidence rate was observed in developing countries of Central America, Asia, and Africa [2], [3], [4], [5], [6], [7]. However, the growth of population infestation with pediculosis, primarily head pediculosis, has also been noted in prosperous socio-economic countries: the UK, Norway, the USA, and others [8], [9], [10], [11]. In Russia, up to 300 thousand cases of pediculosis are registered every year. According to Rosstat, the number of reported cases of pediculosis in 2010 amounted to 272.8 thousand people, in 2015 — 243.5 thousand, and in 2016 — 212.7 thousand. Pediculosis takes fourth place in the structure of infectious morbidity of children aged 0 – 14 years. In 2010, the number of reported cases of pediculosis among children aged 0 – 14 years was 52.0 thousand people, in 2015 — 58.2 thousand, and in 2016 — 52.1 thousand [12]. The number of cases of pediculosis is much greater since only the cases reported by the patients to medical institutions, or identified by routine inspections in kindergartens, schools, hostels, orphanages, nursing homes for the elderly, etc. are considered [13]. Because of the aforesaid, analysis of the effect of medical social factors on the pediculosis incidence rate is very relevant and requires both reassessing the effect of these factors and finding ways to optimise the organisation of the preventive measures in the modern conditions.

Material and Methods

To identify the medical and social factors that affect the spread and prevention of head pediculosis, a sociological study was performed, for which three questionnaires had been developed: for the

population — 20 questions, for pharmaceutical specialists — 18 questions, and for dermatologists — 10 questions.

The questions were presented technically as closed, semi-closed, or open, in terms of the kind as qualitative, where, depending on the question, the respondents were asked to choose one or several variants of the answer, or write their own opinion. The questioning was performed anonymously in writing. The results of the study were processed using the tools of mathematical statistics.

The study involved three groups of the respondents: the first group — 580 inhabitants of Moscow and the Moscow region (23.5% males and 76.5% females) in all age groups: 12% — 18 to 20 years of age; 47% — 21 to 30 years of age; 20% — 31 to 40 years of age; 17% — 41 to 50 years of age; 3% — 51 to 60 years of age; and 1% — older than 61 years; the second group — 115 pharmacists that were members of pharmacy organizations of Moscow and the Moscow region. The competence of the specialists in the topic of the study was determined by two criteria: occupied position — qualified pharmaceutical chemist (51%) and pharmacist (49%); work experience in pharmacy organizations — most respondents (32%) had 5 – 10 years of experience, 27% — 11 to 20 years, 25% — under three years, 16% — over 20 years. The third group were doctors of the dermatovenerologic dispensary that covered 220 thousand people of the population of a city in the Moscow region — eight dermatovenerologists (100% sampling) with the work experience of 10 to 20 years — 25%, over 20 years — 25%, and under 10 years — 50%.

Results

As a result of the sociological study of the population, it was found that 62% of the respondents had had head pediculosis. Most respondents (44%) were infected with head pediculosis at school, 36.5% — in the kindergarten, 10% — at professional educational institutions (higher educational institution, college, etc.), and 9.5% — on vacation. The immediate circle of 67% of the respondents also had head pediculosis, namely: brother/sister — 29% of the respondents; parents — 18%; children — 22%; and friends — 31%. The information obtained is an evidence of the high epidemicity of the pediculosis disease among minors, which is consistent with the judgment of practicing dermatovenerologists, qualified pharmaceutical chemists, and pharmacists of pharmacies. According to the opinion of 33.5% of the respondent doctors and 46% of the pharmaceutical specialists, patients with head pediculosis are children up to seven years of age, according to the opinion of 55.5% of the respondent doctors and 27% of the pharmaceutical specialists — children between 7 and 18 years of age, according to the opinion of 11% of the respondent doctors and 25% of the pharmacy specialists — women, according to 2% of the pharmaceutical specialists — men.

The next module of questions was dedicated to the progress of the disease in those who had had pediculosis; therefore, analysis of this module did not contain the respondents who stated that neither they nor their immediate circle had had faced this problem.

For timely treatment and reducing the risk of spreading scalp pediculosis, early diagnosis of the disease is of paramount importance. A significant majority of the respondents (41%) found head pediculosis by experiencing one of the symptoms — increased itchy scalp [14]. Public prevention of head pediculosis consists of active identification of patients through preventive medical examinations — using this method, head pediculosis was detected in only 16% of the respondents. Preventive inspection at home identified pediculosis in 27% of the respondents; and in 1% of the respondents, pediculosis was detected by a hairdresser.

Despite the fact that in 15% of the respondents pediculosis was found after obtaining information that people from their inner circle had lice, the survey showed that upon infestation with pediculosis, one third of the respondents had not informed anyone about the disease (28%), which had been one of the reasons for epidemics development. Another third of the respondents informed only their inner circle about the disease: friends — 9%, parents of children from their inner circle — 16%; and classmates — 11%. Only one-third of the respondents informed officials (class teacher/tutor — 16%; health care worker at school/kindergarten/college — 20%) about the disease, timely action of whom might have a significant effect on reducing the epidemic of head pediculosis at a particular school or an educational institution.

The reasons for officials' low awareness about pediculosis and nondisclosure of this information revealed the next set of questions aimed at identifying the psychological state of a patient with pediculosis.

The results of the survey showed that 61% of the respondents bothered whether people around them would learn about their infestation with lice. This circumstance is explained by the reaction of other people to the respondents at the time they were infected with lice. Forty-three percent of the respondents experienced a negative attitude to themselves in the form of contempt (3%), disgust (15%), and apprehension (25%). Twenty-seven percent of the respondents were sympathized with, i.e., were understood and shared their negative emotions with. Only one-third of the people around treated the patients kindly (7%), or neutrally (23%).

Negative feelings and emotions in case of

pediculosis were experienced by 86.5% of the respondents: upset state – 25.5%; shyness — 20%; shame — 19.5%; fear of becoming an outcast — 9%; fear of being mocked at — 12.5%, and only 10% of the respondents didn't care, and 3.5 % remained calm (Figure 1).

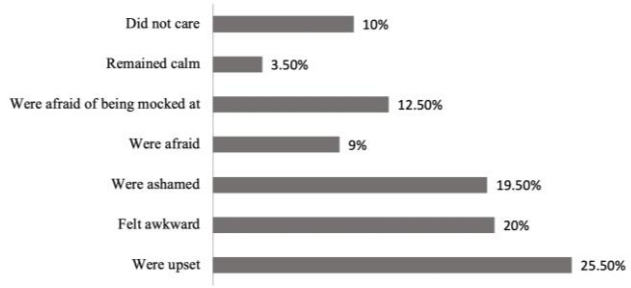

Figure 1: Feelings of the respondents with pediculosis

Questioning the population has shown how the attitude of the respondents to a person with pediculosis changes: 10% will forever cease contacting and communicating with the patient; 45% will reduce communication and contacts for the period of the disease; only 45% of the respondents will not change their attitude to the patient.

The obtained data are consistent with the opinion of the pharmaceutical specialists, 81.5% of whom indicated that pharmacy visitors asking for a pediculicidal product had negative emotions: embarrassment (35%), shame (13.5%), and anxiety (33.5%).

The time for which respondents or their relatives infected with pediculosis ceased attending school/kindergarten/job: one day — 6% of the respondents, 3 – 7 days — 31%, 10 – 14 days — 16%; with that, 35% of the respondents returned to their normal life immediately after the single treatment of the head. 12% of the respondents did not interrupt the educational/work process, which fact speaks of low awareness of the respondents about the epidemiological component of this disease.

The results of questioning show that most (78.0%) of the respondents preferred self-treatment of pediculosis, and did not seek qualified medical aid in case of infestation. The respondents only sought medical advice in case they failed to solve the problem themselves: in case of repeated infestation with lice (9%) and chronic nature of pediculosis (11.5%). Questioning of dermatovenerologists confirmed the low rate of seeking skilled medical care by patients with pediculosis [15], [16]. Fifty percent of the responding doctors noted that patients with this disease came to the clinic once a month, 37.5% — not more than twice a year.

Due to the high level of pediculosis self-treatment, it was important to find out whose recommendations the patients followed when buying pediculicidal products. According to the results of questioning, 31% of the respondents based their choice on the advice of friends and acquaintances, 29% — on recommendations of pharmacists at pharmacies, 12% — on the information from Internet sources, and 8% — on the information from the literature. 20% of the respondents bought the preparation on prescription, which coincides with the number of visits to doctors on this issue.

According to pharmaceutical specialists, the most important criteria for the consumers choosing pediculicidal product are speed and reliability of the therapeutic effect, dosage form, age limitations, and price.

Seventy three percent of the respondents infected with pediculosis used nonpharmacological methods of getting rid of pediculosis, namely, shortening or shaving off the hair — 13%; scalp treatment: with kerosene or turpentine — 18%, with salt/vinegar/alcohol solution — 11%, with cranberry or pomegranate juice — 6%; with essential oil (cranesbill, etc.) — 6%, with an infusion or a decoction of herbs — 6%, and with coal-tar soap — 13%. The inefficiency of these methods was indicated by 13% of the respondents, efficiency — by 29%, but if the method of shaving the hair off the head (which was used by 13% of the respondents) is considered, pediculosis will definitely be defeated. 18 % of the respondents noted the efficiency of the comprehensive use of the traditional medicine and pediculicidal products, which prevents characterizing the real efficiency of each method.

Discussion

The survey revealed that only 67% of the respondents were cured after a single application of a single pediculicidal product; at the same time, 20% received a prolonged treatment, with a change of the preparation; repeated infection was observed in 13% of the respondents. The obtained results may be associated with the development of resistance in lice to certain groups of pediculicidal products [4], [16].

To exclude the possibility of repeated infection and spread of pediculosis, it is important to promptly and correctly use the methods of treating clothes and household items of a patient with pediculosis [17], [18], [11]. The analysis revealed low awareness of the respondents of this issue: 13 % of the respondents did not process clothes and household items of the patient, and continued using them for other purposes; 22% thrown them away or destroyed, which was economically not sound, considering the existing treatment methods; 38% washed them normally (at 30 – 40 degrees), which was not an efficient method, since lice and nits were killed at the temperatures above 50 degrees Celsius

[19]; 14.5% put items into a refrigerator or kept them outside in the winter, this method may be used if items are placed in a freezer of a refrigerator with the temperature below 20 degrees; 8% packed items into plastic bags for two weeks, 4.5% treated the items with an iron.

Given the entirety of the respondents, pediculosis is dangerous due to the following factors: prevalence rate — 32%, epidemicity — 18%; social and psychological isolation of patients — 17%, repeated infection rate — 17%; and being the carrier of typhoid fever pathogen — 17%. Comparison of the results of questioning the doctors and population on this issue revealed unanimity of opinion (in percent) on such factors as prevalence rate, emerging socio-psychological isolation, the risk of infection with typhoid fever, but there was also a difference: the doctors interviewed did not specify the factor of epidemicity, which fact confirmed the small number of patients seeking medical help in case of pediculosis.

Stereotype "pediculosis is the disease of the poor" exists in the society, i.e., only the lowest social groups may have pediculosis; respondents were asked how true this statement was in their opinion. 84% of the respondents did not agree with this statement, moreover, a tendency to the dependence of the choice of the positive response to this assertion on the presence of pediculosis in the anamnesis of the respondent was discovered. The questioning does not prove the relevance of this stereotype in the modern circumstances either [20]: 58% of the respondents have higher professional education, 31% have secondary specialized education, the average family income of 43% respondents is 30 to 50 thousand rubles per month, the income of 25% is 50 to 100 thousand rubles per month.

To identify the level of public awareness about the physiological properties of head lice and the ways of infestation with pediculosis, respondents were asked to choose the statements that were correct in their opinion. The result showed weak awareness of the population on this topic: 46% of the respondents believed that head lice jump on to the head; 6.5% were sure that head lice could fly; 42% believed that one could catch head lice swimming in a pond, or dealing with animals; 12% were sure that only untidy people got infected; and 28% believed that people with long hair got infected more frequently. The research has shown that public awareness in the issues of pediculosis is not associated with the level of the respondents' education.

Measures were determined, which, according to the respondents, would help reduce the prevalence rate of pediculosis in the community. Seventy three percent of the respondents believed that the most efficient measure was preventive checkups in kindergartens and at schools; but as mentioned above, pediculosis was detected this way in only 16% of the respondents, which shows low efficiency and insufficient timeliness of scheduled inspections in kindergartens and in educational institutions. 39% of the respondents recommended detective combing at home in order to not only rely on preventive examinations. 56% of the respondents mentioned personal hygiene, 43% of the respondents believed that an important measure of prevention was early informing the people around about possible infection, but given the negative socio-psychological perception of the disease, it would be possible to change the situation only by modifying the stereotype of perception of pediculosis; the same measure was mentioned by 16% of the respondents. 5% of the respondents supported the opportunity of obtaining a free pediculicidal product.

Given the high prevalence rate of pediculosis and high percentage of self-treatment of this disease by the population, it was paramount to determine the level of pharmaceutical advice upon the sales of pediculicidal products.

Unfortunately, the level of pharmacists' competence in the issues of pediculosis proved to be inefficient.

In case of recommending a pediculicidal product, the age of the patient is not considered by 34% of the respondents, sensitivity to the components of the product — by 53%, presence of an asthmatic component or bronchial asthma in the diagnosis — by 29%, repeated infection — by 73%, and previously used pediculicidal products — by 81% of the respondents.

In oder to reduce the resistance to pediculicidal products, the Federal Budget Institution of Science Research Institute of Disinfectology of Rospotrebnadzor recommends certain sequences of using various products for treating pediculosis; only 7% of the pharmaceutical specialists chose the correct sequence of treatment, 38% did not choose the correct sequence, and 55% believed that the sequence was unimportant.

Instructions for the medical use of some pediculicidal products contain an indication that it is necessary to address a medical specialist about the rules of performing detection combing; questioning of the pharmaceutical specialists also showed that 25% of pharmacy visitors had asked to be advised about detection combing. The results of questioning showed low level of knowledge about the rules of detection combing by the respondents, a significant percentage of errors was about the frequency of combing and the combing procedure.

Ethics of pharmaceutical workers identified the need for consulting the patients not only about treatment but also about prevention of the disease. To reduce repeated infection and avoid the spread of pediculosis, it is crucial to inform the population about the appropriateness of treating clothes and household items of a patient with pediculosis. Only 35% of the

pharmacists reported the need for and methods of processing items of the patient upon every purchase of a pediculicidal product, 51% revealed this information only to the questions of consumers, the remaining 14% did not speak on the subject, either because they were not aware of it (7%), or believed that this issue was outside their competence (7%).

In the opinion of 88% of the pharmaceutical specialists, the most important measure to help reduce the prevalence rate of pediculosis is preventive examination in kindergartens and at schools; 56% stand for early informing of people about the possibility of infection; 52% stand for handing out an information factsheet with proper actions of the infected; and 30% believe in changing the attitude of the population to pediculosis.

In order to reduce the incidence rate of pediculosis, it is necessary to change the stereotype existing in society about this disease as a disease of socially disadvantaged people with poor personal hygiene. In the modern world, children from any social environment may have pediculosis, with that, most of them experience negative psychological state and have no desire to inform the people around about the disease. The study has shown that even a long time after the disease in childhood, adult people retain a negative impression about the disease for the entire life. Insufficient awareness about the physiological properties of head lice, the methods of infestation with pediculosis, methods of treatment and prevention, and the high degree of ignorant self-treatment are the result of the perception of this disease by the society.

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Clinical Science

Management of Maxillofacial Fracture: Experience of Emergency and Trauma Acute Care Surgery Department of Sanglah General Hospital Denpasar Bali

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Abstract

BACKGROUND: Maxillofacial fracture is one of the major trauma; the cases were increasing because of the high number of motorcycles in Bali. The goal of the treatment is proper positioning of the occlusion, and it depends on rapid diagnosis and proper treatment.

AIM: This study aims to know the characteristics of the maxillofacial fracture patients in Sanglah General Hospital Denpasar Bali.

METHODS: A retrospective study, based on medical record were concluded, samples taken in Sanglah General Hospital from January to June 2015. Total recorded patient were 35 patients. The data obtained include age, gender, type of the fractures, and operation management.

RESULTS: The injury was more common in male compared to female (80% vs 20%). Age 20 to 40 years old were more common (48.57%), followed by the child to adolescent (aged 0 to 20 years old) were 31.43%, and adult to elderly (aged 40 to 60 years old) was 20%. The mandibular fracture was most common (51.43%), other fractures such as a zygomatic fracture (31.43%) and maxillary fracture (17.14%). Internal fixation was the gold standard of the treatment (65.71%), and the other was an arch bar (34.29%).

CONCLUSION: Diagnosing the right injury to the facial bone is a key step in determining a treatment plan. Rapid diagnosis and proper treatment lead to good occlusion, both internal fixation and arch bar were an effective treatment. The importance of dealing with almost all maxillofacial fracture problems in the first surgery.

Introduction

Emergency and Trauma Acute Care Surgery has been established since 2001 at Bali, where various trauma cases are expected to be seen. Maxillofacial trauma is the highest cases seen at Sanglah Hospital Bali. Maxillofacial fracture is one of the major traumas; the cases were increasing because of the high number of motorcycles in Bali. The standard operating procedure of the management maxillofacial fracture in Sanglah General Hospital is using open reduction and internal fixation (ORIF) using mini plates and screws. This procedure has proven to be the most effective procedure associated with early mobilisation and minimal morbidity. Maxillofacial trauma also knew as facial trauma can be classified into three parts, such as the upper face (the frontal bone and frontal sinus), the midface (the nasal, ethmoid, zygomatic, and maxillary bones), and the lower face (the mandible) [1].

Fixation procedure can be performed with intermaxillary fixation (IMF) or maxillomandibular fixation (MMF) with arch bars, 4-point fixation, and mini plates [2], [3]. There are advantages and disadvantages to both methods of fixation. The closed reduction does not endanger vessels and lower costs for patients. However, this is related to a significant

period of oral immobilisation and closure and requires intact teeth. Unlike ORIF, it can get direct visualisation, reposition fractured bone segments, and restore occlusion of mandibular and maxillary patients [5]. One important factor to consider is patient compliance. About 60% of patients with facial fractures may not adhere to treatment [4], [6], [7]. The IMF or MMF is a technique to immobilise the mandibular segment by locking the occlusion externally, using the teeth as a point of stability [3], [4], [5], [7].

The aim of this study is to know the characteristics of the maxillofacial fracture patients in Sanglah General Hospital Denpasar Bali.

Methods

A descriptive retrospective study, samples taken in Sanglah General Hospital from January to June 2015. Inclusion criteria were all patients with maxillofacial fractures who underwent surgery. The total recorded patient was 35 patients with maxillofacial fractures. The data obtained from general primary data include age and gender, the type of the fractures derived from radiology expertise, and physical examination. All data is taken from medical records and surgeons records during the operation. The type of fixation in the maxillofacial fracture depends on the type of fracture. Orbital rim fractures, zygomaticomaxillary fractures, and mandibular fractures used ORIF miniplate. Simple loose teeth and alveolar process fractures used interdental wiring (IDW) and if multiple alveolar process fractures used the arch bar. If multiple fractures of mandibular bone or multiple fracture maxilla and mandible, we can immobilise the mandible with MMF. All data were analysed descriptively.

Results

The injury was more common in male compared to female (80% vs 20%). Age 20 to 40 years old were more common (48.57%), followed by the child to adolescent (aged 0 to 20 years old) were 31.43%, and adult to elderly (aged 40 to 60 years old) was 20% (Table 1).

Table 1: Distribution according to gender

Variation	n (%)
Gender	
Male	28 (80)
Female	7 (20)
Age	
0-20 years old	11 (31.43)
20-40 years old	17 (48.57)
40-60 years old	7 (20)

In Table 2, we showed a variation of maxillofacial fracture in Sanglah General Hospital. The mandibular fracture was most common (51.43%), other fractures such as a zygomatic fracture (31.43%) and maxillary fracture (17.14%).

Table 2: Distribution according to the side of the fracture

Variation of fractures	n (%)
Mandibular bone	18 (51.43)
Alveolar process of mandible	6 (33.33)
Parasymphisis mandible	9 (50)
Angle of mandible	2 (11.11)
Segmented	1 (5.56)
Zygomatic bone	11 (31.43)
Maxillary bone	6 (17.14)
Displaced	1 (16.67)
Alveolar process of the maxilla	2 (33.34)
Zygomaticomaxillary bone	3 (49.99)

Based on the mechanism of injury (Table 3), the most common cause of the maxillofacial fracture was a traffic accident (90%) followed by violent activities (8.57%).

Table 3: Mechanism of injury

Cause of injury	n (%)
Traffic accident	32 (91.43)
Violent activity	3 (8.57)

Operation technique of maxillofacial fracture in Table 4. Internal fixation was the gold standard of the treatment (65.71%), and the other was an arch bar (34.29%).

Table 4: Distribution according operation technique

Operation technique	n (%)
ORIF miniplate	23 (65.71)
Arch bar + IDW	3 (8.57)
Arch bar + MMF	5 (14.29)
ORIF miniplate + Arch bar	4 (11.43)

ORIF: open reduction and internal fixation; IDW: interdental wiring; MMF: maxillomandibular fixation.

Discussion

The common cause of maxillofacial fracture is due to a traffic accident [2], [4]. Male is predominant in our study due to their high-risk activities such as driving vehicles and their social life involving alcohol and violent activity. The age-related of maxillofacial injuries in 20-40 years old, this relates to a more active and productive situation in the life period [2], [4]. In extreme age groups, very young or old, the incidence of maxillofacial fractures will be lower due to limited activity.

Maxillofacial is the prominent site of the human body, and this makes the region is prone to suffer from trauma [7], [8]. Low level of awareness among the population to wear a full-face helmet. Indonesian standard of the helmet with chin can reduce the number of maxillofacial injuries, especially in Bali. The anatomy features of maxillofacial and all

problem mentioned above are the main factors that make the number of maxillofacial injuries quite high in Indonesia. There are several ways to diagnose maxillofacial fractures such as history taking to know the mechanism of injury and physical examination to evaluate any airway obstruction, deformity, lost teeth, and malocclusion. On palpation of maxillofacial, we evaluate false movement and pain at the temporomandibular joint during movement due to dislocation. Radiological examination such as skull anteroposterior or lateral and panoramic are the cheapest and the most commonly done. It does not just to evaluate mandible alone, but this photo can describe many parts of facial bones. More sophisticated examination such as CT (computed tomography) scan and MRI (magnetic resonance imaging) have become more popular. CT Scan can construct facial bone in three-dimension reconstruction, where we can observe in detail the type of fracture. In a developed country, MRI has more role as radiological examination in maxillofacial fracture, and in our centre, it is start done routinely. MRI primarily is used to evaluating the damage of soft tissue especially in complex fracture [8].

There are several choices of treatment in maxillofacial fractures, but the proper treatment is depending on many factors such as treatment cost, availability of tools at the hospital, doctor's skills, patient's willingness to obey post-surgical treatment. The choice of treatment can be different in each country or region [3], [4], [5]. In our hospital, most of the patients underwent closed reduction treatment with arch bar fixation and some patients were done with ORIF miniplate. ORIF miniplate has been reported to be the gold standard of the treatment [3], [4]. However, this form of treatment become quite famous at Sanglah General Hospital due to the effectiveness of diagnosing and readily available of tools and local insurance assist. It makes the cost of treatment affordable. There are some of the surgical approaches in maxillofacial, such as intraoral sublabial approach, coronal approach, transcutaneous approaches, and transconjunctival approach [9], [10]. The transconjunctival approach does not familiar with Sanglah General Hospital. The main goal of reduction is to restore proper functions of maxillofacial. We need to correct function of chewing and speaking, to stabilise and correct occlusion, to obtain a pain-free mandibular range of motion, to restore the contour of the maxillofacial, and to offer enough stability to ensure the union of bones, and to reduce the risk of infection [11].

In early 2001, the insurance does not cover ORIF miniplate and arch bar fixation as well as any medical devices for open and closed reduction. This makes the delay in the management of maxillofacial fracture. But today, since the new agreement between hospital and insurance provider was made, the choices of a method in maxillofacial fracture management is more flexible [9], [10]. The high rates of infection could be described as the use of closed reduction with MMF. Not only oral hygiene is a major concern for patients after surgery, but also adequate nutrition needs to be considered and plays an important role in wound healing [6].

Orbital fractures often occur periorbital oedema, ecchymosis, conjunctival bleeding, limited movement of the eye, and diplopia. The orbital fracture can be associated with zygomaticomaxillary fractures, nasal fracture, and it can cause tripod fracture. Tripod fracture includes zygomatic arch, lateral orbital rim, and inferior orbital rim. Immobilisation techniques use ORIF miniplate. Subcilliary incision approach is often used in our department to correct the inferior orbital fracture. The zygomaticomaxillary fracture can use a combination approach of subcilliary and intraoral.

Mandibular fractures are often characterised by a malocclusion. Dentoalveolar trauma is often observed with mandibular fractures, include trismus, pain with mastication, the floor of the mouth hematoma, facial asymmetry, and paresthesia of the third trigeminal division [11]. Immobilisation technique of mandibular fracture uses ORIF miniplate. If mandibular or maxilla fracture associated with dentoalveolar trauma, we can use an arch bar or IDW to secure loose teeth and alveolar bone. IDW can be used for simple alveolar process fracture. MMF is used as an aid for the appropriate anatomical reduction of bony segments. We can use rubber or wire MMF to immobilise mandible.

Early fracture immobilisation can reduce the risk of infection. Because of the high risk of bacterial contamination in maxillofacial fractures, the antibiotic drug should be given to all maxillofacial fractures. Teeth adjacent to the fracture site must be evaluated and should be preserved to increase stabilisation of the fracture area.

Proper maintenance of oral hygiene, both before and after surgery, is an important treatment in the management of maxillofacial fractures. Loss of tissue barriers due to bacterial invasion due to maxillofacial fractures, loose or missing teeth, gingival tears, hematoma, oedema, and disorders with natural cleansing mechanisms will increase the risk of infection. Appropriate oral hygiene uses saline, peroxide, or drugs (chlorhexidine gluconate) should be encouraged. Increasing the frequency of brushing teeth must be educated to patients and families, and the use of pulsatile irrigation devices is very helpful for patients.

Eating right and maintaining important nutritional status for postoperative care. Mandibular immobilisation with MMF for 4-6 weeks will make nutrition intake more difficult, and weight loss cannot be avoided [10]. Our experience, MMF can be maintained for 3-4 weeks to get good occlusion. While patients with MMF, they need to keep oral hygiene with antiseptic gargle and get a liquid meal.

However, many choices of nutritional supplements are available for patients in liquid form, which will reduce weight loss and malnutrition. Patients can be asked to pay attention to weight gain and calories as needed. The position of the patient and bedside suction device can simplify the patient's ability to manage oral secretions and bleeding after surgery. Elevating the head of a 45-degree angle bed allows the patient to cleanse secretions effectively. Postoperative steroids and the use of ice packs can be effective in reducing oedema [10].

After the MMF was removed, the patients can eat soft food and liquid meal. In maxillofacial fracture which immobilised with an arch bar in maxilla and mandible, the maxillary arch bar can be removed after 6-8 weeks and after 12 weeks for the mandibular arch bar. They need to avoid hard food for approximately 12 weeks.

In conclusion, maxillofacial trauma has become often reported in Sanglah General Hospital, despite rapid diagnoses and proper treatment, maxillofacial trauma becomes challenges to manage due to demanding skill and high level of expertise. Based on this study, we must be more vigilant to comply with traffic signs and use of personal protective equipment when driving. Rapid and proper management of maxillofacial fractures can reduce patient morbidity.

Diagnosing the right injury to the facial bone is a key step in determining a treatment plan. Surgeons must have sufficient knowledge of facial anatomy and physiology to be able to reconstruct broken segments. Deformities after facial trauma are difficult to repair in the second operation. So, the importance of dealing with almost all maxillofacial fracture problems in the first surgery is pretty clear to all traumatologists.

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The Effect of Oral Probiotic on the Interleukin-10 Serum Levels of Acne Vulgaris

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Abstract

BACKGROUND: Acne vulgaris is a chronic inflammatory skin disorder that commonly found in pilosebaceous units which can have an impact on the patient's psychological burden. The relationship between dermatology and mental health is increasingly understood by the evidence shows that functional integrity and microbes in the gastrointestinal tract may play a role in mediating skin inflammation and emotional behaviour. The gut-brain-skin theory was first described in 1930 by Stokes and Pillsbury, became the basis of many current studies that look for clinical implications of the relationship between the gastrointestinal tract, brain and skin in acne vulgaris. Probiotics are live microorganisms which can provide a healthy effect to the hosts when consumed in adequate amounts.

AIM: To determine the effect of oral probiotic on the interleukin-10 serum levels in acne vulgaris and also to determine the side effect of oral probiotic on acne vulgaris.

METHODS: This is a pre-experimental clinical study with a pretest-posttest design involving 33 subjects with acne vulgaris. The subjects in this study were measured for IL-10 serum levels before and after oral probiotic was given for 30 days. This research has been approved by the Health Research Ethics Commission of the Faculty of Medicine, Universitas Sumatera Utara.

RESULTS: This study found an increase in serum IL-10 levels after oral probiotic in acne vulgaris. The value of serum IL-10 levels before oral probiotic administration was 5.27 ± 1.49 pg/ml, while the value of serum IL-10 levels after oral probiotic administration was 6.19 ± 1.68 pg/ml) with p values obtained through Wilcoxon test was 0,0001 ($p < 0.05$). The side effect of oral probiotic found in this study is bloating that was found in 2 subjects within the first week using oral probiotic.

CONCLUSION: Oral probiotic trigger elevated IL-10 serum levels of acne vulgaris. This study supports previous studies that suggested oral probiotic can be considered as adjuvant acne vulgaris therapy and its side effect is quite safe and tolerable.

Introduction

Acne vulgaris is a chronic inflammatory skin condition which has an impact on the psychological burden. The relationship between dermatology and mental health is increasingly understood by the evidence that functional integrity and microbes in the gastrointestinal tract may play a role in mediating skin inflammation and emotional behaviour [1]. The intestinal microbiota consists of trillions of microbes that affect normal physiology and alter host susceptibility to disease [2], [3]. Intestinal microbiota is important for immune system maturation, normal intestinal development as a natural barrier to foreign matter and bacteria, and also the synthesis of vitamin K and B12 [4]. Changes in the intestinal microbiota or exposure to specific bacteria in the intestine can stimulate the central nervous system and peripheral nervous system in animals causes changes in brain function and shows the presence of intestinal microbiota and brain axis [3], [5], [6].

The gut-brain-skin theory was first described in 1930 by John H. Stokes and Donald M. Pillsbury, became the basis of many studies that look for clinical implications of the relationship between the gastrointestinal tract, brain and skin particularly in acne [1], [7], [8]. Based on evidence that as many as 40% of acne patients experience hypochloridia, namely a decrease in gastric acid; Stokes and Pillsbury elucidate the hypothesis that insufficient gastric acid might induce migration of colon bacteria to the distal portion of the small intestine and interfere with normal intestinal microflora. Furthermore, changes in stressed microbial flora can increase intestinal permeability which in turn, stimulates systemic inflammation and also local skin inflammation. They also recommend the direct introduction of acidophilic organisms in cultures such as *Bacillus acidophilus* to overcome stress-triggered cycles [1], [8].

Probiotic are living microorganisms that when consumed in adequate amounts can give a healthy effect on hosts [9]. Probiotic have been extensively investigated because of their effects on the gastrointestinal system and digestive function, but these microbes can be applied more broadly based on evidence of the gut-brain-skin theory which was stated for 80 years ago. The first report on probiotic originated in 1907 by Elie Metchnikoff which illustrates the relationship of consuming acid producing bacteria in yogurt with longevity [10]. Probiotic acts against pathogenic bacteria, support barrier functions and contribute to the regulation of natural and acquired immune responses [9], [10].

Methods

This is a pre-experimental clinical study with a pretest-posttest design involving 33 subjects with acne vulgaris. The subjects in this study were measured for IL-10 serum levels before and after oral probiotic was given for 30 days. The oral probiotic that was given is L-Bio® and consumed by participants as much as 2 sachets per day mixed with 500 cc of water before breakfast in the morning. One L-Bio® sachet contains rice starch, malodextrin, *B. lactis W51*, *B. lactis W52, L. acidophilus W55, L. casei W56, L. salivarius W57, L. lactis W58* with total bacterial cells > 10^8 cfu (*colony forming unit*). The serum IL-10 levels were examined by Quantikine ELISA method, using the human IL-10 kit obtained from *R & D Systems catalog* D1000B.This research has been approved by the Health Research Ethics Commission of the Faculty of Medicine, Universitas Sumatera Utara.

Results

From a total of 33 subjects, female gender was 20 subjects (60.6%), more common than male gender 13 subjects (39.4%). The majority of the research subjects were 17-25 years old (90.9%). Based on the severity of acne vulgaris, we found the highest number of subjects was severe acne vulgaris, namely 12 subjects (36.4%), followed by mild acne vulgaris 11 subjects (33.3%) and moderate acne vulgaris 10 subjects (30.3%).

Table 1: Characteristics of the research subject

Characteristics	Number (n)	Percentage (%)
Gender		
Male	13	39.4
Female	20	60.6
Age		
17-25 years old	30	90.9
26-35 years old	3	9.1
Acne severity		
Mild	11	33.3
Moderate	10	30.3
Severe	12	36.4

In this study, we found the mean value of serum IL-10 levels before administration of oral probiotic was 5.27 ± 1.49 pg/ml while the mean serum IL-10 level after administration of oral probiotic was 6.19 ± 1.68 pg/ml. The *p* value obtained through the Wilcoxon test is 0.0001 ($p < 0.05$). This shows a significant difference in serum IL-10 levels before and after administration of oral probiotic.

Table 2: Comparison of serum IL-10 levels before and after probiotic administration

Acne vulgaris	serum IL-10 (pg/ml) levels					
	n	Mean	SD	Min	Max	*p*
Before	33	5.27	1.49	3.41	11.30	0.0001
After	33	6.19	1.68	3.43	12.60	

The side effect of oral probiotic found in this study is bloating that was found in 2 subjects (6.1%) within the first week using oral probiotic.

Table 3: The side effect of oral probiotic on clinical subjects

Side effect	Cases	
	n	(%)
Bloating	2	6.1
No present	31	93.9

Discussion

Probiotic are living microorganism that when consumed in adequate amounts can give a healthy effect on hosts [9]. Probiotic has been extensively investigated because of their effects on the gastrointestinal system and digestive function, but can be applied more broadly based on evidence of the gut-brain-skin theory by John H. Stokes & Donald M. Pillsbury which is the basis of the many studies

currently looking for clinical implications of the relationship between the gastrointestinal tract, brain and skin in acne vulgaris [1], [7], [8].

Evaluation of the effect of probiotic on serum IL-10 levels in patients with acne vulgaris has never been studied before. Previous studies evaluated the effect of probiotic on IL-10 levels on the immune cell supernatant by Livingstone et al., [11]. The study of Weid et al looked-for certain strains of lactic acid bacteria in inhibiting T-helper cell function in vitro and research by Hepburn et al aimed to examine the effect of daily probiotic supplementation on the cytokine profile of 20 healthy volunteers [12], [13].

Clinical trials that evaluating the effects of oral probiotic on acne are still limited. The first clinical trial was carried out by Siver in 1961 involved 300 patients where 80% of patients experienced improvement especially in inflammatory lesions [1], [8], [10]. Recent clinical trials by Jung et al., in 2013 showed that antibiotics and oral probiotic can provide a synergistic effect, especially in inflammatory acne. The study involved 45 patients aged 18-35 years who were randomly divided into 3 groups which are only probiotic supplement, minocycline group and probiotic plus minocycline groups. In this study, it was found that the group using probiotic and minocycline had a better reduction in the total number of lesions compared to the other two groups [14], [15]. The latest clinical trials were conducted in 2016 involved 57 patients with facial papulopustular lesions diagnosed with acne, seborrheic dermatitis and rosacea, which was randomly divided into 37 patients as the experimental group and 20 patients as a control group. The experimental group was given additional oral probiotic supplement *E. coli* Nissle every day for 1 month. The results of the study found 89% of the experimental group experienced significant improvement and complete recovery compared to the control group (56%) ($p < 0.01$).

Probiotic are useful for acne because they can help inflammatory regulation by stimulating synthesis of regulatory cytokines (IL-10) which has been shown in many studies [10], [17], [18]. Interleukin-10 is the most important anti-inflammatory cytokines where the main function is to limit and eliminate the inflammatory response and regulation of differentiation and proliferation of many immune cells such as T cells, B cells, NK cells, APC cells, mast cells and granulocytes [19].

This study found that the mean serum IL-10 level before oral probiotic administration was 5.27 ± 1.49 pg/ml while the mean serum IL-10 level after oral probiotic administration was 6.19 ± 1.68 pg/ml (p = 0,0001); showed that oral probiotic had an effect on increasing serum IL-10 levels. The side effect of oral probiotic found in this study is bloating that was found in 2 subjects (6.1%) within the first week using oral probiotic.

In conclusion, oral probiotic trigger elevated IL-10 serum levels of acne vulgaris which is antiinflammatory cytokine. This study supports previous studies that suggested oral probiotic can be considered as adjuvant acne vulgaris therapy and its side effect is quite safe and tolerable.

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Risk Factors Associated with Mild Cognitive Impairment among Apparently Healthy People and the Role of MicroRNAs

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Abstract

BACKGROUND: Mild cognitive impairment (MCI) is a stage between the expected cognitive decline of normal ageing and the serious decline of dementia.

AIM: To identify risk factors and role of miRNAs associated with mild cognitive impairment (MCI) among employees.

SUBJECTS AND METHOD: A cross-sectional study was carried out on 186 employees aged between 40 and 65 years. Cognitive function was evaluated using ACEIII, MoCA, and Quick cognitive tests. Medical history and lifestyle were assessed. Family 132 & 134 miRNA expressions were assessed by real-time PCR.

RESULTS: MCI was detected among 14 / 186 (7.5%). miRNA 132 expression was the only significant miRNAs to detect MCI with low sensitivity and specificity (70%). The logistic analysis revealed that higher miRNA132 expressions, low monthly intake of; vegetables, unroasted nuts, low education and higher ALT levels were predicting factors for MCI with AOR 1.1 (1.01-3.3), 1.2 (1.04-1.43), 0.8 (0.8-0.98), 2.7 (1.9-7.4) and 1.6 (1.1-2.3) respectively.

CONCLUSION: MiRNAs expression showed low sensitivity and specificity in detecting MCI; only miRNA 132 might be used. Several modifiable factors seem to reduce the risk of MCI.

Introduction

Mild cognitive impairment (MCI) is a stage between the cognitive decline of normal ageing and dementia. MCI involves the onset and evolution of problems related to memory, language, and thinking beyond those expected for age. People with MCI are at greater risk of later developing dementia. However, some of them never get worse, and a few get better [1].

Detecting intervenable risk factors is a key component of preventive strategies for mild cognitive impairment [2]. Medical and lifestyle factors that have been related to an increased risk of cognitive impairment include hypertension, diabetes, elevated cholesterol, depression, infrequent participation in mentally or socially stimulating activities, and smoking [3], [4].

MicroRNA (miRNA), is a small non-coding RNA molecule that functions in the regulation of gene expression. It plays an important role in the pathogenesis of many diseases. It has been reported that miR-132 is linked with cognitive impairment [5]. MiRNAs are actively secreted and expressed in body fluids, such as plasma and cerebrospinal fluid (CSF) [6], [7], [8].

This study aimed to assess the potential risk factors of MCI among employees at the National Research Center (NRC) and the role of family 132 and 134 miRNAs expression.

Subjects and Methods

Study design

A cross-sectional study was carried out on 186 adults aged between 40 and 65 years recruited from NRC employees. Recruitment of participants and data collection was carried out along one year during the period between June 2016 and December 2017. Employees with diabetes, neurological and psychiatric illness, major organ failure and cancer were excluded from the study.

Ethical statement

This work has been carried out by The Code of Ethics of the World Medical Association (Declaration of Helsinki). Each study participant provided informed written consent after acknowledgement about the research and the study was approved by the Ethical Committee of the National Research Centre (registration number 15131).

Methods

Assessment of risk factors for MCI

A closed-ended questionnaire module was designed to cover data on demographic characteristics and full medical history. Data included age, gender and education. Data on tobacco smoking, physical activity, dietary habits were also collected to identify risk factors associated with MCI. The questionnaire was tested for the clarity & flow of the questions, and the time required to complete it. A consent form was also signed by all the participants before the interview.

Assessment of cognitive function with its domains

The case definition of MCI was derived from the National Institute on Aging–Alzheimer's Association recommendations [9]. It includes the following clinical criteria for the diagnosis of MCI.

Subjective Concern as regards the change in cognition

We measured subjective cognitive concerns through two questions, according to Lara et al., [10].

"How would you most describe your memory at present?", with answer options being very good, good, moderate, bad or very bad, and "Compared to 12 months ago, would you say your memory is now better, the same or worse than it was then?".

Participants were evaluated to have memory weakness if they answered "bad" or "very bad" to the former question and / or "worse" to the latter.

Objective Impairment in one or more cognitive domains

Changes can occur in a diversity of cognitive domains, including memory, executive function, attention, language, and visuospatial skills. To assess global and specific cognitive domains, we choose the most reliable and validated Arabic forms scales or easily translated cognitive scales and freely available to be applied to the studied subjects. The following tests were used to assess MCI:

A) Montreal Cognitive Assessment test (MoCA)

It comprises five neurological domains: executive function, attention, short-term memory, language, and visuospatial [11]. It is available in Arabic version [12].

B) Addenbrooke's Cognitive Examination III (ACE III)

It is available in Arabic [13]. It assesses MCI and early stages of dementia [13], [14].

C) The Quick Mild Cognitive Impairment (Quick MCI)

It assesses the five domains of cognitive function: registration, orientation, delayed recall, clock drawing and verbal fluency (VF) [15], [16]. It is easily translatable (linguistically and culturally) and has alternative forms, which allow follow up of cognitive function over time [17].

The recommended cut-off points for each studied cognitive scale were utilized to detect objective cognitive impairment

The cut-off point for ACE III score < 88 [18], for MoCA score < 26 [19] and for Quick MCI score < 67 [20].

Objective MCI was considered if two or all of the three studied cognitive tests were below the cut-off points.

Preservation of independence in daily functional abilities, such as paying bills, preparing a meal, or shopping.

Clinical Assessment

All studied participants were subjected to thorough clinical examination. The anthropometric assessment included weight, height, waist and hip circumference, and body mass index (BMI) was calculated. Blood pressure was measured**.**

Laboratory analysis: - Fasting peripheral blood samples (10 ml) were withdrawn from each participant under complete aseptic conditions and after 10 fasting hrs. Part of the blood sample was anticoagulated with EDTA for assessment of the glycated haemoglobin (HbA1c) using Labona check™ HbA1c analyser and measurement of selected MicroRNAs by real-time PCR. The other part of the blood sample was left to clot and sera were separated immediately for analysis of fasting blood sugar, lipid profile, liver and kidney functions by Erbaxl -300 Mannheim Gmbh Germany; and - MicroRNA isolation and real-time RT-PCR.

MicroRNA was isolated according to the manufacturer's instructions using miRNeasy Mini Kit (Cat. No. 217004) and QIAzol Lysis Reagent supplied by QIAGEN, Germany. Concentration and purity of the yield were determined using Nanodrop 2000, USA. Synthesis of cDNA was performed via thermal cycler (Verity, Applied Biosystems, USA) using the TaqMan® Advanced miRNA-cDNA synthesis kit (cat. No A28007) supplied by Applied Biosystems, Life Technologies, USA.

The relative quantity of (miRNA-128, miRNA-132, miRNA-874) and (miRNA-134, miRNA-323, miRNA-382) in plasma were measured by qRT-PCR via TaqMantechnology using QuantStudio™ 12K Flex real-time PCR system (Applied Biosystems, USA). MicroRNAs' levels were determined by the comparative CT (ΔΔCT) method via the Expression Suite Software using miRNA-491 and miRNA-370 levels, respectively for normalisation.

Data analysis

Data entry was carried on excel sheet, and statistical analysis was done using Statistical program for social science (SPSS) version 18 for windows SPSS; Inc, Chicago IL. Continuous data were expressed as mean and standard deviation Number and percent were used to describe categorical data. Chi square test was used for comparing between two qualitative variables. T-test was used for comparing between two means. Non-parametric tests were used in cases of not normally distributed data. The Bivariate correlations procedure (Pearson correlation) was used to calculate the pairwise associations for a group of variables. Receiver-Operating Characteristic (ROC) curves were formed and the area under ROC curves (AUC) was determined to assess sensitivity and specificity of various miRNAs biomarkers. The cutoff points on the ROC curves, where the accuracy of MCI detection is greatest, were determined. P-value was considered statistically significant if $p < 0.05$ and considered statistically highly significant if $p < 0.01$.

Results

Regarding socio-demographic data, there were 73 (36.6%) males and 113 (63.4%) females, their ages ranged from 40 to 65 years with a mean of 51.3 ± 4.1 years. About 41% of the studied individuals had secondary education, 42% had a university education, and 17% had a post-graduate level of education. As regards the subjective complaint of memory impairment, about 60% of all participants complained of frequent forgetfulness. Comparing their current memory to that of the previous year, 31.9% of them reported impaired memory. MCI was detected among 14 / 186 (7.5%) of the studied individuals depending on both subjective complaint and objective detection with at least two positive cognitive tests. The mean score of ACE III (88.14 ± 2.5), MOCA (25.29 ± 2.09) and Quick MCI (63.42 ± 9.78) tests was significantly lower among individuals with confirmed MCI compared to those with normal cognition (94.5 ± 4.5), (27.9 ± 2.2) and (77.6 ± 10.09) respectively, $P < 0.01$.

The percentage of subjects with MCI was significantly higher among current or ex-smokers compared to nonsmokers and among individuals with sedentary life compared to those with physical activities, $P < 0.05$. Mean BMI was significantly higher among individuals with MCI compared to those with normal cognition, $P < 0.01$ (Table 1).

Table 1: Socio-demographic, medical and physical history of the studied participants about MCI

Variable	Total	Cognitive function		Odds Ratio (CI 95%)
		MCI (N = 14) N (%)	Normal (N = 172) N (%)	
Age in years				
< 50	65	5 (7.8)	60 (92.2)	®
50 -< 55	64	6 (9.4)	58 (90.6)	1.2(0.3-4.3)
55 -< 60	42	3 (7.1)	39 (92.9)	0.9(0.2-4.1)
60-65	15	0 (0.0)	15 (100.0)	
Gender		6 (8.2)	67 (91.8)	1.1 (0.3-3.5)
Males	73	8 (7.1)	105 (92.9)	®
Females	113			
Education				2.6 (0.8-8.2)
Secondary	79	9 (11.3)	70 (88.7)	®
University and Master / MD	107	5 (4.7)	102 (95.3)	
Smoking				3.1(0.96-10.1) *
Current or ex-smokers	31	5 (16.1)	26 (83.9)	®
Non-smokers	155	9 (5.8)	146 (94.2)	
Marital status				3.7 (0.5-29.1)
Married	150	14 (9.3)	136 (90.7)	®
Single or widowed	36	1 (2.7)	36 (100.0)	
Family history of dementia				0.8(0.2-3.1)
Yes	45	3 (6.6)	42 (93.4)	®
No	141	11 (7.8)	130 (92.2)	
History of head Trauma				
Yes	44	3 (6.8)	41 (92.3)	0.8(0.2-3.2)
No	142	11 (7.7)	131 (92.3)	®
Physical activities				
No	40	6 (15.0)	34 (85.0)	4.2(1.2-14.5)*
Several times/month	123	5 (4.1)	118 (95.9)	®
Systolic blood pressure (mean ± SD)	186	120 ± 14.1	118 ± 14.9	P > 0.05
Diastolic blood pressure (mean ± SD)	186	78.5 ± 7.7	77.5 ± 10.7	P > 0.05
BMI (mean ± SD)	186	35.5 ± 7.2	30.8 ± 5.6	P < 0.01
Waist/Hip ratio (mean ± SD)	186	0.88 ± 0.08	0.87 ± 0.07	P > 0.05

® Reference group; CI: confidence interval; * Significant P value < 0.05.

The mean monthly intake of important food for good memories like vegetables, dark green

vegetables and unroasted nuts were significantly higher among those with normal cognition compared to those with MCI, P < 0.01. The mean monthly hours spent on the internet, playing intellectual games and going to the museum / cinema was significantly lower among participants with MCI (P < 0.01) (Table 2).

Table 2: Monthly frequency intake of some food items, social and mental activities of the studied participants about MCI

Variable	Cognitive function		P-value
	MCI	Normal	
	N = 14 (Mean ± SD)	N = 172 (Mean ± SD)	
Main Meal contain (vegetables- carbohydrates- protein)	11.3 ± 9.4	21.1 ± 27.9	0.249
Red meat	5.0 ± 3.0	5.4 ± 4.5	0.748
Egg	8.5 ± 10.9	12.6 ± 10.0	0.201
Dairy products	23.5 ± 9.3	25.4 ± 12.3	0.603
Fish	5.7 ± 8.3	4.6 ± 7.5	0.623
Canned tuna	0.9 ± 0.8	1.9 ± 4.1	0.446
Bean	8.0 ± 11.3	15.4 ± 12.3	0.059
Whole brown Grain	1.3 ± 2.5	4.3 ± 10.2	0.336
Vegetable	4.2 ± 8.7	19.4 ± 16.1	0.000*
Dark green vegetables	1.0 ± 1.1	4.9 ± 7.7	0.000*
Fruits	12.7 ± 9.6	21.1 ± 14.1	0.055
Unroasted nuts	0.4 ± 0.5	3.6 ± 7.1	0.000*
Dark chocolate	0.4 ± 0.5	2.8 ± 6.5	0.219
Social activities			
Going to Club	1.0 ± 1.6	2.4 ± 6.8	0.520
Going to Museum/ Cinema/ parties	0.0 ± 0.0	0.2 ± 0.5	0.000*
Mental activities (In hours)			
Watching TV	79.1 ± 70.1	51.3 ± 50.4	0.088
Using Internet	21.8 ± 14.0	36.9 ± 33.3	0.007*
Playing intellectual games	0.7 ± 0.3	6.2 ± 14.9	0.000*

P significant at < 0.05.

None of the studied laboratory tests was found to be significantly associated with MCI (P > 0.05) (Table 3).

Table 3: Lipid profile and other laboratory analysis of the studied participants and MCI

Laboratory Test	Cognitive function		P-value
	MCI	Normal	
	N = 14 Mean ± SD	N = 172 Mean ± SD	
Alanine aminotransferase (ALT)	21.4 ±14.1	19.2±10.5	0.467
Albumin (ALB)	4.5 ± 0.2	4.6 ± 0.2	0.128
Creatinine	0.9 ± 0.1	0.9 ± 0.1	0.539
Fasting Blood Glucose (FBG)	117.8 ± 31.2	96.5 ± 31.2	0.134
Glycosylated Hemoglobin (HbA1c)	6.2 ± 1.5	5.5 ± 1.2	0.620
Cholesterol	181.7 ± 16.8	189.5 ± 14.2	0.073
Triglycerides	107.1 ± 81.5	120.7 ± 49.0	0.401
High Density lipoprotein (HDL)	42.3 ± 14.5	46.3 ± 13.6	0.348
Low Density lipoprotein (LDL)	122.7 ± 22.1	125.1 ± 20.3	0.092

P non-significant > 0.05.

ROC curve analysis was done for miRNA 128, miRNA-132, miRNA-874, miRNA-134, miRNA-323 and miRNA-382 expressions in plasma of the studied subjects. Upon analysis, only miRNA-132 can significantly differentiate between MCI and normal cognition with AUC = 0.69 with a 95% confidence interval 0.52 – 0.86 (P = 0.04) (Figure 1).

These biomarker pairs for family miRNA 132 expressions differentiated individuals with MCI from those with normal cognition with 60%-70% sensitivity and 57.9%-68.6% specificity.

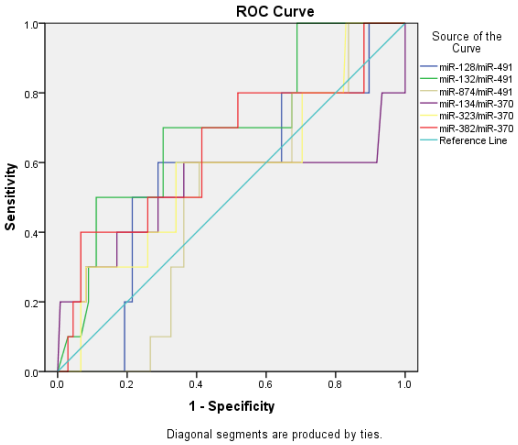

Figure 1: Receiver-Operating Characteristic (ROC) curve for differentiation between MCI and normal cognition among apparently healthy individuals obtained with different miRNAs biomarker pairs

Family miRNA 134 expressions differentiated individuals with MCI from those with normal cognition with 40%-60% sensitivity and 65.2%-83.3% specificity (Table 4).

Table 4: Sensitivity, specificity and ACU of MiRNA biomarker pairs in detecting MCI

Family / normalizer	AUC (95% CI)	Sensitivity	Specificity	Accuracy	P-value
miRNA-128 / miRNA-491	0.56 (0.37-0.74)	60%	68.6%	64.3%	0.5
miRNA-132 / miRNA-491	0.69 (0.52-0.86)	70%	68.6%	69.3%	0.04
miRNA-874 / miRNA-491	0.49 (0.34-0.63)	60%	57.9%	58.9%	0.9
miRNA-134 / miRNA-370	0.52 (0.26-0.78)	40%	83.3%	61.6%	0.8
miRNA-323 / miRNA-370	0.57 (0.38-0.77)	60%	65.2%	62.6%	0.4
miRNA-382 / miRNA-370	0.64 (0.44-0.84)	50%	71.9%	60.9%	0.1

AUC: area under the curve.

Logistic regression analysis revealed higher miRNA132 expressions, little vegetables, little nuts, lower level of education and a higher level of ALT are predicting factors for MCI with AOR 1.1 (1.01-3.3), 1.2 (1.04-1.43), 0.8 (0.8-0.98), 2.7 (1.9-7.4) and 1.6 (1.1-2.3) respectively (Table 5).

Table 5: Logistic regression analysis for predicting the risk of MCI

Variable	Adjusted Odds Ratio	95% confidence interval		P-value
		Lower	Upper	
miRNA132	1.12	1.01	3.387	0.007
no vegetables	1.2	1.04	1.43	0.01
no nuts	0.88	0.80	0.98	0.02
secondary education	2.7	1.96	7.4	0.04
ALT lab	1.586	1.079	2.332	0.019
Constant	1.459E23			0.017

Discussion

The risk of dementia is higher in individuals with MCI (10-15%) compared to others with normal cognition (1-2%) [21], [22], [23].

In the present study, the prevalence of MCI was 7.5%. In a study done among doctors and workers at Zagazig University Hospital, Egypt, with a

similar age group, MCI was found among 9% of them [24]. In China, in Tianjin city, MCI was detected among 9.7% of normal individuals according to the Petersen's criteria of MCI [25]. Ding and Zhao [26] documented MCI prevalence of 20.1% among normal Chinese individuals aged ≤ 60 years in an urban community of Shanghai using Mini-Mental status test. Moreover, Salama et al., [27] found that the prevalence of MCI was 11.6% among non-obese aged 40-60 years. The lack of homogeneity in the reported data about the prevalence of MCI may be due to the differences in the test used for the detection of MCI, the varieties in the application of MCI diagnostic criteria, the demographic characteristics of the shared populations, the education level and the age range of the participants.

The current study aimed to identify the demographic factors associated with cognitive function. The results showed that the prevalence of MCI was insignificantly higher at the age of 50-55 years ($P > 0.05$), which contradicts previous results that showed a trend that the prevalence of MCI increased with increasing age [28]. This could be attributed to the fact that all the participants at higher age group had post-graduate education. We found that all 32 participants with post-graduate education had normal cognition. A previous study found that education was an effective preventive factor against MCI [29]. People with a lower level of education were more likely to develop MCI. Possible reasons may be: high level of education can make changes to the brain metabolism and the degree of biological neural synaptic connections so that the brain can tolerate functional or structural defects in the brain cells to a certain extent. Instead, for people with lower levels of education, there is a lack of knowledge to stimulate the brain, causing massive loss of neurons [30]. Another possible reason is the health; poverty and socio-economic status which are associated with lower education level [31].

Our findings revealed no difference as regards the prevalence of MCI among males (8.2%) compared to females (7.1%), $P > 0.05$. Similarly, Ferreira et al., [32] reported that there were no sex differences as regards the rates of cognitive decline in the normal ageing process. On the contrary, Caracciolo et al., and Roberts et al., [33], [34] found that the incidence of MCI was higher among men. The pathological mechanisms that could explain these patterns precisely are still concealed. However, the pathophysiological changes regulated by endocrinal transition states, such as the menopause may have a role [35].

Midlife obesity has consistently been considered as a risk factor for the decline of cognitive function [36]. Our findings revealed that the mean BMI was significantly higher among participants having MCI (35.5 ± 7.2) compared to those with normal cognition (30.8 ± 5.6), $P < 0.01$. Similarly, Salama et al., [27] found that the prevalence of objective MCI was 42.6% among obese compared to 11.6% among non-obese. In a population-based study, participants who were obese had twice the risk of developing dementia (OR = 2.10) [36].

In the current study, the prevalence of MCI was significantly higher among smokers (16.1%) compared to non- smokers (5.8%), with OR 3.1 (0.96-10.1). It was reported that cognitive dysfunction accounted for 75.6% among smokers, compared to only 52.5% among nonsmokers, suggesting that smoking is a risk factor for MCI. During smoking, carbon monoxide fumes, as well as tobacco nicotine, tar and other harmful substances are produced, which will cause vascular endothelial damage, myosin contraction, and increase vascular permeability to accelerate atherosclerosis. Smoking may impair cognitive function through its effect on the arterial wall, leading to thickening of arterial plaque, increasing plasma viscosity and fibrinogen levels, platelet aggregation, hypertension and increases the risk of stroke [30].

A history of traumatic brain injury may possess a higher risk of having neurodegenerative diseases such as MCI and dementia across the life span [37]. The relation between traumatic brain injury and the risk of dementia later in life has been repeatedly established [38], [39]. In the present study, the prevalence of MCI was not significantly different between subjects having previous head trauma and those without, $P > 0.05$. May be that the reported head trauma in the current study was mild and did not lead to brain injury.

The present study assessed the effects of lifestyle habits and leisure activities on cognition. Participants with normal cognition were found to be monthly consuming a significantly higher level of vegetables, fruits and unroasted nut compared to MCI subjects, $P < 0.05$. It was found that eating a healthy balanced diet that contains fruits, vegetables, unrefined cereal grains and nuts, and avoiding fatty and high-calorie foods was directly related to normal cognition [40]. The Mediterranean diet was reported as a preventive factor against MCI and AD diseases [41]. A study done by Pasinetti and Eberstein [42] found that polyunsaturated fatty acids, vegetables, and a high Mediterranean-diet score may improve cognitive activity. A balanced diet with fewer calories and low glucose may enhance carbohydrate metabolism and decrease obesity which will effectively reduce the risk of hyperglycemia and thus avoiding Alzheimer's disease [43]. On the other hand, persons with Alzheimer's disease reported high consumption of animal protein and sugar in comparison to healthy controls who consumed more vegetables, fruits and whole grains [44]. Prevention of the deterioration of cognitive functions and dementia is directly related to the intake of certain nutrients or dietary antioxidant supplements [45].

Increased level of engagement in physical

activities is an important factor that increases the cognitive reserves [29]. We found that the prevalence of MCI was significantly higher among inactive subjects (15%) compared to those who were practising physical exercise (4.1%), OR 4.2 (1.2-14.5). In a population-based study, and frequency of moderate-intensity exercise had a protective effect on MCI. Those findings were consonant in both sexes. Light and intense physical activities were not significantly associated with MCI [46].

There is growing evidence demonstrating that participation in different forms of leisure activities, either mental or social activities have a good effect on well-being, especially for conserving functional capacity during the ageing procedure and decreasing the risk of chronic diseases [47], [49]. In the present study, the mean time in hours/week spent in intellectual games, reading and social activities were significantly higher among participants with normal cognition compared to subjects with MCI, $P < 0.01$. Several studies agreed with the results of the current study and reported that computer activities, reading, dancing, playing games and musical instruments decrease the risk of MCI and dementia [50], [51], [52].

In the present study, there was no significant association between lipid profiles and FBS and the risk of MCI. Similarly, other studies reported that lower concentration of HDL was associated with lower cognitive function, suggesting a positive correlation between HDL and cognitive function [53], [54].

A study done by Mielke and his colleagues [55] concluded that high cholesterol level at old age was associated with reduced risk of dementia. However, Yaffe et al., [56] and He et al., [57] found that cholesterol and triglyceride levels were significantly higher among individuals with MCI subjects compared to those with normal cognition. Increase in serum cholesterol might cause damage to the brain capillary endothelial cells and accelerate atherosclerosis, then reduce cerebral blood flow, leading to the impaired cognitive function [30].

Evolution of reliable and non-invasive procedures for detecting subjects with MCI is important. It could help in increasing the efficiency of existing and new therapies and observing the advancement of disease [58] (Michael-Titus et al., 2010). Sheinerman et al., in 2012 [59] performed a pilot study for selecting promising miRNA biomarkers for detecting MCI. Two sets of biomarkers (the miR-132 and miR-134 families paired with miR-491-5p and miR-370) showed high specificity and sensitivity in recognising MCI subjects. Moreover, Sheinerman et al., [60] validated these two sets of 50 MCI patients and 50 controls. They reported that these two sets could distinguish MCI from normal controls with 84%-94% sensitivity and 96%-98% specificity for miRNA-132 family and 74%-88% sensitivity and 80%-92% specificity for miRNA-134 family.

In the present work, we aimed at validating these two miRNA-132 and miR-134 families (paired with miR-491-5p and miR-370 respectively), as biomarkers for the detection of MCI**.** Surprisingly, these two sets revealed much lower sensitivity and specificity in detecting MCI. Family miRNA 132 expressions differentiated individuals with MCI from those with normal cognition with 60%-70% sensitivity and 57.9%-68.6% specificity**.**

For family miRNA, 134 expressions differentiated individuals with MCI from those with normal cognition with 40%-60% sensitivity and 65.2%-83.3% specificity. However, only miRNA 132 expression was significantly upregulated among individuals with MCI compared to those with normal cognition, with a significant AUC 0.69 (0.52-0.86), 70% sensitivity, 68.6 specificities and accuracy 69.3%.

These results preliminarily indicated that miRNA-132 might be a potential biomarker for the diagnosis of MCI. A significantly upregulated expression of miRNA-132 was reported among patients with MCI (n = 66) in comparison to their age-matched controls (n = 76) [61]. Moreover, Weinberg et al., [62] assessed miRNA 132 in the frontal and inferior temporal cortex of postmortem brains of patients with MCI and normal individuals and found that miR 132 was significantly down-regulated in MCI. Many studies have reported the value of miRNA-132 in managing dendritic morphology. MiR-132 is important for the functional integrity of adult neurons reduces synaptic transmission between neurons in the hippocampus and has a key role in controlling cognitive functions [63], [64], [65]. While, Dhahbi et al., [66] reported that ageing is associated with an increase in the levels of miR-134 and miR-874 expression.

The accuracy of miRNAs as diagnostic and/or prognostic biomarker of MCI is inconsistent among several studies which could be attributed to different factors including different sample collection techniques regarding temperature, freezing and centrifugation of the drawn blood sample, different sample size, difference in selection of the participants as some studies matched cases and controls depending on gender and age while others relied on gender and ethnicity, different analytical methods and publication bias (where negative studies may be difficult to be found when searching on usual databases) which may overestimate the connection between miRNAs and MCI [67], [68].

In conclusion, the risk of MCI could be reduced by targeting modifiable risk factors. MiRNAs showed low sensitivity and specificity. However, miRNA 132 expression might be used as a minimally invasive test for detection of MCI.

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Case Report

Hoarseness of Voice as a Rare Presentation of Tuberculosis: A Case Report Study

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Abstract

BACKGROUND: Having hoarseness of voice as the first clinical manifestation of tuberculosis is rare. This atypical presentation causes some confusion since other more common conditions, such as laryngeal carcinoma, present similarly and might require more invasive tests to confirm the diagnosis.

CASE PRESENTATION: A 38-year-old male presented to the otorhinolaryngology clinic with a four-month history of change in voice. Laryngoscopy demonstrated a right glottic mass, raising suspicion of laryngeal cancer. The computed tomography showed a mass and incidental finding of opacities in lung apices. Chest x-ray demonstrated findings suggestive of tuberculosis. Polymerase chain reaction and culture of sputum samples confirmed the diagnosis and the patient was started on anti-tuberculosis treatment.

CONCLUSION: Despite accounting for only 1% of pulmonary tuberculosis cases and having a similar presentation to laryngeal carcinoma, we recommend considering laryngeal tuberculosis when evaluating hoarseness of voice in endemic areas.

Introduction

According to the World Health Organization, Saudi Arabia estimated a yearly incidence rate of tuberculosis of 10 per 100,000 population and a total of 3004 cases in 2016 [1]. Laryngeal tuberculosis (LTB) is an uncommon complication of pulmonary tuberculosis. It is estimated to occur in 1% of those infected with pulmonary tuberculosis [2]. Also, having hoarseness of voice as the first clinical manifestation of tuberculosis is rare. This atypical presentation causes some confusion since other more common conditions, such as laryngeal carcinoma, present similarly and might require more invasive tests to confirm the diagnosis. Although laryngeal tuberculosis is not a common condition, it should be considered in the evaluation of hoarseness of voice in areas where tuberculosis is endemic, such as southern Saudi Arabia [3].

Case Report

This is a case of a 38-year-old previously healthy Saudi male who presented to our otorhinolaryngology clinic complaining of a four-month history of hoarseness of voice.

Four months before his clinic visit, the patient started complaining of a noticeable change in his voice. It started gradually and was later associated with a cough, occasional sputum, night sweats, and decrease of weight three months afterwards. There was no history of hemoptysis, fever, or loss of appetite. Moreover, there was no history of contact with sick patients, particularly those with Tuberculosis (TB). He has been a smoker for at least 15 years. The patient was well-oriented and vitally stable. Laryngoscopy demonstrated congestion in the hypopharynx and larynx as well as a right glottic mass extending to the supraglottic area involving the arytenoid cartilage. Remaining ear, nose and throat

examinations were unremarkable. Examination of the cervical lymph nodes showed two masses on level two and three on the left side of the neck. After that, the patient was admitted as a case of laryngeal mass and was booked for Computed Tomography (CT) of the neck with intravenous contrast and labs were taken. Also, an operating room was booked for the next day for the potential need for biopsy taking. The CT demonstrated a soft tissue mass involving the right true vocal cord causing mild narrowing of the airway (Figure 1).

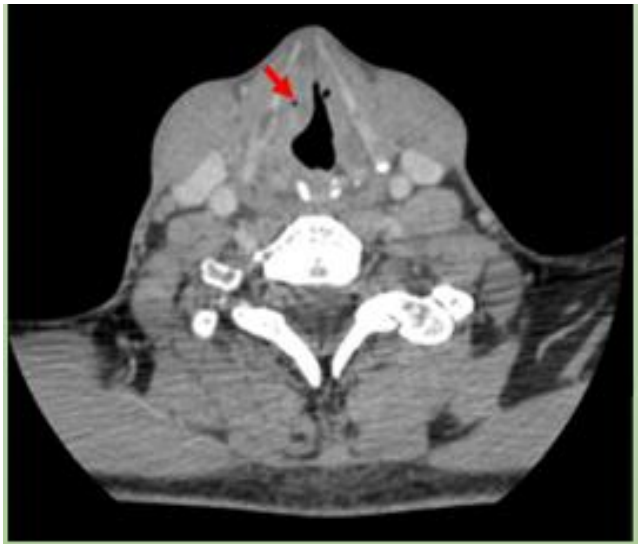

Figure 1: Computed Tomography of the neck showing soft tissue mass involving the right true vocal cord or glottis region (arrow)

Multiple bilateral cervical lymph node enlargements were noted with some showing central necrosis. Lung apices showed heterogeneous opacities with parenchymal destruction (Figure 2).

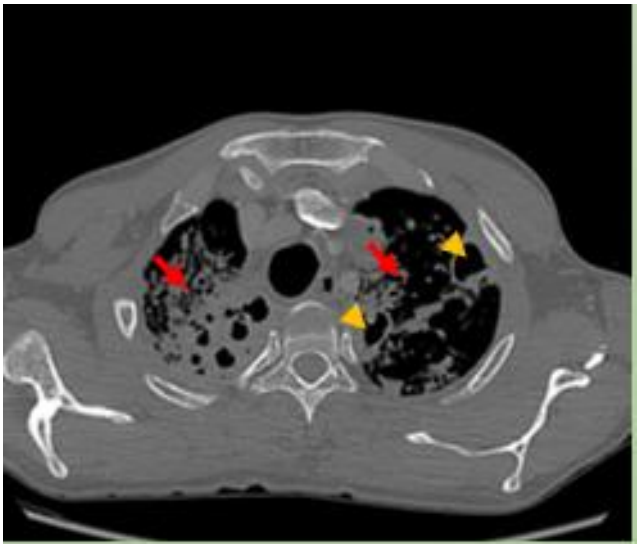

Figure 2: Computed Tomography of the neck with lung apices showing heterogenous opacities with parenchymal destruction (arrow) and cavitary lesions (arrowhead)

As a result, a Chest x-ray (CXR) was ordered and showed findings suggestive of TB. Subsequently, a sputum sample was taken for Acid Fast Bacilli (AFB) testing, AFB bacterial culture, and Polymerase Chain Reaction (PCR). Results were positive, and a diagnosis of TB was established. The Patient was transferred to isolation and started on anti-TB therapy (ATT) including Rifampicin, Isoniazid, Ethambutol, and Pyrazinamide with Pyridoxine. During his stay, the patient was stable and tolerating the medications well. However, due to noncompliance with isolation protocols, it was agreed that the patient continues treatment with home isolation.

Additionally, an outpatient follows up visit was appointed and the patient was discharged. On six-month follow up, the patient's symptoms of cough, dysphagia and night sweat resolved. His main complaint of hoarseness of voice improved but persisted. On laryngoscopy, there was the resolution of the previously noted mass. However, generalised oedema and hypertrophy of the false vocal cord were seen.

Discussion

In a previous case report by Junaid M et al., a 76-year-old man presented with dysphagia and gradually progressive hoarseness of voice. Due to the high suspicion of malignancy, the patient was admitted for laryngoscopy and biopsy. However, a chest x-ray was done as part of the preoperative assessment and revealed classical findings of pulmonary TB. It was decided to shift the patient to an isolation room and later proceed with direct laryngoscopy and biopsy with the addition of bronchoalveolar lavage. Results confirmed TB and the patient were started on ATT with symptoms resolution on one-month follow up [4].

In an additional case report by Suhail A et al., a 40-year-old man complained of persistent hoarseness for two months. Laryngoscopy revealed findings suspicious of carcinoma of the larynx and CXR showed findings suggestive of pulmonary TB. However, it was decided to proceed with laryngoscopy and biopsy. The findings confirmed TB and the patient was commenced on four ATT drugs. On five-months follow-up, the symptoms were resolved. In these two cases as well as our case, the initial suspected diagnosis was laryngeal cancer and a laryngoscopy and biopsy were initially planned [5].

Furthermore, a case reported by Fsadni P et al., described the clinical course of an 85-year-old woman with a six-month history of hoarseness of voice, she underwent bronchoscopy and a lesion was found and a biopsy was taken. Histopathology showed a granuloma, which is suggestive of TB. She was started on ATT and had complete resolution at

six-months follow up [6].

An additional study by Swain SK et al., retrospectively reviewed 11 cases of primary laryngeal TB. They found that the most common symptom was hoarseness of voice. Besides, an endoscopic examination was non-specific and had findings similar to laryngeal cancer. Confirmation of the diagnosis included bacteriological and histopathological tests. All patients had an excellent outcome after six-months of ATT [7].

However, in our case, a neck CT was performed and revealed a laryngeal soft tissue mass, cervical lymphadenopathy and apical lung abnormalities. Subsequently, a CXR was done and revealed classical findings of TB. After that minimally invasive test to confirm TB was performed, such as PCR and sputum AFB and culture. In other cases, the diagnosis was confirmed by obtaining a biopsy under anaesthesia by laryngoscopy guidance.

In conclusion, LTB should be considered in the initial differential workup of hoarseness of voice, especially in endemic areas of TB. The diagnosis may be confirmed by minimally invasive testing, such as PCR and culture.

Conference Presentation

Part of this paper was presented at the 11th International Saudi Otorhinolaryngology Conference, 2019 Feb 14-16, Alkhobar, Saudi Arabia.

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Dental Science

Awareness of Dental Interns to Treat Pregnant Patients

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Abstract

BACKGROUND: Pregnancy causes major changes in maternal physiology and metabolism, which may lead to increased susceptibility to oral infection.

AIM: Aim of this study is to assess the awareness of dental interns regarding the management of the dental needs of pregnant patients.

METHODS: A cross-sectional questionnaire survey was conducted among 188 interns of a private dental college in Saudi Arabia. The questionnaire comprised of 14 knowledge-based questions regarding their training, awareness and practice management of the pregnant patient in dental clinics. Four questions to record and evaluate their training, the number of pregnant patients treated by them and their confidence level in the dental management of the pregnant patient. Excel spreadsheet was used for mathematical calculations.

RESULTS: Almost 62% of our participants never treated a pregnant female during their training. About 65% of the interns knew using antibiotics, almost 55% have a clear idea of the safest NSAIDS, and 43% regarded local anaesthesia to be safe when used among pregnant females. Conversely, about 50% of the participants had no clear knowledge of the FDA category of drugs. Only 24% considered dental radiographs to be safe in pregnant patients. 57% thought to postpone the dental treatment in an acute active dental infection in expecting mothers. Results also showed a lack of confidence among interns to provide dental care to gestating female.

CONCLUSION: On analysing the results, we found that there is a need to improve the knowledge, awareness and confidence levels among the interns who are the future dentists treating these patients.

Introduction

Pregnancy is a distinctive phase in a woman's life, complemented by a variety of anatomic, physiologic, and hormonal fluctuations that can indirectly affect oral health. These comprise changes in the respiratory, cardiovascular, and gastrointestinal systems, as well as changes in the oral cavity and increased susceptibility to oral infection [1], [2]. Though these variations are normal for a pregnant female, they dictate the consideration and modifications in the treatment by any dentist in performing the treatment or prescribing medication.

Injudicious use of medications during pregnancy can sometimes be lethal to the fetus. The wise choice should be made to determine the medical condition of the mother and the fetus, and if the medical treatment is unavoidable, only then the permitted drugs should be prescribed for a pregnant lady [3], [4]. All physicians and the general public should bear in mind that certain untreated dental conditions can as well be dangerous for both the mother and the baby [3]. Some dentists have a false belief that the dental procedures might cause bacteremia which might lead to spontaneous abortions or preterm labour. Although, few dental procedures are contraindicated in certain complicated pregnancies [1], [5], [14] The need to minimise systemic infection and disease is of utmost importance during this period. Different mechanisms have been suggested for this effect of periodontal disease on the fetus; one such proposed mechanism is seeding of urinary tract infections with bacteria from periodontal disease in mother. Dental hygiene

procedures, such as prophylaxis, deep scaling, or root planning are permitted in any trimester of normal pregnancy [1], [6], [7], [8], [9]. In case of deep dental caries causing severe pain or acute infection in an otherwise healthy gestational woman, the dentist should offer required dental care no matter what the patient's phase of pregnancy.

Many patients and dentists have the misconception about radiation exposure in dentistry. Regarding the dental radiographs, it is safe to be taken for pregnant patients by following all the protective measures like using high-speed films, paralleling technique, covering with a lead apron and thyroid collar. It is expected that the average full-mouth dental survey may expose the fetus to 1 X 10-5 rads of radiation, far below the teratogenic risk to the unborn child [8], [9], [10].

It is indicated by many researchers that comprehensive oral examination and routine oral health maintenance of pregnant patients is mandatory to improve the overall outcome of the patient and the fetus [11], [12], [15], [16]. So, the present interns who are the future dentists play a vital role in treating pregnant patients in society. The aim of the present Knowledge, Attitude and Practice (KAP) study is to know the knowledge, awareness and attitude of the dental interns in treating the pregnant patients in Riyadh.

Material and Methods

Our cross-sectional survey was conducted using a self- structured questionnaire among the dental interns between December 2105 to March 2016 in a private dental college in Riyadh, Saudi Arabia. Some of them who just entered the internship and some of the participants were about to finish the postings. The questionnaire comprised of multiple-choice questions on knowledge, attitude and awareness towards doing's and don'ts in the treatment of pregnant patients. The content authenticity was pretested on a random sample of 30 population to ascertain feasibility, strength and rendition of responses. It was developed in consultation with an oral medicine specialist and gynaecologist to improve its content validity.

The first section of the questionnaire contained the demographic data of the interns (Figure 1). Followed by the queries regarding the treatment management of a pregnant patient on different occasions in a dental clinic. The survey questionnaire was distributed as hard copy randomly to about 200 Saudi dental interns in Alfarabi dental college, and the participates were instructed to choose only one correct and appropriate answer which they feel is correct for each particular question, the filled forms were collected after half an hour. The participants who were willing to participate in the study were included, and only those who completely answered the questionnaire were considered in the study. All the 200 interns available in the university were approached, 188 (94%) participated in this study, among which 83 (44%) were males while 105 (56%) were females. Few participants (6%) submitted incompletely filled forms. So, they were excluded from the study.

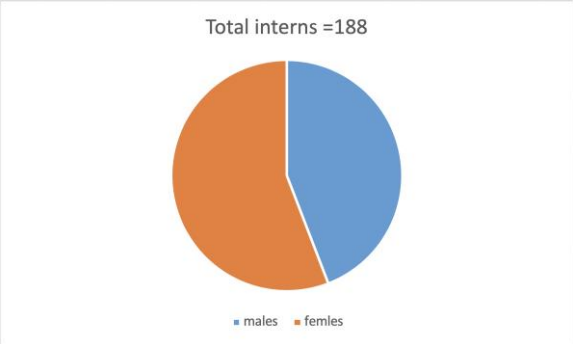

Figure 1: Demographic data of the participants

Statistical Analysis

Analysis of survey results was performed using the Statistical Package for the Social Sciences SPSS Version 18 (SPSS Inc; Chicago, IL, USA) differences between the percentage of responses for all the questions separately for male and female interns' students were statistically assessed with Chi-square test (Significance level was set at $P \leq 0.05$).

Results

The study is conducted among 188 interns in Al Farabi dental college at Riyadh, among which 83 were males, and 105 interns were females. When asked about the knowledge-based question 35% interns answered that the curriculum centred training to treat a pregnant patient in dental college was not sufficient. A group of 33% of the interns suggested that they depend on lectures and books for the treatment concerned to a pregnant patient and 55% interns rely on internet resources for the dental management of pregnant patients.

When posed for the experience in treating a pregnant female, 33% interns admitted that they never treated any gravid female in dental setup and over 57% of interns treated at least (1 to 5) pregnant patients during their course of study. For the question which probed the confidence level of the interns in managing a pregnant female, 55% of interns disclosed that they were not confident to treat a pregnant patient for dental treatment.

About 83% of interns know that the best period to do dental treatment for a pregnant female is during the second trimester. About 50% of interns were not aware of whether it is safe to use mercury restorations in a pregnant female. And 30% of the participants never heard of FDA classification of the 5-categories of drugs which determine fetal risks of medications. 12% of males are aware of the FDA classification of drugs and their safety. Only 8% of females have clear knowledge about the FDA classification of drugs (Table 1).

Table 1: Awareness of dental interns to treat pregnant patients

Sl. no	Question	Options	Total (n = 188)	M	F	P Value
1	For pregnant patients, is it safe to use mercury restorations?	Yes	30%	8%	22%	0.057
		No	20%	12%	8%	
		I do not know	50%	23%	27%	
2	What is the best period to treat pregnant women?	First trimester	10%	5%	5%	0.03*
		Second trimester	83%	33%	50%	
		Third trimester.	7%	4%	3%	
3	Are you aware of the FDA classification of the 5-categories of drugs which determine fetal risks of medications?	Yes, aware of.	20%	12%	8%	0.054
		Never heard before	30%	20%	10%	
		Little knowledge	50%	20%	30%	
4	Which antibiotic you prefer for pregnant women?	Tetracycline	20%	13%	7%	0.045*
		Amoxicillin	65%	32%	33%	
		Cefixime.	15%	5%	10%	
5	Which analgesics you prefer to use for pregnant women	Diclofenac	7%	2%	5%	0.071
		Paracetamol	55%	23%	22%	
		Ibuprofen.	38%	13%	25%	
6	Most common oral disease in pregnant patients?	Periodontitis	15%	8%	7%	0.065
		Gingivitis	55%	28%	27%	
		Ulcers.	30%	13%	17%	
7	What is the best comfortable position for pregnant women to sit in a dental chair for treatment?	Supine position	40%	24%	16%	0.061
		An upright position and turning to the right side	33%	10%	23%	
		Semi Reclined position with a pillow under right hip.	27%	11%	16%	
8	If pregnant women in 3rd trimester develop supine hypotension in the dental chair, what should be done?	Make the patient sit upright	40%	24%	16%	0.068
		Roll the patient to left side	35%	12%	23%	
		Raise the legs up	25%	12%	13%	
9	Does periodontal infection in pregnant women cause any effect on the fetus?	May lead to Preterm birth & low birth weight babies.	30%	12%	18%	0.13
		No effect on the fetus	35%	22%	13%	
		Congenital deformities.	35%	20%	15%	
10	An active dental infection like a dentoalveolar abscess in pregnant women requires?	Immediate treatment	23%	10%	13%	0.045*
		Postponed treatment till delivery.	57%	32%	25%	
		Only symptomatic treatment.	20%	8%	12%	
11	Diagnostic X ray in pregnant women is:	Permitted to take.	40%	26%	14%	0.17
		Absolutely contraindicated	36%	22%	14%	
		I do not know	24%	14%	10%	
12	Do pregnant women have a high risk of dental caries?	Yes.	40%	18%	22%	0.086
		No.	38%	27%	11%	
		Don't know.	22%	15%	7%	
13	Is local anesthesia safe to be used in pregnant patients in any trimester?	No	22%	15%	7%	0.048*
		Yes	43%	23%	20%	
		Don't know.	35%	20%	15%	
14	The best way to treat anxiety and fear for dental treatment during pregnancy is by?	Counseling and non-pharmaceutical methods.	46%	21%	25%	0.06
		Benzodiazepines and other sedatives	21%	15%	6%	
		Nitrous oxide sedation.	33%	21%	12%	

Majority of the participants (65%) opted Amoxicillin as the drug of choice to be prescribed for antibiotic coverage for pregnant patients, and the results were highly significant with P-value < 0.05. Almost 55% of interns assumed paracetamol to be the safest analgesic in gravid females, whereas 38% of the participants chose ibuprofen as the analgesic to be prescribed for expecting mothers. A nearly equal number of participants (28% males and 27% females) know gingivitis as a common oral disease among gestating females. The results showed a statistical significance where P-value < 0.05. The question regarding the best chair position for the pregnant patient during dental treatment, only 27% had the awareness that the semi-reclined position with a pillow under the right hip, is the best advice for a gravid female. And only 35% of the participants were aware that if a pregnant woman in 3rd trimester develops supine hypotension in the dental chair, the immediate care is to roll the patient to the left side. Merely 18% of females and 12% of males were mindful of the fact that periodontal infection can lead to preterm birth & low birth weight babies.

Almost 57% of the participants (with noteworthy p-value = 0.045) desired to postpone the treatment till delivery when there is a severe dental infection like a dentoalveolar abscess in a prenatal woman. Just about 35% interns (significant p-value = 0.048) were not having a clear idea if local anaesthesia was safe to be used in any trimester among expecting mothers. A group of 36% of the interns believed taking a radiograph is an absolute contraindication for pregnant women. For the query, the best way to treat anxiety and fear for dental treatment during pregnancy; 21% males and 25% females answered to use non –pharmacological methods.

Discussion

Recently there were few studies done on dental interns in different parts of the world to assess the knowledge and awareness to treat the pregnant female. We wanted to know the response from our interns and to help them have better confidence in treating a gravid female. Usually, many of them work in private practice after their graduation, so they need to be competent about the emergencies and have enough knowledge regarding what kind of prescription they can advise and what treatment is permitted in an expecting mother.

With this aim, the study was conducted to evaluate the knowledge and increase the awareness

Among dental interns to treat pregnant patients, it is crucial to be aware of the oral health needs of the pregnant patient and preventive care, dental treatment and drugs that can be provided safely during pregnancy. The results give us a picture of interns, in terms of how they are equipped to treat a

pregnant patient. However, the difference between male and female interns and their confidence levels could be due to the difference in the amount of exposure to patients. Also, the results of this study may not have external validity. About 60% of surveyed dental interns think that the information that was thought about dental management of pregnant women in dental school was not enough or it is just little. The results were in correlation with a similar study done where 70% of participants declared that the knowledge and training received through their curriculum was not enough [16]. About 62% of our participants never treated pregnant women during their training. And 10% of our interns have the experience of treating at least 5 or more expecting mothers in the dental clinics. Almost 55% of our participants were not confident in treating a pregnant mother in a dental clinic. In a study conducted among the interns in India, 35% of the participants never treated a pregnant patient, and only 57% participants treated less than 5 pregnant patients, and only 21% of the participants were not confident to treat such patients [16].

Approximately the similar results were recorded Tantradi P et al., in a study conducted on general dentists where two-thirds of the participants were interested in receiving continuing dental education (CDE) regarding dental care in expecting mothers [16]. In our study, 60% of the respondents were eager to update their knowledge through CDE programs. Nearly 55% of our interns were aware that gingivitis was the most common oral manifestation among gestating female, and the results were in contrast to previous two different studies where 92% and 81% participants respectively agreed that pregnancy increases chances of gingival inflammation [17], [18], [19] the disparity in the results could be attributed to the sample population in one study being the medical doctors, and in another study the sample of dental interns were from different universities and hospitals. Around 83% of our participants thought that 2nd trimester is the safest for performing any dental treatments; analogous results were recorded in previous research where 87% of interns stated 2nd trimester being safe for a pregnant lady to undergo any dental treatment [16], [18].

Regarding the diagnostic dental X-ray in pregnancy, 36% believed it is contraindicated,24% did not have a clear idea about the safety of dental radiation. It shows that most of them were not having the proper knowledge about the dose of dental radiation. In a previous study, almost 63% of the participants considered using the diagnostic dental x-ray among pregnant patients. As the fetus sizes grow, there is elevated discomfort for the patient in the supine position [1]. For gestating females, it is advised not to position them in the supine position during dental treatment to avoid supine hypotension and deep venous thrombosis.

Contrary to the previous study where 33% of the interns were aware of the right patient position during dental treatment, only 27% of our participants were aware that semi-reclined position with a pillow under right hip is best advocated for these patients [16]. 35% of our interns were aware that if a pregnant patient develops supine hypotension during dental treatment, it is best to roll the patient to the left side. It is always recommended to keep the scheduled dental appointments short and allowing these patients to resume the semi-reclined position and encourage for frequent change in positions.

Fortunately, most of the drugs used in a dental clinic are generally considered safe for both expecting mother and the fetus [1], [2], [6], [7] Almost 43% of our interns were of the opinion that local anaesthesia is safe to be used in pregnant patients in any trimester, whereas 59% of the participants in a previous study conducted among interns had agreed that local anaesthesia has the least risk when used in pregnant females. Nearly 30% of our participants were mindful of the fact that periodontal infection can lead to preterm birth & low birth weight babies. Contrasting results were identified in another study where 59% of interns were aware of the fact that periodontitis causes preterm low birth weight babies [17]. Few clinical trials have recorded that non-surgical periodontal therapy like plaque control instructions, scaling, polishing and root planing under local anaesthesia can reduce the risk of preterm low birth weight babies [13], [19], [20], [21].

Food and Drug Administration (FDA) has classified drugs into five categories of safety for use during pregnancy. Acetaminophen is the safest NSAID and the drug of choice to prescribe for an expecting mother [9], [12]. Only 55 % of our participants knew the safest NSAID among pregnant females. There were varied results in another study with 90% of participants being aware of the safest NSAID [17]. Ibuprofen, when given in first and second trimesters, considered as category B analgesic, but because it has been associated with lower levels of amniotic fluid, premature closure of fetal ductus arteriosus and inhibition of labour when taken during the third trimester, it is a category D drug during this phase [1].

If dental caries is a source of pain because of acute infection in an otherwise healthy pregnant woman, it is the dentist responsibility to provide prompt care irrespective of the patient's phase of pregnancy [1], [2]. Because untreated active infection can cause greater risk than the hazard caused by performing the treatment, also, febrile illness and sepsis can precipitate a miscarriage [12]. Unfortunately, only 23% of interns were aware that acute dentoalveolar infection should be treated immediately. The results were in contrast with the other study were 37% interns were willing to provide immediate treatment for active dental infection for a pregnant patient. With varying emotional phases, fears, and phobia, these pregnant patients delay or

avoid their dental treatment. Anxiety may lead to transient increases in blood pressure, gastrointestinal upset, uterine cramping or hyperventilation. Often, counselling and addressing the cause of the patient's fears help relieve these symptoms. In our study, only 46% of the participants thought that counseling and non- pharmaceutical methods are sufficient to treat anxiety for a pregnant patient in a dental clinic. The limitations of our study are the sample size being small, and we have conducted only in one university who are the representatives of dental interns in Saudi Arabia. We also need to consider regarding the fluency in English language, since participants were not the native speakers.

In summary, we found that a significant number of the participants were not having adequate awareness to treat the pregnant patients. On analyzing the results, it is established that most of the interns (55%) lacked confidence to manage the dental needs of pregnant patients. While 35% expressed that they were somewhat confident to do any dental treatment for a gestating female. Despite the limitations in the study there is a definitive need to improve their knowledge. The confidence level among the interns can be instigated by implementing curriculum to strengthen their experience to face a pregnant patient in dental clinic during their training period, and to have in-depth foundation on the knowledge of oral health needs and treatment options for gestating females, also by encouraging the interns to update their knowledge by attending the CDE programs periodically.

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Dental Science

The Comparative Evaluation of the Post-Antimicrobial Effect of MTAD ® and 2% Chlorhexidine against *Enterococcus faecalis* of Permanent Teeth with Necrotic Pulp

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Abstract

AIM: *Enterococcus faecalis* is one of the most resistant bacteria in necrotic teeth. That's why the goal of this study was to determine the post-antibiotic effect of MTAD®& 2% Chlorhexidine® as root canal irrigating solution on clinical isolates of *E. faecalis* from infected root canals of permanent teeth, using the spectrophotometric technique.

MATERIAL AND METHODS: The antibacterial efficacy of Chloramphenicol 30 mcg, Nitrofurantoin 300 mcg, Vancomycin 5 mcg, Amoxicillin/clavulanic acid 30 mcg and Ofloxacin 5 mcg against E. faecalis was compared using the Disc diffusion method. Patients were selected for this study with permanent necrotic teeth. The sterile paper point was inserted inside the infected root canal and left for 60 seconds; to obtain the microbiological sample. Postantibiotic effect of MTAD® and 2% Chlorohexidine® on *E. faecalis* was compared. The absorbance of bacterial growth was examined for both irrigating solutions during the first 10 hours with an hour interval, and then tested at 48, 72, 96 up to 240 hours.

RESULTS: The results showed that during the first 10 hours, MTAD® showed immediate antibacterial effect and maintained its higher antibacterial activity than 2% chlorohexidine®. After 48, 72, 96 and 240 hours, both MTAD® and 2% chlorohexidine® showed the same prolonged action of post-antibiotic effect against *E. faecalis* with a non-significant difference. According to Antibiotic sensitivity, the results revealed MTAD® is the most effective antimicrobial drug, showing the highest zone of inhibition, followed by 2% Chlorhexidine and Nitrofurantoin 300 mcg which showed the same inhibitory activity

CONCLUSION: From the current study, it can be concluded that MTAD® has a strong bactericidal effect against *E. faecalis* and showed the highest zone of inhibition.

Introduction

The short- and long-term success of *endodontic treatment* depends on the elimination of bacteria from the *root canal* system and prevention of reinfection. This can be achieved with both mechanical debridement and using of the suitable irrigating solution with strong bactericidal properties especially against the most resistant type of bacteria in the necrotic teeth which is the *Enterococcus faecalis* which humper the success of endodontic treatment [1].

E. faecalis is considered a pathogen responsible for persistent apical periodontitis as it can tolerate extreme conditions and survive in the root canals and periapical tissues without the support of other bacteria [2].

That's why it is considered one of the most resistant bacteria in necrotic teeth, and its persistence causes the failure of the root canal treatment. And it requires different visits and using intracanal medications in-between visits to eradicate this bacteria from the root canal, So it is very important to find an irrigating solution which has a strong bactericidal effect of getting rid of bacteria and improve the success rate of root canal treatment of necrotic teeth [3].

An antimicrobial agent that has a prolonged Post antibiotic effect (PAE) has several potential advantages, among them, decrease the frequency of using the antimicrobial irrigant, decrease the number of visits, and increase the time between visits. All of

these will result in reduced cost, less toxicity, time-saving for the endodontist and the patient and better compliance among patients. The major clinical relevance of the PAE pertains to its impact on antimicrobial dosing, where agents inducing a long PAE may be (used with less frequency without loss of efficacy or affecting the results) [4].

In this study, the persistent suppression of bacterial growth following brief exposure to an antibiotic (Postantibiotic effect) [PAE] has been examined in vitro for antibiotic containing irrigating solutions, MTAD® and 2% Chlorhexidine®, against clinical isolates of oral Enterococci. This examination was done using the spectrophotometric technique.

The antimicrobial susceptibility was also measured to Chloramphenicol 30 mcg, Nitrofurantoin 300 mcg, Vancomycin 5 mcg, Amoxicillin / clavulanic acid 30 mcg and *Ofloxacin 5 mcg* by using the Disc diffusion method.

The goal of this study was to determine the post-antibiotic effect of MTAD® and 2% Chlorhexidine® as root canal irrigating solution on clinical isolates of *E. faecalis* from infected root canals of permanent teeth, using the spectrophotometric technique.

Compare the antibacterial efficacy of Chloramphenicol 30 mcg, Nitrofurantoin 300 mcg, Vancomycin 5 mcg, Amoxicillin / clavulanic acid 30 mcg and Ofloxacin 5 mcg against *E. faecalis* using the Disc diffusion method.

Material and Methods

The clinical procedure of microbiological samples

Patients were selected for this study with permanent teeth with necrotic pulp. Local Anesthesia was given to the patients. Necrotic teeth were isolated using a rubber dam to prevent further contamination of the tooth or the microbiological samples. Caries removal and access cavity preparation using round bur and flaring using endo Z bur. The sterile paper point was inserted inside the infected root canal and left for 60 seconds; then sterile tweezer was used for removal of the paper point from the canal with the microbiological sample and inserting it into airtight vials containing thioglycolate media and the sample transported to the lab immediately in the icebox.

Purification and identification of the recovered isolate

E. faecalis was recovered from clinical specimens of patients suffered from infected root canals of permanent teeth. All clinical samples were streaked on the surface of Blood agar plates. The inoculated plates were incubated aerobically at 37˚C for 24 to 48 hours. The colonies of Enterococci appeared on Blood agar plates with no hemolysis and white colonies (Figure 1).

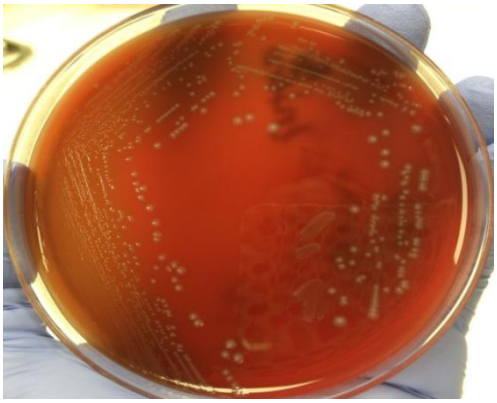

Figure 1: Growth of Enterococcus faecalis on Sheep Blood Agar (Gamma hemolysis)

E. faecalis isolates were isolated and identified by traditional methods. Identification relies on phenotypic identification of the *E. faecalis* using Gram staining, culture, and biochemical processes. Furthermore, molecular biology method was obtained by polymerase chain reaction identification of *E. faecalis* (GenBank: ASDA01000011.1).

Antimicrobial irrigating solution preparation

The first experimental irrigant used in this study was BioPure® (MTAD®), which is a mixture of doxycycline, an acid (citric acid) and detergent (tween 80). It is provided in the form of a powder (bottle) and liquid (syringe). MTAD® should be freshly mixed immediately before use. The liquid syringe was fixed to the powder bottle, and the liquid was injected into the bottle and left for mixing for 60 seconds tell the powder completely dissolves in the liquid. After that, the solution was drawn into the 5 ml delivery syringe and attached to the needle to be ready for use.

The second Experimental irrigant was 2% Chlorohexidine was supplied as a liquid, ready for use.

Post-antibiotic effect (PAE) experiments

Postantibiotic effect of MTAD® and 2% chlorhexidine® on *E. faecalis* was compared using the spectrophotometric technique by measuring the absorbance of the Optical density (OD) of bacterial growth at 590 nm, at different time intervals up to 240 hours. The absorbance of bacterial growth was examined during the first 10 hours with a one-hour interval and then tested at 48, 72, 96 up to 240 hours.

Determination of PAE

One of the most widely cited *in-vitro* methods, described in details by Dominguez *et al.,* (4). PAE was induced by exposing new cultures on the broth of Muller-Hinton medium in the logarithmic phase to the tested chlorhexidine® or MTAD® for 5 minutes at 37°C in an incubator shaker. After incubation for 5 minutes, the antimicrobial agent is removed by repeated washing (at least three times) of the bacterial cells by saline then centrifugate at 13000 rpm for 20 minutes in 15 ml Falcon tubes. After removing the supernatant, the bacterial cells are re-suspended in a new broth of Muller-Hinton to characterise the growth kinetics. In general, to ensure that the process of removal of antimicrobial agent is not contributing to the PAE, an untreated control culture undergoes a similar process of antimicrobial agent removal, subsequent incubation, and absorbance determination. This negative control culture is used as a reference for comparison of the growth of both control and treated culture.

The duration of PAE was calculated by using the formula (PAE = T-C), where T was the time required for the relative optical density of the exposed cell suspension to reach the 0.05 absorbance level after removal of the irrigant, and C was the time required for the relative optical density of the irrigant-free control cell suspension to reach the same absorbance level. Thus T-C expressed the time in which the antibacterial agent was capable of causing growth suppression of the organism following limited exposure to the irrigant.

Disk diffusion test

Antibiotic susceptibility test of *E. faecalis* isolates was determined on Muller Hinton agar plates by Kirby-Bauer disc diffusion method. Antibiotic discs were purchased from Himedia, Mumbai, India. The antibiotics tested were Chloramphenicol (30 mcg), Nitrofurantoin (300 mcg), Vancomycin (5 mcg), Amoxicillin / clavulanic acid (30 mcg) and Ofloxacin (5 mcg). The clinical isolate of *E. faecalis* was declared as sensitive or resistant according to the zone of inhibition following the criteria of the Clinical Laboratory Standards Institute.

A One-way Analysis of Variance (ANOVA) test was used to analyse the bacterial growth of MTAD, CHX and control group, where the P-value is < 0.0001.

Results

Ten clinical samples were obtained from infected root canals, and the following microorganisms were isolated; 5 isolates of *E. faecalis,* 3 isolates of *Candida albicans,* 4 isolates of *Actinomyces* species and 2 isolates of *Streptococcus mutans. E. faecalis* strain was successfully identified and isolated from clinical samples of infected root canals. Antimicrobial susceptibility test of MTAD® and 2% chlorhexidine were examined for the isolates of *E. faecalis* (Table 1). Isolates no.4 was the most potent one, so it was chosen to determine the PAE.

Table 1: Antimicrobial susceptibility test of MTAD® and 2% chlorhexidine for five clinical isolates of *Enterococcus faecalis*

Number of isolates of *Enterococcus faecalis*	Zone of inhibition of MTAD	Zone of inhibition of 2% chlorhexidine
1	19	10
2	13	15
3	13	10
4	22	20
5	16	18

Determination of PAE

The PAE of MTAD®and 2% chlorhexidine against *E. faecalis* isolate was determined by the spectrophotometric technique as shown in Figure 2. The obtained data showed that MTAD® and chlorhexidine against *E. faecalis* isolate induced prolonged PAE at different time intervals up to 10 days.

In the control group, the absorbance of bacterial growth was 0.5 during the first 4 hours, then increased to reach 1.3 at 5 hours. In 2% Chlorohexidine group, the absorbance of bacterial growth was 0.3 during the first 10 hours. MTAD® showed immediate antibacterial effect and prolonged action after its application on *E. faecalis*, and higher percentage of bacterial growth inhibition and minimal absorption of bacterial growth during the first 10 hours, which was measured spectrophotometrically. This indicates that MTAD has prolonged PAE in comparison to 2% chlorhexidine® which showed weak antibacterial effect within the first 4 hours and high absorption of bacterial growth when measured by spectrophotometry as shown in Figure 2A.

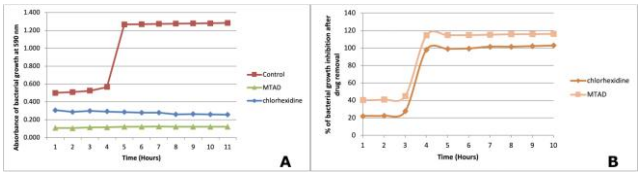

Figure 2: A) Absorbance of bacterial growth of Enterococcus faecalis to 2% chlorhexidine and MTAD, to determine the PAE after 5 min. of exposure to the irrigating solutions during the first 10 hours; B) Percentage of bacterial growth inhibition of Enterococcus faecalis to MTAD and 2% Chlorohexidine to determine the PAE after 5 min. of exposure to the irrigants during the first 10 hours

There was non-significant difference between 2% Chlorohexidine group and MTAD group during first 10 hours, where MTAD showed least absorbance of bacterial growth indicating its strong antibacterial activity in comparison to 2% Chlorohexidine.

During the first 10 hours, MTAD® maintained its higher antibacterial activity than 2 % chlorhexidine®, which indicates the prolonged post-antibiotic effect of MTAD®, as shown in Figure 2.

Measuring the absorbance of bacterial growth for *E. faecalis* during a period of 10 days after irrigation with MTAD or 2% chlorhexidine, showed no absorbance starting from the second day up to the next 10 days, due to no growth of bacteria. This indicates the complete death of bacteria on the second day which continued for 10 days, with the non-significant difference between MTAD and 2% Chlorohexidine group. These results showed that both MTAD and 2% Chlorohexidine irrigating solutions have prolonged action of post-antibiotic effect against *E. faecalis* and also they have bacteriocidal effect after exposure of the bacteria to the irrigating solutions for 5 minutes as shown in Figure 3.

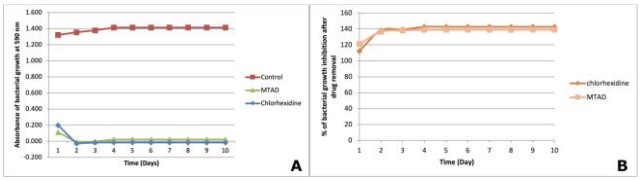

Figure 3: A) Absorbance of bacterial growth of Enterococcus faecalis to 2% chlorhexidine and to MTAD, to determine the PAE after 5 min. of exposure to the irrigating solutions within 10 days; B) Percentage of bacterial growth inhibition of Enterococcus faecalis to MTAD and 2% Chlorohexidine to determine the PAE after 5 min. of exposure to the irrigating solutions within 10 days

Antibiotic Sensitivity test

According to Antibiotic sensitivity, the results revealed MTAD® as the most effective antimicrobial irrigant, the zone of inhibition (22 mm), while Amoxicillin/clavulanic acid 30 mcg showed no effect against *E. faecalis.*

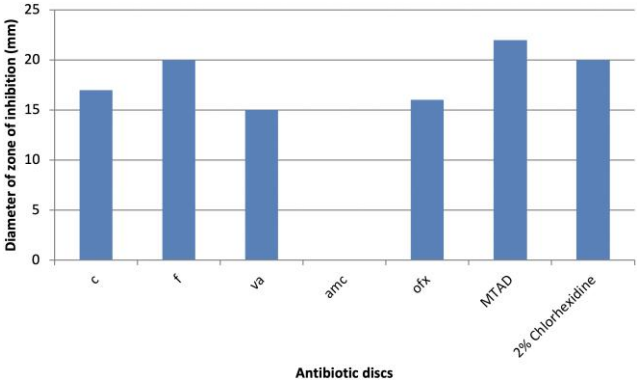

Figure 4: Antibiotic susceptibility test results for the isolated Enterococcus faecalis. Chloramphenicol (C) 30 µg, Nitrofurantoin (F) disc 300 µg, vancomycin (VA) 30 µg, Amoxycillin/clavulanic acid 2:1 30 µg, Ofloxacin (OFX) 5 µg, MTAD® and 2% Chlorhexidine®

Two percent Chlorhexidine and Nitrofurantoin 300 mcg showed the same inhibitory activity (20 mm) against *E. faecalis* clinical isolate, as shown in Figure 4 and Table 2.

Table 2: Antimicrobial Activity against *Enterococcus faecalis* by Disc diffusion Method

Antibiotic discs	Zone of inhibition (mm)
MTAD®	22
2% Chlorhexidine	20
Chloramphenicol 30 mcg	17
Nitrofurantoin 300 mcg	20
vancomycin 5 mcg	15
Amoxicillin/clavulanic acid 30 mcg	Resistant
Ofloxacin 5 mcg	16

Discussion

Different techniques of root canal preparation leave areas of the canal walls untouched by the instruments. So irrigating solutions have a significant role in debridement and cleaning of these areas of the root canal walls. That's why it is very important to search for the most suitable irrigating solution which can reach these untouched areas and has a strong antibacterial action against resistant bacteria [5].

E. faecalis is the most persistent pathogen that makes it play the most critical role in the persistence of ofperiradicular lesions after root canal treatment [6], [7]. Therefore, *E. faecalis* is usually used as a model organism in the testing of the efficacy of irrigants and intracanal medicaments.

Different irrigating solutions have their share of limitations, that makes searching for an ideal root canal irrigant continues with the development of newer materials and methods. In the current study, MTAD and 2% Chlorhexidine were used, to evaluate their post antimicrobial effect against *E. faecalis* and the persistence of this effect for different durations.

Microbiological samples were taken from patients with permanent teeth with necrotic pulp, as *E. faecalis* is the most persistent type of bacteria in necrotic teeth, as stated by Kamberi *et al.,* [3].

Due to the composition of MTAD which is (citric acid, Tween 80 and doxycycline hyclate) [8], it was found to be highly effective intracanal irrigant comparing to other commonly used root canal irrigants having excellent disinfection of the entire root canal system [9]. Citric acid is a crystalline organic acid, which has an antimicrobial property and helps in removal of smear layer in different concentrations, thus helping deeper penetration of doxycycline into the dentinal tubules and exerting its antibacterial action. While Tween 80 (polyoxyethylene sorbitan monooleate) is a detergent present in MTAD and a non-ionic surfactant. Therefore, it helps in reducing the surface tension of distilled water, EDTA, NaOCl, thereby enhancing the flow and penetration of irrigating solutions deeper into the dentinal tubules and thus wholly disinfecting the canal spaces. Doxycycline Hyclate, is an isomer of tetracycline, they differ in structure but not in composition. It is a broad-spectrum antibiotic effective against a wide range of microorganisms. Tetracyclines act by inhibiting protein

synthesis and reversibly binding to the 30s ribosomal subunits of susceptible microorganisms [10]. All these components may explain why MTAD has a prolonged PAE for more than ten days in the current study. Because of the combination of actions of different antimicrobial agents. On the other hands, Gomes *et al.,* [11], Vianna *et al.,* [12] were in agreement with results of the current study as they found that the 2% Chlorohexidine and Cetrexidin were significantly more effective against *E. faecalis* than the 5.25% NaOCl at both time periods.

MTAD showed immediate and strong antibacterial action against *E. faecalis* compared to chlorohexidine. And its antibacterial activity is sustained for an extended period up to 10 days. Chlorhexidine succeeded in reaching the same antibacterial effect but after a more prolonged period. These results are by Mohammadi and Shahriar [13] who measured the residual antibacterial activity of chlorhexidine and MTAD and found that the substantivity of MTAD was significantly greater than chlorhexidine and NaOCl.

Also, Giardino *et al.,* [14] and Mohammadi *et al.,* [15] found that MTAD and Tetraclean showed the larger area of bacterial inhibition of *E. faecalis* compared to NaOCl. White *et al.,* [16] found that the antibacterial activity of chlorhexidine lasted for 72 hours. Also, Leonardo *et al.,* [17] concluded that the residual antibacterial activity of chlorhexidine lasted for 48h in the root canal system. While, Khademi *et al.,* [18] found that antibacterial substantivity of chlorhexidine was greater than doxycycline and NaOCl where these results are in contrast with the results of the current study.

In this study, the obtained results showed that MTAD® induced prolonged PAE period (more than ten days) than 2% chlorhexidine against *E. faecalis*. These data are in agreement with Mohammadi and Shahriari [13] who compared the antimicrobial effect of MTAD®, 2% chlorhexidine and 2.6% NaOCl on *E. faecalis* in human root dentin. Their findings showed the MTAD® was more effective than the other solutions and was retained in the root canal dentin for at least 28 days. These findings are consistent with results of the current study and those of other researchers Royal *et al.*, [19] and Tay *et al.*, [20] who have reported the superior efficacy of MTAD® against *E. faecalis.* In another said, Davis *et al*., [21], used experiments *in vitro* to show that 2% chlorhexidine and 5.25% NaOCl both exhibited less antimicrobial efficacy against *E. faecalis* than MTAD®, demonstrating that MTAD® is a viable medicament against *E. faecalis*. These data are in agreement with the results of the current study.

Pathogenic bacteria in root canals can generate resistance to doxycycline because of the topical use of MTAD as a root canal irrigant. Therefore, for endodontic specialists, the development of a highly efficient root canal irrigant is an essential precondition for improvement in the success rate of root canal treatment. Muchmore, Clinical isolates of *E. faecalis* displayed greater sensitivity to MTAD than *E. faecalis* ATCC 29212 in the minimum bactericidal concentration (MBC) assay [22], [23].

It can be recognised that the concept of a PAE is not only inhibition of regrowth but additional effects, such as morphological and physiological changes [24], [25], [26], which might be of clinical significance. It should be clear that a PAE is not the only post-exposure event that should be evaluated. An antibiotic inducing sublethal damage to bacteria might produce increased susceptibility to host defences, which might contribute to recovery from infections, at least in an immunocompetent host. However, it should be evident that the single most important parameter for the antimicrobial effect of an antibiotic must be its bactericidal activity rather than the unpredictable elements of a PAE (or postantibiotic sub-MIC effect) or reduction of virulence. The data presented in this study reveal that MTAD had significantly greater bactericidal activity and a longer PAE (240 h).

Enmd *et al.,* [27], found high sensitivity and resistant of *E. faecalis* to different antibiotics, which is similar to results of the current study which showed sensitivity of *E. faecalis* strains to vancomycin, on the other hand, Johnson *et al.,* [28] found resistance of some strains of *E. faecalis* against vancomycin and ciprofloxacin.

With the limitations of the current study, it can be concluded that both MTAD and Chlorohexidine have a powerful anti bactericidal effect against *E. faecalis* in contaminated root canals by producing extended PAE affect more than 120 hours after removing of MTAD or even chlorhexidine (2%).

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Dental Science

Comparison of Accuracy in Determining the Root Canal Working Length by Using Two Generations of Apex Locators – An In Vitro Study

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Keywords: Apex locators; Apical foramen; Root canal; Working length

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Abstract

AIM: The present in-vitro study aims to compare the accuracy of root canal working length determination between the third generation and fourth generation electronic apex locators.

MATERIAL AND METHODS: Fifty extracted single-rooted single canal teeth were selected for the study, and a definite coronal plane was prepared. Actual working length (AL) was measured using a stereomicroscope under 8X magnification. Electronic working length measurements were recorded using Root ZX (EL1) and Elements Diagnostic Unit (EL2) apex locators. One-way ANOVA test was carried out to analyse the data among the experimental groups.

RESULTS: The results of the one-way ANOVA test showed that difference in the working length determined by either apex locators (EL1 and EL2) and actual length determined under a stereomicroscope (AL) was statistically not significant. The independent 't' test comparing between groups EL1 and AL; and EL2 and AL showed that working length determined by either of the apex locators (EL1 and EL2) and actual length determined under a stereomicroscope (AL) was statistically not significant.

CONCLUSION: In this in vitro study, the Root ZX and Elements Diagnostic Unit apex locators are equally accurate for determination of working length when compared to actual working length.

Introduction

The prerequisites for successful endodontics are a proper access opening, complete debridement and biomechanical preparation of the root canal to an accurate predetermined length and three-dimensional obturation of the prepared root canal space. Out of these three prerequisites, the latter two cannot be accomplished accurately unless the working length is determined precisely [1].

The working length is defined as "the distance from a coronal reference point to the point at which canal preparation and obturation should terminate". Accurate working length determination is a crucial part of successful endodontic treatment. Determining the working length accurately decides the apical end-point for the instrumentation and obturation [3].

Grove [4] in 1930 stated that, 'the proper point to which root canal should be filled is the junction of the dentin and the cementum and that the pulp should be severed at the point of its union with the periodontal membrane'. The cement-dentinal junction (CDJ) is a landmark where the periodontal ligament begins, and the pulp ends [5]. The cement-dentinal junction is a histological landmark that cannot be located clinically or radiographically. The cement-dentinal junction does not always coincide with the apical constriction. Hence, the apical constriction is regarded as an ideal apical end-point for instrumentation and obturation in root canal therapy [6].

The most common methods for working length determination are radiographic methods and electronic methods. Other methods like digital tactile sense, apical periodontal sensitivity, and paper point measurements have also been used, but are unreliable and subjected to marked intra-subject differences [8].

The idea of electronic determination of working length is not new and has a history, long back when Custer [9] in 1918 first reported that the root canal length could be determined by using the electrical conductance. Authors have performed a series of experiments on patients and reported that the electrical resistance between the mucous membrane and the periodontium was consistent regardless of the age of the patients or the shape and type of the teeth [8], [10]. Since then, many electronic apex locators under various generations were developed, with every new unit being somewhat superior and overcoming the drawbacks of earlier ones.

The third-generation apex locators measure the impedances of 8 kHz and 400 Hz at the same time, calculates the quotient of the impedances and expresses this quotient in terms of the position of the file inside the canal. This quotient is barely affected by the electrical conditions inside the canal. Also, it is not necessary to calibrate this device each time because the microprocessor automatically controls the calculated quotient. This device has been exhaustively tested and reported to be quite accurate in various conditions [8], [11].

The fourth-generation device breaks impedance down into its primary components (resistance and capacitance) and measures them independently during use. This eliminates erroneous readings because different combinations of these properties provide the same impedance reading. This prevents apex locator from being jumpy and erratic. Multiple frequencies are still used to compensate for canal conditions. The Elements Diagnostic Unit apex locator (Sybron Endo, Sybron Dental, Orange, California, USA) does not make calculations internally as third-generation units do. Instead, all combinations of capacitance and resistance relating to a location within the canal have been loaded into a matrix database within the unit. This decreases processing time, making the displayed information much more stable [12].

The aim of the present *in vitro* study is to compare the accuracy of working length determination between two generations of electronic apex locators; between third generation and fourth generation apex locators.

Material and Methods

Fifty extracted, single-rooted, single canal teeth, with mature apices, were used in this study (Figure 1). The criteria for tooth selection included intact enamel without caries, restorations or surface anomalies. Teeth were kept in 5% sodium hypochlorite (Septodont health care India Pvt. Ltd) for 2 hours to remove the periodontal remnants and then stored in sterile 0.9% saline solution (Baxter India Pvt. Ltd) until use. A definite coronal plane prepared with carborundum disc (Dentorium, New York, USA) fixed on slow speed straight handpiece (Marathon, Korea) provided a fixed, stable surface for the adaptation of rubber stopper. This helped to avoid measuring errors resulting from different interpretation of the coronal reference point.

Figure 1: Fifty single-rooted teeth with definite coronal plane

The access cavity was prepared using No. 2 round bur (Mani, Japan), patency of the canal established using No.10 k-file (Dentsply, Maillefer, USA). Gates Glidden drills no. 5 and 6 (Dentsply, Maillefer, USA) were used to flare the coronal one-third of each canal. The canals were cleansed of debris by irrigating with 5% sodium hypochlorite (Septodont health care India Pvt. Ltd) after which canal patency was evaluated using a size 10 K file (Dentsply, Maillefer, USA).

Working length determination

Group AL-Actual length determination under the stereomicroscope **(**Magnus**)**.

Group EL1-Electronic working length

determination by third-generation electronic apex locator (Root ZX, J. Morita Co., Kyoto, Japan). Group EL2-Electronic was working length determination by fourth-generation electronic apex locator (Elements Diagnostic Unit Apex locator).

Actual length determination

The actual length (AL) was measured with the aid of a stereomicroscope (Magnus Opto Systems India Pvt. Ltd) under 8 X magnification by introducing a no. 15 K-file until it emerged at the apical foramen. The file was then withdrawn until its tip was tangential to the apical foramen (Figure 2). After adjusting the silicone stopper to the flattened reference point, the file was removed, and the distance between the file tip and the stopper was measured with digital callipers. To record the actual root canal length (AL), 0.5 mm was subtracted from it. This determined length served as the control group.

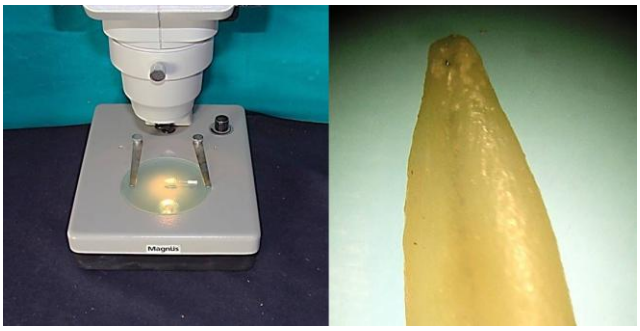

Figure 2: Actual working length determination under the stereomicroscope

Working length determination using third-generation apex locator

The file was advanced within the root canal to just beyond the foramen, as indicated by the flashing APEX bar and the solid tone. The file was then withdrawn to a flashing bar halfway between APEX and 1, and that measurement was recorded.

Working length determination using fourth-generation apex locator

The file was advanced into the canal to just beyond the foramen, as indicated by the '0.0' mm mark on the LCD. The file was then withdrawn until the reading showed a consistent '0.5' mm mark with the corresponding symbol and audible signal, and the measurement was recorded

In order to reproduce clinical conditions involved in the electronic measurement of the root canal length and to complete the circuit apical third of each tooth is immersed in 0.9% saline bath in a glass beaker with a rubber lid and the lip clip is attached to the lid is in contact with the saline (Figure 3).

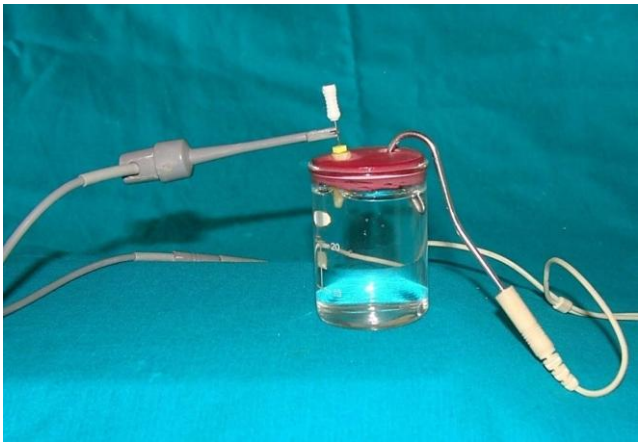

Figure 3 Apparatus for working length determination using apex locators

The statistical analysis was performed using a commercially available software program SPSS version 12. One-way ANOVA test was carried out for comparing the three groups, whether significant differences existed among the tested groups.

Results

The mean working length obtained from third-generation apex locators, Root ZX (Group EL1) at 14.72 mm and from fourth-generation apex locator, Elements diagnostic unit apex locator (Group EL2) at 14.66 mm were comparable to the actual length determined under a stereomicroscope (Group AL) at 14.76 mm (Table. 1).

Table 1: Mean ± SD values of actual length and length determined by apex locators

GROUP	N	Mean ± SD (mm)	Minimum (mm)	Maximum (mm)
Group AL	50	14.76 ± 1.84	11.64	20.74
Group EL1**	50	14.72 ± 1.85	11.51	20.72
Group EL2 ***	50	14.66 ± 1.84	11.62	20.70

*AL = Actual length determination under stereomicroscope; **EL1=Electronic working length determination by third-generation electronic apex locator; ***EL2=Electronic working length determination by fourth-generation electronic apex locator.

The results of the one-way ANOVA test showed that difference in the working length determined by either apex locators (EL1 and EL2) and actual length determined under a stereomicroscope (AL) was statistically not significant. (Table. 2)

Table 2: Results of One-way ANOVA test comparing the three groups

GROUP	N	Mean ± SD	F	p-value
Group AL*	50	14.76 + 1.84	0.0370	p = 0.963
Group EL1 **	50	14.72 + 1.85		
Group EL2***	50	14.66 + 1.85		

[c]Statistically significant difference p ≤ 0.05; *AL = Actual length determination under stereomicroscope; **EL1=Electronic working length determination by third-generation electronic apex locator; ***EL2=Electronic working length determination by fourth-generation electronic apex locator.

The independent '*t*' test comparing between groups EL1 and AL; and EL2 and AL showed that test statistic p-value was, 0.9214 and 0.7882 respectively. This showed that working length determined by either of the apex locators (EL1 and EL2) and actual length determined under a stereomicroscope (AL) was statistically not significant, (Table 3).

Table 3: Results of Independent 't' test comparing the individual groups

Comparing groups	*t'*	p-value
Group EL1 and Group AL	0.0989	p = 0.9214
Group EL2 and Group AL	0.269	p = 0.7882
Group EL1 and Group EL2	0.170	p = 0.8656

[c]Statistically significant difference p < 0.05; *AL = Actual length determination under stereomicroscope; **EL1=Electronic working length determination by third generation electronic apex locator; ***EL2=Electronic working length determination by fourth generation electronic apex locator.

The test statistic p-value obtained for independent 't' test between groups EL1 and EL2 was 0.8656 (more than 0.05), this showed that working length determined by third-generation electronic apex locator, Root ZX (Group EL1) and by fourth-generation electronic apex locator, Elements diagnostic unit apex locator (Group EL2) was statistically not significant.

Discussion

An accurate working length determination is one of the critical steps for successful endodontic treatment [2]. The radiographic method has disadvantages like more radiation exposure, time-consuming, and in most cases, the cement-dentinal junction does not coincide with the point 0.5 mm short from the radiographic apex because of cementum deposition. Also, it is only able to give a two-dimensional image and provides reliable information on the location of the radiographic apex [15].

In recent years, electrical devices have been developed for determining the length of the tooth without resorting to radiography. Here, the working length is determined by comparing the electrical resistance of the periodontal ligament with that of the gingiva surrounding the tooth, both of which should be similar by measuring the depth of insertion of the file one may determine the exact working length of a root canal [12].

Third generation apex locators have set a landmark inaccurate location of tooth apex and are now considered an essential tool in the endodontic armamentarium [16]. The new fourth-generation apex locators claim to be even more accurate regarding accuracy in apex location.

Root ZX (J. Morita Co., Kyoto, Japan) is a third-generation multi-frequency apex locator which uses two waveforms, 8 kHz and 400 Hz. Studies have shown it to be accurate in the range of 64% to 100% [16]. If 1.0 mm difference is deemed acceptable, the accuracy reported at 100% [18]. Lesser deviations from the apical constriction are reproducible [11], [13]. Elements diagnostic unit apex locator, (Sybron Endo, Sybron Dental, Orange, California, USA) is a fourth-generation apex locator which breaks impedance down into its primary components (resistance and capacitance) and measures them independently during use. This eliminates erroneous readings because different combinations of these properties provide the same impedance readings, and also prevents the apex locators from being "jumpy" and erratic [14]. Since this generation of apex locator guarantees better accuracy than the third generation, this apex locator has been used in the present study.

To reproduce clinical conditions involved in the electronic measurement of root canal length and to complete the circuit various laboratory models have been suggested: immersion in agar solutions or gels [22], or embedding in alginate [23], or a sponge soaked with saline solution [24]. In the present study, a 0.9% solution of NaCl was used according to a study conducted by Kobayashi and Suda [21] to obtain good contact with the K-file. The electrode-electrolyte interface impedance when the electrolyte is a biological tissue is similar to 0.9% NaCl, and this solution has become a benchmark since its ionic content is equivalent to that of blood plasma [25]. The disadvantage of this model is that it is not able to completely simulate the in vivo conditions [16].

Comparison between third-generation apex locator and actual length showed statistic p-value as 0.9214, and that between fourth-generation apex locator and actual length showed statistic p-value as 0.7882. Thus, statistically, both third generation and fourth generation apex locators are equally accurate for determination of working length.

The results of the study are in agreement with the study done by Plotino G *et al*., [8], where the accuracy of three electronic apex locators was tested and was found that the accuracy of the Elements diagnostic unit apex locator was not significantly different from the accuracy of Root ZX. The accuracy in determining the working length for both the apex locators are comparable with the previous studies by Baruah Q *et al.,* [16] and Pagavino G *et al.,* [18].

In conclusion, inaccurate measurement of working length leads to inappropriate biomechanical preparation and obturation of the root canal, which in turn fails treatment. Electronic root canal length measuring devices were developed to improve the accuracy of the root canal length measurement, reduce the number of radiographs during the treatment, and to save time.

In the present study, we compared the accuracy of working length determination of two generations of apex locators and found that there is no statistically significantly difference in working length measurement between the two apex locators,

Root ZX and Elements diagnostic unit apex locator. Statistically both third generation and fourth generation apex locators are equally accurate for determination of working length when compared to actual working length.

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Public Health

Moderating Role of Demographic Characteristics in Breast Cancer Awareness and the Behavioural Disposition of Women in Ogun State, Nigeria

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Abstract

BACKGROUND: Breast cancer incidence is fast increasing, posing a significant threat to the health of women of all races globally. In Nigeria, breast cancer causes the most cancer-related deaths among women each year as a result of inadequate awareness.

AIM: This study is aimed at examining the moderating role of demographic characteristics in facilitating breast cancer awareness among women, and how it relates to their behavioural disposition to the disease.

METHODS: The study adopted the descriptive (survey) and cross-sectional research designs to elicit information from women of adult age selected across five Local Government Areas in Ogun state. The data, collected through questionnaire were analysed through the use of a variance-based SEM Partial Least Square (PLS).

RESULTS: The result shows that demographic characteristics (age and education) has a significant positive effect and jointly explain 74.9% of the variance in the breast cancer awareness and behavioural disposition among women in the study area. The findings revealed that a significant number of women with breast cancer had not acquired useful knowledge that could potentially be used to diagnose, prevent, and manage the disease. Unfortunately, the practice of Breast Self-Examination is grossly low among Nigerian women, as a consequence, only 20-30% of the women in study areas, including professionals, are aware of the benefits of BSE and only a smaller percentage practice BSE.

CONCLUSION: There is, therefore, a need to educate women on the benefits of this simple life-saving procedure through the consistent use of media platforms.

Introduction

Breast cancer is fast becoming a global epidemic, constituting a significant threat to the health of women of all races. Statistics revealed that it accounts for about 23% of all cancer incidences [7] [22], with Over 1 million breast cancer cases reported each year. It has also been reported that over 411,000 deaths occur annually representing 14% of female cancer-related deaths worldwide [6].

Going by all of these statistics, it is evident that the prevalence of breast cancer is increasing worldwide, and a steady upsurge in its incidence has been observed in both developed and developing countries [5], [7], [22], [30]. In most developed countries, medical care is personalised with patients being offered scientific and evidence-based treatment. Unfortunately, in developing countries like Nigeria, a significant number of women may not benefit from evidence-based best practice due to the multi-faceted and multi-sectoral problems ravaging the country's health care delivery system. With the current economic downturn, the health care sector is worse off with regard to public healthcare expenditure [4].

There are significant challenges associated with accessing quality and affordable health care in Nigeria. For instance, health care services are largely inaccessible and unaffordable especially for the urban

poor and women residing in rural areas. The whole gamut of health care delivery system in the country is almost in a state of comatose as a result of inadequate funding, poor maintenance culture and a lack of genuine commitment by the government [2], [25]. Health care in Nigeria is characterised by poor and inadequate basic infrastructure, obsolete and dilapidating medical equipment, poor and inadequate distribution of health workers, poor quality of healthcare services, lack of essential drug supply, and, corruption. [4], [13], [14]. Radiotherapy and chemotherapy treatments for breast cancer in Nigeria are expensive and unaffordable [28], complicated by poor awareness about the disease among women. This may explain why many women are left with the option of alternative and unorthodox treatment, and it may also be one of the reasons for late presentation to hospitals at a more challenging stage with attendant poor prognosis leaving the patient with little or no hope for survival [32], [28].

Most breast cancer patients often present with the advanced stage of the disease with high-grade infection load on the breast and metastasis [10], [33], [20]. Available treatment facilities are few and unevenly distributed, with the presence of old and broken radiotherapy machines and equipment [28], [9]. Accessibility and cost are major limiting factors why many African women cannot access simple lifesaving treatments [26].

Extant literature has identified a direct link between breast cancer awareness and behavioural disposition. For instance, studies by Justo, Wilking, Jönsson, Luciani and Cazap (2013), and that of Ganiyu and Ganiyu (2012) have shown that the most pressing element influencing the relationship between knowledge creation and performance could be as a result of socio-demographic factors which may include gender, age, education, locality and a number of other factors. However, the relationship between and among these variables have not been fully explored and established. This study, therefore, shall attempt to focus on the moderating role of some identified demographic variables in the facilitation of breast cancer awareness, and the behavioural disposition of women to the disease in Ogun state.

Problem Statement

While most women are aware of breast cancer, many lack adequate knowledge about the steps to take to detect the disease in its early stage [3]. In the late 80s and early 90s, studies on the level of knowledge and awareness of breast cancer revealed that women generally are oblivious of the disease, especially its risk factors and treatment options. Recent studies, however, have shown an increase in the knowledge level of breast cancer, especially among women in more developed societies. But the same cannot be said of women in developing countries, especially those in the Sub-saharan region of Africa where awareness is still relatively low with limited access to timely and accurate information

In Nigeria, breast cancer has been reported to have accounted for about 56.6% of all cancer diagnosis [35], with about 70% of Nigerian women presenting late with the advanced stage of the disease. In low-and-middle-income countries like Nigeria, one of the identifiable causes for late presentation is ignorance, because “women are still afraid to know of their diagnosis”, coupled with poor health education [23]. According to Jedy-Agba *et al.,* 2017 “, the stage at diagnosis is a reflection of the degree of awareness of the disease in the population”. A recent projection perceives global cancer burden doubling between this current time and the year 2030 with the majority of this burden falling on low income and lower-middle-income countries (LMCs) because of limited resources to deal with the disease and partly because of low levels of awareness [15].

Literature Review

In the past, there was a great deal of shame surrounding breast cancer. This was because it affects what was considered a “private part” of a woman’s body which is so closely tied to her sexuality, maternity and feminine identity. Women were ashamed to disclose their diagnosis publicly and may even outrightly refuse to be examined or diagnosed at all [6]. In the present time, women are gradually becoming more open to the discussion of breast cancer and are relatively aware of the disease. Breast awareness among women is central to the correct and accurate recognition of breast cancer symptoms which is believed will help in expediting action for treatment, thereby increasing the hope of survival [19].

Death rates from breast cancer have reduced significantly in the United States largely because of better screening and early detection, increased awareness and improvement of treatment options [7]. This has translated to an increment in the rate of survivors, given that well over 3.3 million breast cancer survivors are alive in the US today [6]. The situation is, however, unfortunately, different in Nigeria, where women still have limited knowledge about the disease and eventually die as a result of ignorance.

Many women, especially women in rural areas in Nigeria, think breast cancer was a mere infection that could be easily cured with drugs [36]. This revelation is an indicator of the fact that breast cancer knowledge level, especially among rural women, is still very questionable. Women living in rural areas constitute an underserved population and are characteristically riddled with a lot of health challenges. Reports from past studies have shown that health and media facilities are limited in most

rural communities in Nigeria, making rural women less likely to have adequate health information [38]. This situation may impede their access to adequate information on breast cancer and put them at risk of the disease.

It was reported in one study that, only 1.9% of rural women acknowledged a painless lump as an early warning sign of breast cancer [37]. It was also revealed in a recent study that, 80.4% of 316 women respondents claimed to have ever heard of breast cancer while only a few displayed a good knowledge of its causes (12.6%) and symptoms (13%) [21]. Knowledge and awareness of breast cancer were, however, appreciable among women living in urban centres. A sample of the opinion of women about their knowledge of breast cancer symptoms, risk factors, prevention methods and treatment options showed that urban women (55.61%) were more knowledgeable than rural women (47.81%) [38]. Knowledge is indeed central to the curtailment of breast cancer as knowledge empowers and liberates from ignorance. For women to present early to the hospital, they need to be breast aware, and they must be able to recognise the symptoms of breast cancer [18], [34], and for this to happen they need to be equipped with accurate and timely information. The right information about breast cancer will enable women to detect their symptoms early and seek medical attention on time.

Lack of early detection has been documented in the literature as the bane of most breast cancer deaths [11], especially in the sub-Saharan region of Africa. This, in turn, has been blamed on poor awareness. As noted by Badar *et al.,* (2007), knowledge about breast cancer is a fundamental element necessary for its early detection, prevention and treatment. According to Omaka-Amari, Ilo, Nwimo, Onwunaka and Umoke (2015), knowledge was conceived as the possession of an accurate understanding of breast cancer, its symptoms, risk factors, prevention and treatment options. To them, adequate knowledge will equip women with the ability to observe and correctly identify symptoms of breast cancer promptly before it begins to metastasize. It will also empower women to seek medical assistance promptly [8]. Knowledge of breast cancer risk factors will also help in its prevention by the adoption of appropriate measures and lifestyles.

Before a country like Nigeria can begin to experience an appreciable rise in the rate of breast cancer survival, women must possess the basic knowledge about breast cancer prevention. This involves the adoption of three screening approaches as suggested by Modeste, Caleb-Drayton and Montgomery (1999). These approaches are Breast Self-Examination (BSE), Clinical Breast Examination (CBE) and Mammography. BSE is a monthly procedure usually recommended for women starting from age 20. CBE is to be observed by women between the ages of 20-39 years every 3 years. It is however expected that women who are 40 years and above should have it done every year [11]. Mammography is a procedure recommended for women who are above 40 years and is usually advised to be done every one or two years.

The knowledge of different options available in the treatment of breast cancer is also central to the curtailment of the disease. Essentially, women should be equipped with accurate information about these treatment options to be able to make informed decisions about their well-being. The four basic treatment options for breast cancer are surgery, chemotherapy, radiation and hormone therapy [1]. The knowledge about available breast cancer treatment centres is another key factor that can further reduce breast cancer fatalities in Nigeria [38]. When women are equipped adequately with these information, they will be better informed about their options which will, in turn, influence their health-seeking behaviour

Methodology

The study adopted the descriptive (survey) and cross-sectional research designs. The descriptive (survey) method was adopted for this study because of its high propensity for representativeness and its ability for wider coverage of respondents. Quantitative data were thus elicited from women in their reproductive years and post-menopausal women aged 15 – 69 years.

The area under investigation with regards to this study is Ogun State. It is located in the South-western region in the Federal Republic of Nigeria. Ogun state is one of the 36 states in the country with a total land area of 16,980.55 sq.km [29]. It consists of 3 senatorial districts, 9 federal constituencies, 27 State constituencies and 20 Local Government Areas [31]. The population of interest with regard to this study are women selected across the 20 LGAs of Ogun State. Although previous studies have reported that breast cancer peaks between age 45 and 55 years [12], [41], this study covered women between age 15 – 69 years because findings from the literature suggest that these age groups are the most vulnerable to breast cancer and vulnerability increases with age.

The multi-stage sampling technique was adopted in the selection of respondents for this study. The sample population was drawn from over 880,970 regular households distributed unevenly across the 20 LGAs in Ogun State [29]. The first stage of the sampling process involved the stratification of the 20 LGAs according to their population size. The second stage involved a random selection of 5 out of the 12 densely populated LGAs. The lottery method was used in making this selection. The 5 selected LGAs are Ado-Odo Ota; Abeokuta South; Sagamu; Obafemi Owode and Ijebu Ode. The third stage involved the

selection of one ward from each of the selected LGAs using the lottery method. Out of the ward selected from each of the LGAs, streets were selected using the purposive sampling method, while, the systematic sampling method was adopted in the selection of residential houses in selected streets. One household was selected in every 5th house to ensure everyone was given an equal non-zero chance of being selected. The final stage of the sampling process involved the selection of respondents in selected households. All women aged 15-69 years were given equal chances of being selected in every household sampled. A sample size of 280 female respondents was randomly selected from the total population of 901, comprising 31% of the total population. The questionnaire titled *"Awareness and Behavioural Disposition to Breast Cancer among Women"* (ABCWQ) was used for data collection.

The questionnaire was administered to all females that fall between the ages of 15-69 years, and the completed questionnaire was collected on the spot. The services of field assistants were sought in this regard, and they have been duly trained appropriately before the commencement of the data collection. To ensure data quality, the collected questionnaire was checked and edited for coherence and consistency before sending them for analysis.

Ethical Consideration

Approval was sought from the Ethical Committee of the University of the researchers, and verbal informed consent of participants was duly obtained. Participants were debriefed about the aim and objectives of the study before the data collection process commenced. Participants were also assured of privacy, anonymity and confidentiality of the information supplied.

Reliability and Validity of Research Instrument

The research instrument was presented to management experts in the field for validity check while the test and re-test method was used to establish construct reliability [16] and validity as presented in Table 1.

Table 1: Construct Reliability and Validity

	Cronbach_ Alpha	rho_A	Composite Reliability	Average Variance Reliability
Threshold	> 0.7	> 0.7	> 0.7	> 0.5
Awareness level of Breast Cancer	0.962	0.970	0.982	0.971
Behavioural Disposition	0.851	0.888	0.942	0.893
Demographic characteristics	0.711	0.728	0.741	0.597
Through breast self-examination	0.744	0.801	0.954	0.941
Through health service provider	0.759	0.765	0.849	0.760
Through media	0.799	0.805	0.961	0.878
Through Relatives	0.715	0.722	0.770	0.629

Five research questions and hypotheses were tested using inferential statistics such as measurement and structural models. The analyses were done through the use of a variance-based SEM Partial Least Square (PLS) as suggested by Fornell and Larcker (1981).

Results

The PLS-SEM path model was adopted to establish the awareness level of breast cancer (through breast self-examination (H1), the health service provider (H2), Media (H3) and Relatives (H4), and the impact on the behavioural disposition of women. The moderating role of demographic characteristics (H5) was also assessed to explain the relationship between the variables as presented in Figure 1.

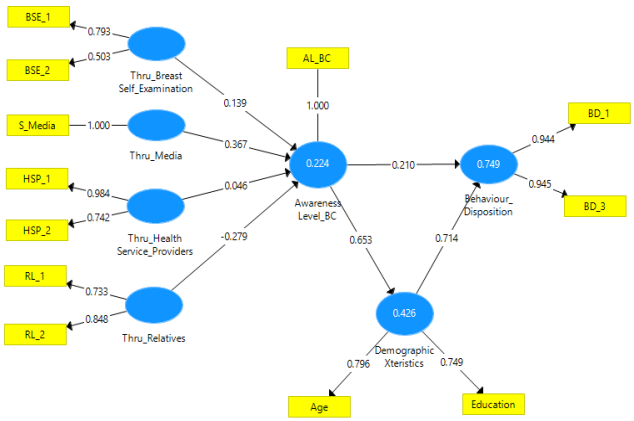

Figure 1: PLS-SEM Path Co-efficient Model

Figure 1 showed the moderating role of demographic characteristics (age and education) on the relationship between the awareness level of breast cancer and behavioural disposition of women using variance-based structural model. Based on the statistical result, it was found that there was a significant direct relationship between the awareness level of breast cancer and behavioural disposition among women, though the correlation coefficient (0.210) is weak. All the findings except "through relatives" were held significant at the 0.05 probability level. Findings also revealed that only 20-30% of the women in study areas, including professionals, know of the benefits of Breast Self-Examination (BSE) and only a smaller percentage practice it.

Essentially, demographic characteristics (age and education) had a very strong significant and positive effect on the awareness level of breast cancer among the women in the study area. In the same vein, demographic characteristics (age and education) had a stronger significant relationship in moderating the

awareness level of breast cancer and behavioural disposition to the disease among the women. Importantly, the total variance explained by the model as a whole was 74.9%. This also implies that the tested measures of demographic characteristics (age and education) jointly explain 74.9% of the variance in the level of awareness on breast cancer and behavioural disposition among the women in the study area.

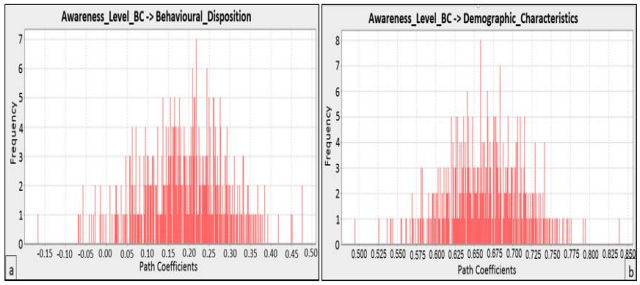

Figure 2: A) Path Coefficients Histogram for Awareness Level of Breast Cancer and Behavioural Disposition among Women; B) Path Coefficients Histogram for Awareness Level of Breast Cancer among Women and Demographic Characteristics

Under each null hypothesis, bootstrapping re-sampling, which indicates the statistical power of the proposed tests and their sensitivity concerning the size of the co-efficient has been performed to obtain the bootstrap approximation using the histogram path co-efficient. Hence, the histograms of the bootstrap approximations of the GoF distributions under the null hypotheses for Test 1, Test 2, Test 3, Test 4 and Test 5 were presented in the Figures 2 and 3.

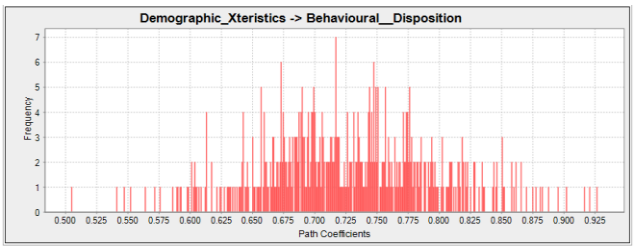

Figure 3: Path Coefficients Histogram for Demographic Characteristics and Behavioural Disposition among Women

The idea of standardisation was extended to a multivariate system, which possesses many properties, called partial regression coefficients. The term "path coefficient" indicates the use of a diagram-based approach (see Figure 1) to consider the possible causal link between a variable assumed to be a cause, and another variable assumed to be an effect as presented in Table 2.

Table 2: Path coefficients

	R -Square (R^2)	R -Square Adjusted	Standard Deviation (STDEV)	P Values	Decision
Awareness Level_BC	0.224	0.184	0.393	0.000	Significant
Behaviour_Disposition	0.749	0.743	0.404	0.001	Significant
Demographic Xteristics	0.426	0.419	0.353	0.026	Significant
R-Square			0.675		
R-Square Adj.			0.665		
Chi Square			301.307 SRMR = 0.071 NFI = 0.922		

Discussion

The findings from this study have reverberated those of Mbuka-Ongona and Tumbo (2013) [17] and, Ganiyu and Ganiyu (2012) [26]. They all asserted that there is a high-level relationship between the strategies employed for creating awareness about breast cancer and the behavioural disposition of women to the disease in Nigeria. The persistent increase in the prevalence of breast cancer in Nigeria with seemingly non-existent solution has necessitated the need to evaluate women's level of awareness about their behavioural disposition thereby ascertaining the relevance of demographic characteristics in this regard. Several factors have contributed to the increase in the incidence of breast cancer especially in the sampled areas. According to the findings, a significant number of women in the study area have not acquired useful knowledge that could potentially be translated into diagnosing, preventing, and managing the disease.

It is therefore recommended that the government and the private sector should intensify efforts in heightening awareness creation through various media platforms to disseminate accurate information about breast self-examination. Provision should be made to make available better incentives for healthcare workers in Nigeria to optimise breast cancer service delivery. There is also a need to ensure the adequate training of Nigerian health workers on basic health education particularly concerning the early detection of breast cancer.

Compulsory, targeted and age-specific breast cancer screening should become an integral part of the healthcare services for women in Nigeria allowing for early detection and prompt intervention. The government and other major stakeholders must champion aggressive awareness campaigns on the advantages of early detection through the simple life saving procedure (BSE). Women should be made to realise the dangers of late presentation and should be encouraged to report any unusual changes in their breast to any health care facility closest to them for a professional assessment.

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Public Health

Radiogenic Components of Limestone Samples Collected from Ewekoro SW Nigeria: Implications for Public Radiological Health Risks Assessment and Monitoring

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Keywords: Medical geology; Public health assessment; Radiogenic composition; Natural radioactivity; Ewekoro limestone; Cancer risks

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Abstract

AIM: This research presents the radiogenic components in thirteen limestone samples from a quarry site in Ewekoro, southwestern Nigeria.

METHODS: The distributions of natural radionuclides (^{238}U, ^{232}Th and ^{40}K) in the limestone samples were determined by gamma spectroscopy using a well-type thallium-doped sodium iodide detector. Also, estimated associated radiological hazards are presented and compared with the standard threshold values.

RESULTS: The activity concentrations for ^{238}U, ^{232}Th and ^{40}K radionuclides range 18.09 ± 3.43-239.50 ± 25.74 Bqkg-1, 8.33 ± 0.83 - 360.01 ± 21.33 Bqkg-1 and 11.28 ± 0.81-735.26 ± 0.95 Bqkg-1 respectively. The radium equivalent activity concentration in the samples ranges 58.857-758.832 Bqkg-1 with samples S3, S4 and S11 values higher than the threshold limit of 370 Bqkg-1. Estimated dose rate and annual effective dose rate (AEDE) from the samples have ranges 28.754-330.917 nGyh-1 and 35.26-405.84 μSvy−1 respectively greater than the standard limit of 59 nGyh-1 and 70 μSvy−1 respectively for all samples except S9. The estimated external and internal indices are ranging 0.16 – 2.05 and 0.21 – 2.68 respectively, greater than permissible unity in some limestone samples such as S3, S4, S8, S11 and S13. Excess lifetime cancer risk was also computed using a life expectancy of 54.5 years. The results of higher radiological parameters in the limestone samples revealed that the miners have a high probability of contracting induced cancer.

CONCLUSION: A regular check-up is recommended for the miners and staffs within the quarry site. Also, the residents within the environs should be relocated far away from the quarry site, as the particulates from the limestone rock blasting could contaminate the air in the study area.

Introduction

The exploration of the mineral is of economic importance to any nation as it contributes greatly to the nation's wealth. Despite the great and remarkable contributions of mineral prospecting towards the economic growth and advancement, environmental pollution impacts of these exploration activities are of serious concern all over the world. An aspect of geology that is concerned with the detailed understanding of several health implications of geological factors such as the occurrence of mineral deposit (including its exploration and exploitation) on humans, animals and plants is term medical geology [1], [2], [3]. The utmost significance of this area of geology is from the fact that rocks and minerals constitute fundamental building blocks of the planet, and as such, they contain several natural occurring chemical elements that are essential to plant, animals and human in considerable small doses. Hazardous contaminations of these elements in nature are also inimical to human health, since these elements are taken into human body through food, water and air [4], [5], [6], [7], [8]. Weathering of rocks form the soils on which crops and animals are raised. Drinking water percolates through soils and rocks as part of the hydrological cycle. The elements can also be inhaled through atmospheric dusts and gases.

Exposure to mineral dusts in quarry sites can affect the health of the miners and the inhabitants of the community where the minerals are being exploited or utilized [9]. Several human carcinogens have been reportedly associated with mine workers [10], [11], [12]. Huang et al. (2006) reviewed several pneumoconiosis cases among coal worker.

Pulmonary talcosis, silicosis and siderosis associated with inhalation of talc (from kaolinite), silicates and iron oxides respectively. Davies (2010) identified some potential harmful elements (PHEs) from mining operations in Africa, among which are arsenic associated with African Precambrian greenstone belts, mercury associated with the gold mining and Radiation (and radon gas) from radioactive gas formed naturally by the radioactive decay of uranium that occurs in all rocks and soils. Although, there is no place in the world that is free from radiation, natural environmental radioactivity largely depends on the geological rock types and mineral deposits in an area. Several investigations have been carried out on the natural radioactivity levels and their consequent radiological hazards associated with different crystalline rock types and soils [13], [14], [15], [16], [17], [18], [19], [20], [21], [22], tar sand and bitumen deposits [23], [24], [25], [26], [20] as well as phosphate mineral deposits [27], [28], [29], [30], [6].

Quarrying mineral deposits that contain naturally occurring radionuclides that are above the maximum permitted exposure limit can be very dangerous and can pose serious health risks for people residing within the locality where it is being mined for commercial purposes. Gene pool damage is one of the side effects of radiation exposure [31], [32], [33], [19]. Survived victims of an acute radiation exposure disease or any other radiation sickness are posed to high risk of developing cancer later in life. This study, therefore, focuses on the determination of the radiogenic composition of the limestone rock type of the Ewekoro Formation southwestern Nigeria and evaluation of the consequent radiological hazards associated with their commercial exploration and exploitation. The area of study is a limestone quarry site situated between the easting of 3°05' to 3°15' and northing 6°40' to 6°55' located in Ewekoro L.G.A, Ogun State, SW Nigeria (Figure 1).

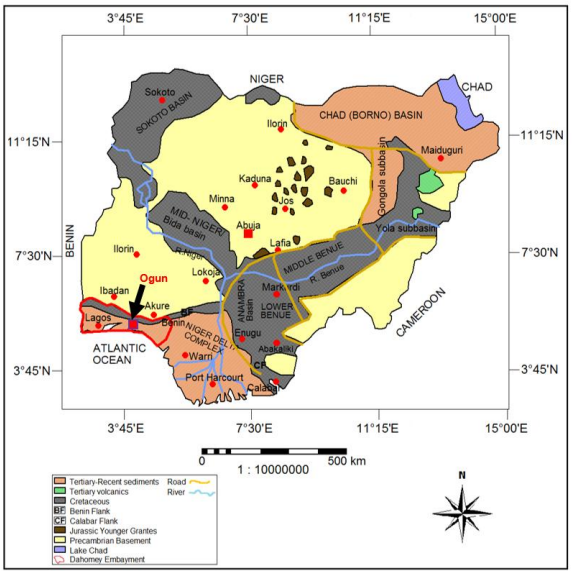

Figure 1: Geological Sketch Map of Nigeria Showing the Major Geological Components: Basement, Younger Granites, and Sedimentary Basins (Modified After Obaje, 2009) [34]

Ogun state is bounded to the North, South, West and East by Oyo state, Lagos state, Benin republic and Ondo state. Figure 2 shows that the study area lies geologically within the Eastern Dahomey Basin with east-westward trend sediments deposition and six lithostratigraphic units comprising Benin, Ilaro, Oshosun, Akinbo, Ewekoro and Abeokuta Formations from youngest to the oldest geological formation. Ewekoro Formation is known to be a Paleocene shallow marine deposit of non-crystalline and non-fossiliferous limestone strata.

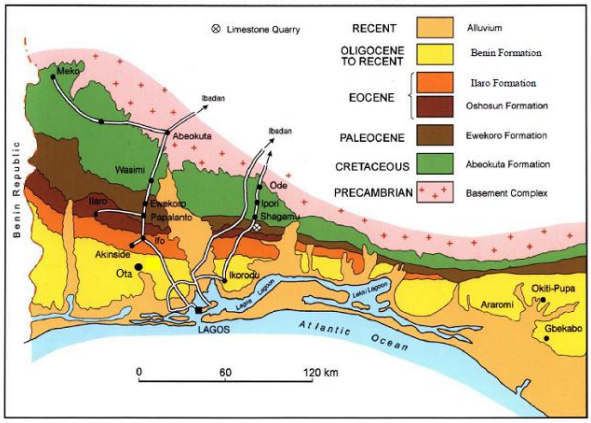

Figure 2: Geological Map of the Nigerian Part of the Dahomey Embayment Showing the Ewekoro Limestone (Modified After Gebhardt et al., 2010) [35]

Methods

Sample Collection, Preparation and Radioactivity measurements

A total of 13 samples were collected from the study area. The limestone samples were picked down to a depth of about 30 cm at each location point using hammer and hand trowel into a sealed polythene bags to prevent the samples from mixing up. In the laboratory, each sample was air-dried, pulverised, homogenised and then sieved using a 2 mm mesh. A 0.2 kg weight of the sieved sample was poured into a standard container (plastic), tightened and sealed to prevent ^{220}Rn and ^{222}Rn gasses from escaping. The sealed samples were left for thirty days to allow for secular equilibrium between parent and daughter nuclei.

The radionuclides activity concentrations were measured using NaI (TI) detector-based gamma spectrometric system where the digiBASE system that combines a miniaturised preamplifier and detector with a powerful digital multichannel analyser and special features for fine time-resolution measurements. The digiBASE incorporates into the NaI (TI) detector provides a gain stabiliser to significantly reduce the sensitivity of the detector to changes in ambient temperature and magnetic fields.

Three gamma-ray lines of interest were 1460 keV, 1764 keV and 2615 k eV which were resolved without much interference. The cylindrical plastic containers of radiation source were of diameters 7 cm. The seven soil samples each of mass 0.2 kg were dried, grinded and kept for more than thirty days in standard plastic containers to reach secular equilibrium were kept above the detector for the counting process.

About 10800 seconds (3 hours) was set as the counting time, which is considered enough for the detector to be able to show clearly and be able to distinguish the desired peak from a spectrum of signals. Multichannel analyser algorithm was used to compute the areas under each peak which represent the count number for a radionuclide in a particular sample. Uranium reference material termed RGU-1 from the International Atomic Energy Agency (IAEA) was used to calibrate the energy of the gamma spectrometer. The reference material was weighed into a standard cylindrical plastic sample container and placed on a NaI detector surface enclosed inside a lead shield of the spectrometer. This was counted for a lifetime of 10800 seconds.

A spectrum was captured, and specifically, three of the energy peaks identified on the spectrum were used in the energy calibration. Corresponding to the locations (channel numbers), the peaks of interest were: 295keV, 1120keV and 1765keV. To convert the count rate (cps) response of the spectrometer to desirable activity (Bq) for each of the three radionuclides (^{40}K, ^{226}Ra and ^{232}Th), the three reference materials RGK-1, RGU-1 and RGTh-1 from International Atomic Energy Agency (IAEA) were used. The *γ*-ray lines of ^{214}Bi at 1764 keV, ^{208} TI at 2014 keV and 1460.8 keV were used to determine the specific activity of ^{226}Ra, ^{232}Th and ^{40}K respectively.

Results

Radionuclides Activity Concentration

The measured activity concentrations of ^{238}U, ^{232}Th and ^{40}K natural radionuclides in limestone samples within the quarry site along with the geographic coordinates of the points are presented in Table 1. Activity concentrations for the radionuclides were estimated in Bq / Kg based on the dry weight. The gamma-ray spectra for samples S10 and S12 are presented in Figure 3.

The activity concentrations of ^{238}U range from 18.09 ± 3.43 Bqkg^{-1} to 239.50 ± 25.74 Bqkg^{-1} with a mean of 121.30 Bqkg^{-1}, ^{232}Th range from 8.33 ± 0.83 Bqkg^{-1} to 360.01 ± 21.33 Bqkg^{-1} with a mean of 112.25 Bqkg^{-1} while ^{40}K ranges from 11.28 ± 0.81 Bqkg^{-1} to 735.26 ± 0.95 Bqkg^{-1} with a mean of 158.47 Bqkg^{-1}.

These results were then compared with the worldwide average activity concentration of 35 Bqkg^{-1}, 30 Bqkg^{-1} and 400 Bqkg^{-1} for ^{238}U, ^{232}Th and ^{40}K, respectively [36], [14], [15].

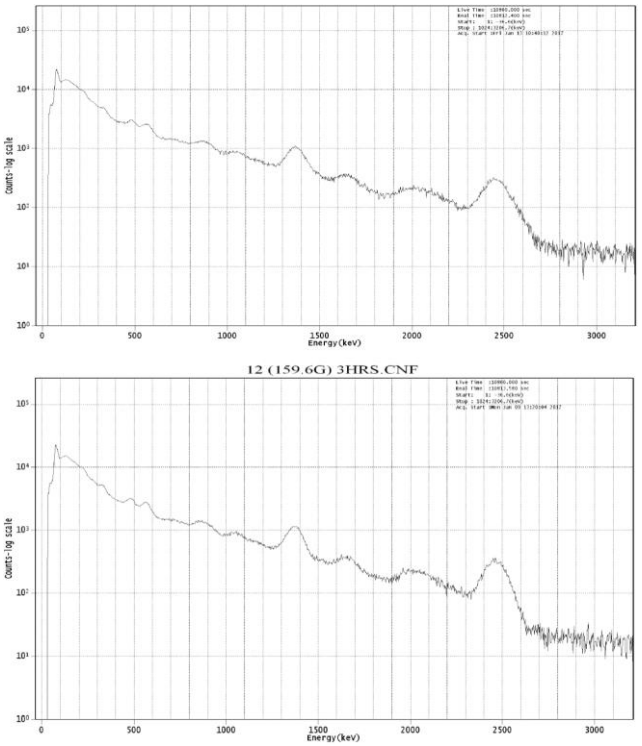

Figure 3: Representative Gamma-Ray Spectra for Samples S10 and S12

The measured activity concentration of ^{238}U was more than the worldwide average value in all of the limestone samples except for samples S2 and S9; ^{232}Th activity concentration levels in the samples were also higher than the worldwide average value except for sample S9. In contrast, ^{40}K activity concentration was below the worldwide average values for all samples considered except S12 (Table 1). Figure 4 shows the highest radionuclides ^{238}U, ^{232}Th and ^{40}K for S3, S4, and S12 respectively.

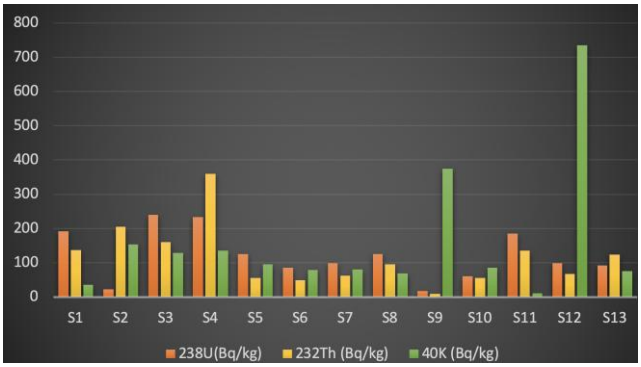

Figure 4: Measured Activity Concentrations at Each Sample Location

Discussion

The radiological maps (Figure 5A, 5B, and 5C) further reveal the higher concentration of ^{238}U localising around the northeastern (NE) and southeastern (SE) regions of the study area, while that of ^{232}Th localised around the SE part of the study area. A good positive correlation with relatively high coefficient is observed between ^{238}U and ^{232}Th radionuclides in Table 2 which imply that they are of the same source since their decay series occur together in nature. However, ^{40}K has weak negative correlation coefficients with both ^{238}U and ^{232}Th radionuclides confirming that K-40 originates from a different decay series. Table 2 also confirms that ^{40}K radionuclide has little contribution to the radioactivity of the limestone samples and consequently, the estimated radiological attributes due to its low activity concentration levels. There are strong positive correlation coefficients of ^{238}U and ^{232}Th with all estimated radiological attributes which imply that the high activity concentration of both ^{238}U and ^{232}Th are the major causative factors of the gamma radiation emission from the Ewekoro limestone. Several radiological parameters are needed to be evaluated to assess various health risks prone to by both the miners and the entire people residing around the quarry area. They include gamma-ray hazard indices, annual gonadal dose equivalence, dose rate and excess lifetime cancer risks.

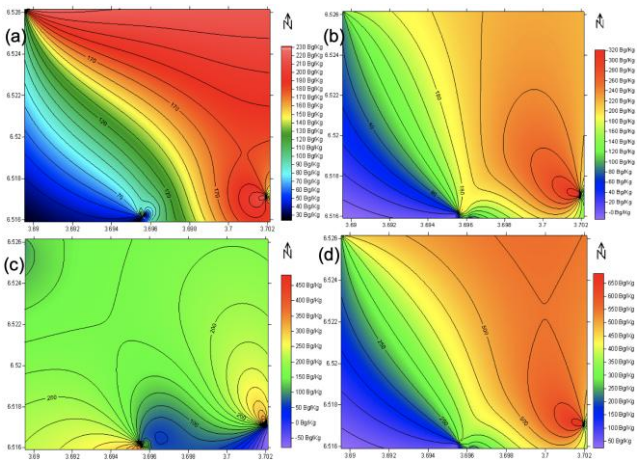

Figure 5: Radiological Map of the Study Area Showing; A) ^{238}U Activity Concentration (Bq/Kg); B) ^{232}Th Activity Concentration (Bq/Kg); C) ^{40}K Activity Concentration (Bq/Kg); and D) Absorbed Gamma Dose Rate (Ngyh^{-1})

X-Ray Radiation Hazard Indices

There is a great need to evaluate the threats of the gamma-ray radiation to both miners and residents of the communities where the limestone deposit is situated. Radium equivalent activity (Ra_{eq}) is used to compare the activity concentration of samples that contain different amounts of ^{238}U, ^{232}Th and ^{40}K natural radionuclides. This radiation index is calculated using equation (1). Where AC_U, AC_{Th} and AC_K are the limestone activity concentration in Bq / kg of ^{238}U, ^{232}Th and ^{40}K radionuclides respectively. The expression of the equation has a assumption that 370 Bqkg^{-1} of ^{238}U, 259 Bqkg^{-1} of ^{232}Th, and 4810 Bqkg^{-1} of ^{40}K will produce the same radiation dose rate (Farai and Ademola, 2005; Usikalu et al., 2015a; Usikalu et al., 2015b)[37], [16], [17].

Table 3 shows the range of radium equivalent activity values in the limestone samples, which varies from 58.857 Bqkg^{-1} in S9 to 758.832 Bqkg^{-1} in S4 with a mean value of 299.96 Bqkg^{-1}. Ra_{eq} values corresponding to samples (S3-4 and S11) are well above the maximum permissible limit of 370 Bqkg^{-1} [36], [14], [15]. These anomalously high radium equivalent values are concentrated around the southeastern region of the study area (Figure 5D). Major contributions to this hazard index are from the measured ^{238}U and ^{232}Th activity concentrations within the samples.

$$Raeq\ (Bqkg^{-1}) = AC_U + 1.43AC_{Th} + 0.077AC_K \quad (1)$$

Table 1: The Measured Radionuclides Activity Concentrations for all the Limestone Samples

SAMPLE NO	EASTING	NORTHING	^{238}U(Bq/kg)	^{232}Th (Bq/kg)	^{40}K (Bq/kg)
S1	3.69561	6.51619	191.92 ± 35.22	137.42 ± 13.67	35.94 ± 2.56
S2	3.69569	6.51621	21.99 ± 2.46	204.36 ± 12.26	153.73 ± 2.07
S3	3.68957	6.52616	239.50 ± 25.74	160.13 ± 9.55	128.39 ± 6.64
S4	3.70195	6.51708	233.59 ± 24.10	360.01 ± 21.33	135.43 ± 7.04
S5	3.70201	6.51703	125.43 ± 11.04	55.43 ± 5.04	95.43 ± 4.04
S6	3.70206	6.51712	85.43 ± 7.04	48.43 ± 3.04	79.36 ± 4.12
S7	3.69612	6.51591	98.43 ± 4.04	61.47 ± 6.04	80.28 ± 4.18
S8	3.69554	6.5162	126.10 ± 13.50	95.37 ± 1.04	69.43 ± 3.04
S9	3.69552	6.51613	18.09 ± 3.43	8.33 ± 0.83	374.75 ± 26.20
S10	3.68955	6.52615	61.04 ± 11.31	55.92 ± 5.57	85.43 ± 7.04
S11	3.70213	6.51707	184.38 ± 34.23	135.43 ± 4.04	11.28 ± 0.81
S12	3.70203	6.51712	98.59 ± 18.28	66.573 ± 3.07	735.26 ± 0.95
S13	3.69606	6.51594	92.43 ± 2.04	124.37 ± 2.07	75.43 ± 7.04
Minimum			18.09 ± 3.43	8.33 ± 0.83	11.28 ± 0.81
Maximum			239.50 ± 25.74	360.01 ± 21.33	735.26 ± 0.95
Mean			121.30 ± 14.80	112.25 ± 6.73	158.47 ± 5.86
*UNSCEAR (2000)			35	30	400

The external and internal hazard indices (H_{ex} and H_{in}) are being used to assess both the external exposure of the limestone miners and the inhabitants within the locality to γ-radiation in the outdoor air and the internal exposure to radon respectively. These indices have to be below unity (1) to have insignificant radiation [36], [16], [17]. They are estimated using equations (2 and 3) respectively. Representative level index I_r is another radiation index that is used to compute γ-radiation level about the measured activity concentrations of ^{238}U, ^{232}Th and ^{40}K radionuclides [38]. It is computed by using equation (4) [39] and its values when less than or equal to unity is the same as the annual effective dose less than or equal to 1 mSv.

$$H_{ex} = \frac{AC_U}{370(Bq/kg)} + \frac{AC_{Th}}{259(Bq/kg)} + \frac{AC_K}{4810(Bq/kg)} \quad (2)$$

$$H_{in} = \frac{AC_U}{185(Bq/kg)} + \frac{AC_{Th}}{259(Bq/kg)} + \frac{AC_K}{4810(Bq/kg)} \quad (3)$$

$$I_r = \frac{AC_U}{150} + \frac{AC_{Th}}{100} + \frac{AC_K}{1500} \quad (4)$$

The estimated H_{ex} values range from 0.16 to 2.05 (Table 3) with an average value of 0.81, while the computed $H_{in\ values}$ range from 0.21 to 2.68 with a mean of 1.14. Samples (S3-4 and 11) had H_{ex} values greater than unity while Samples (S3-4, 8, 11 and 13) had H_{in} values greater than unity. The calculated values for $\mathbf{I_r}$ in the limestone samples range from 0.45 to 5.25 with an average of 2.08. These values are high (except S9) and exceed the upper threshold limit for I_r which is unity (UNSCEAR, 2000) [36]. Results of the γ-Ray radiation hazard indices revealed that exploration of Ewekoro limestone is radiologically hazardous for both the miners and the inhabitants of the area.

Table 2: Correlation Coefficient (r^2) of the Naturally Occurring Radionuclide Concentrations and all the Estimated Radiological Parameters from the Limestone Samples

*Variables	^{40}K	^{238}U	^{232}Th	Ra_{eq}	D	AEDE	H_{ex}	H_{in}	I_r	ELCR	AGED
^{40}K	1										
^{238}U	-0.286	1									
^{232}Th	-0.209	0.5	1								
Ra_{eq}	-0.189	0.802	0.905	1							
D	-0.188	0.797	0.899	0.994	1						
AEDE	-0.188	0.797	0.899	0.994	1	1					
H_{ex}	-0.189	0.802	0.905	1	0.994	0.994	1				
H_{in}	-0.226	0.897	0.822	0.984	0.978	0.978	0.984	1			
RLI	-0.167	0.792	0.909	1	0.994	0.994	1	0.98	1		
ELCR	-0.188	0.797	0.899	0.994	1	1	0.994	0.978	0.994	1	
AGED	-0.106	0.715	0.875	0.953	0.951	0.951	0.953	0.923	0.955	0.951	1

Annual Gonadal Dose Equivalent (AGDE)

The gonads are part of the vital organs in the body that are of interest because they are highly sensitive to radiation. An increase in AGDE has been known to affect the bone marrow and red blood cells. It is calculated using equation (5).

$$AGDE\ (\mu Svy^{-1}) = 3.09AC_U + 4.18AC_{Th} + 0.314AC_K \quad (5)$$

The estimated AGDE values are presented in Table 3. The annual gonad equivalent dose ranged from 208.39 μSvy^{-1} to 2269.16 μSvy^{-1} with a mean of 870.31 μSvy^{-1}. The average value obtained is almost thrice that of the average world value for exposure limit of 300 μSvy^{-1} [36]. Therefore, the radiations emitted from the limestone endanger bone marrow and the bone surface of the miners and residents of the area.

Dose Rate

The absorbed gamma dose rate (D) refers to the amount of radiation energy absorbed or deposited per unit mass of the substance. This radiological parameter is used to characterise the external primordial gamma radiation in the air at about 1 m above the surface of the ground, and it was calculated using equation (6) as proposed by (UNSCEAR, 2000) [36]. The annual effective dose equivalent (AEDE) in μSvy^{-1} resulting from the calculated absorbed dose values (D) was determined using equation (7), where O_C represents the occupancy factor taken as 0.2 and conversion coefficient (F_C) of 0.7 is used to convert absorbed gamma dose rate (D) to AEDE.

$$D\ (nGyh^{-1}) = 0.462AC_U + 0.604AC_{Th} + 0.041AC_K \quad (6)$$

$$AEDE\ (\mu Svy^{-1}) = D\ (nGyh^{-1}) \times O_C \times F_C \times 8760 \times 10^{-3} \quad (7)$$

The absorbed gamma dose rate value ranged from 28.754 $nGyh^{-1}$ in Sample S9 to 330.917 $nGyh^{-1}$ in Sample S4 with a mean of 135.289 $nGyh^{-1}$ (Table 3). All the limestone samples except Sample S9 were above the world average (populated-weighted) adsorbed gamma dose rate of 59 $nGyh^{-1}$ according to UNSCEAR (2000) [36] and Taskin et al., (2009) [40]. The AEDE values in the limestone samples ranged from 35.26 μSvy^{-1} in Sample 9 to 405.84 μSvy^{-1} in Sample S4, with an average of 165.92 μSvy^{-1}. All samples except Sample S9 had relatively high AEDE values greater than the average world value of 70 μSvy^{-1} (UNSCEAR, 1988) [41]. Therefore, based on radiation dose evaluation, the limestone in the study area is unsafe.

Excess Lifetime Cancer Risk (ELCR)

ELCR is the tendency to develop cancer over a lifetime at a given *γ*-radiation exposure limit. It is estimated using the equation (8), where DL is the life expectancy in Nigeria taken to be 54.5 years according to world health organisation report (2015) and the risk factor (RF in Sv^{-1}) of the general public estimated to be 0.05.

Table 3: The Estimated Radiological Parameters from the Limestone Samples Including Annual Gonadal Dose Equivalent AGDE, Absorbed Dose D, Annual Effective Dose AEDE, Excess Lifetime Cancer Risk ELCR, Ra_{eq} Radium Equivalent, I_r Representative Level Index, H_{ex} External Hazard Index, and H_{in} Internal Hazard Index

SAMPLE NO	Ra_{eq} (Bq/kg)	D ($nGyh^{-1}$)	AEDE (μSvy^{-1})	H_{ex}	H_{in}	I_r	ELCR	AGED (μSvy^{-1})
S1	391.2	173.14	212.34	1.057	1.58	2.68	0.58	605.63
S2	326.06	139.9	171.57	0.88	0.94	2.29	0.47	970.45
S3	478.37	212.63	260.77	1.29	1.94	3.28	0.71	1449.71
S4	758.83	330.92	405.84	2.05	2.68	5.25	1.11	2269.16
S5	212.04	95.34	116.93	0.57	0.91	1.45	0.32	649.24
S6	160.8	71.97	88.27	0.43	0.67	1.11	0.24	491.34
S7	192.51	85.89	105.34	0.52	0.79	1.32	0.29	586.3
S8	267.83	118.71	145.58	0.72	1.06	1.84	0.4	810.1
S9	58.86	28.75	35.26	0.16	0.21	0.45	0.1	208.39
S10	147.58	65.48	80.3	0.4	0.56	1.02	0.22	449.18
S11	378.91	167.45	205.36	1.02	1.52	2.59	0.56	1139.37
S12	250.4	115.9	142.15	0.68	0.94	1.81	0.39	813.79
S13	276.09	152.67	187.24	0.75	1	1.91	0.51	871.31
Minimum	58.86	28.75	35.26	0.16	0.21	0.45	0.1	208.39
Maximum	758.83	330.92	405.84	2.05	2.68	5.25	1.11	2269.16
Mean	299.96	135.29	165.92	0.81	1.14	2.08	0.45	870.31
**Limits	370	59	70	1	1	1	0.29	300

The estimated excess lifetime cancer risk range 0.10 to 1.11, with a mean value of 0.45 (Table 3). Only four out of the thirteen samples considered were safe and below the world permissible value of

0.29 (Taskin et al., 2009) [41]. A high level of ELCR within the study area implies a higher probability of induced cancer that a miner or a resident within the study area would be exposed to.

$$ELCR = AEDE \times DL \times RF \quad (8)$$

In conclusion, the assessment of radiological parameters is important to evaluate the corresponding health hazards. The specific activity concentration of ^{238}U, ^{232}Th and ^{40}K in the limestone samples collected from Ewekoro, Ogun State had been determined using gamma-ray spectroscopy method. The activity concentration of ^{238}U and ^{232}Th in the limestone samples were higher than the safe limit except for samples S2 and S9 for ^{238}U and S9 for ^{232}Th. The computed radium equivalent activity values were higher than the global standard limit of 370 $Bqkg^{-1}$ in samples S3, S4, and S11. All the investigated limestone samples except S9 had gamma dose rate values higher than the global average, the estimated radiological hazard parameters than the permissible exposure limits. Hence, the health of the miners and inhabitants of the Communities where the limestone is being mined, processed and utilises is endangered due to the exposure to the radiation. To ensure good quality health, there is a need to protect people from the harmful effects of exposure to ionising radiation. To guarantee a high level of protection for miners, it is recommended that they should wear protective clothing to shield themselves from the radiation and reduce the time of exposure. Also, a routine health check-up should be conducted for the quarry workers and management. It is also recommended that the people should reside far away from the quarry.

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Incentives Motivating Mentors and Criteria for Selecting Mentors in the Pre-Graduation Traineeship of Future Registered Nurses

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Abstract

BACKGROUND: Modern education in the field of healthcare is faced with the challenge of coping with one of the most important tasks – to develop in students a set of competencies which serve as a sound basis for mastering a series of job-specific knowledge, skills and abilities, and which constitute a guarantee for coping successfully with all everyday tasks. The attention of the mentor is focused on finding the most reasonable solution to the various situations that the trainee may find him/herself in, and ensuring emotional comfort.

AIM: To identify the grounds and criteria for selecting mentors for the pre-graduation traineeships of the future registered nurses.

METHODS: To determine the opinion of the mentors, we surveyed 106 mentors in several university hospitals: 'St. Georgi' University Hospital – Plovdiv, MPHAT AD – Haskovo, 'Dr. At. Dafovski' University Hospital – Kardzhali, University Hospital – Stara Zagora.

RESULTS: During the pre-graduation traineeship in a real-life environment, students have the opportunity to develop and consolidate a series of skills, competences and qualities which are an integral part of the modern image of the registered nurse. Logically, the mentor should be the leading factor in the pre-graduation traineeship, yet the tutor has organisational and control functions.

CONCLUSION: Mentors are expected to ensure patient safety, as well to create suitable working conditions for students.

Introduction

The modern world is changing at an unprecedented rate. One of the areas with tangible dynamic transformations in medical education, which has to meet the growing current and future health needs of society. Most urgent among those needs are the processes of demographic ageing which have affected Bulgaria to an alarming extent. Life expectancy is increasing, and so is the proportion of older adults. In this regard, the need for well-trained medical specialists is unquestionable [1].

On the other hand, new technologies have a strong impact on medical education. These new technologies affect every aspect of our lives and cause a fundamental change in the way students communicate, establish and maintain relationships, and study [2], [3], [4]. Lecturers and tutors are expected to make the processes of learning more interesting, challenging and thought-provoking [5]. This applies to both theoretical and practical training of medical specialists.

Modern education in the field of health care is faced with the challenge of coping with one of the most important tasks – the task to develop in students a set of competencies which serve as a sound basis for mastering a series of job-specific knowledge, skills and abilities, and which constitute a guarantee for coping successfully with all everyday tasks. They are of vital importance as they include competencies needed not only for the moment but also for the future. Improving and keeping up the qualifications of registered nurses means striving after improving the quality of their professional work by developing and realising their potential as both personalities and

professionals [6].

In this context, the professional competence of registered nurses is a necessary prerequisite for achieving higher quality healthcare and improving the health status of the population.

During their pre-graduation training, students work independently in the respective hospital ward without the presence of a tutor. Therefore, the traineeship has to be very well organised and controlled by the mentor, who should strictly and closely monitor, support and assess the independent work of the trainees.

The attention of the mentor is focused on finding sensible and efficient solutions to various situations the trainee may find him/herself in, as well as on ensuring emotional comfort.

According to the Dictionary of the Bulgarian Language, a mentor is a person who "tutors and monitors someone, and gives them advice „лице, and synonyms of 'mentor' are 'teacher', 'educator', 'guide' (Dictionary of the Bulgarian Language, 2004). In a broad sense, the mentor is a qualified specialist with sufficient experience in his / her work. The idea of mentorship is widely popular in vocational training in Western Europe, Russia and the USA.

Mentorship is a valuable and popular vocational training method. It is one of the oldest forms of mastering professional skills and enables the trainee to have confidence in his / her skills, to develop the qualities and competencies needed for their future or current profession [7].

Mentorship is a mechanism of sharing experience between two parties, one of whom has a higher status based on knowledge and skills in a certain area. The knowledgeable, competent, capable, established specialists pass on the professional and personal knowledge and skills they have acquired to the newcomers to the profession.

Bulgarian medical education also has traditions in the field of mentorship. Mentors are registered nurses with a Bachelor's or Master's degree in Healthcare Management, working in university hospitals and clinics. They have the required pedagogical training acquired in the respective courses and degrees, and they have also mastered the specific knowledge and skills needed in the process of training [8]. These changes have brought forward mentors alongside tutors.

The international nursing standard defines 8 competences distinguishing the responsibilities of mentors: 1. Mentors are expected to facilitate the progress of the students towards achieving results; 2. Establish efficient working relationships; 3. Facilitate learning; 4. Assess and evaluate the student learning experience; 5. Create a suitable environment for learning; 6. Apply theory into practice, implement scientifically proven practices; 7. Assist in the identification and achievement of the learning objectives of the students; and 8. Participation in the formation of an overall evaluation of the students trained in the conditions of clinical practises guaranteeing the mastering of clinical competences [9].

In the training of Healthcare students, in addition to the necessary qualification and practical experience of the mentor, the latter's personal qualities and teaching skills also play a very important role.

S. Toncheva points out that "the mentor has to be included in the continuous training and improvement system of the healthcare establishment where the mentor works, to keep up his / her professional qualifications up-to-date and to develop his/her personal and professional competences" [10].

Objective: To identify and establish grounds and criteria for the selection of mentors during the pre-graduation traineeship of future registered nurses.

Material and Methods

To determine the opinion of the mentors, we surveyed 106 mentors in several university hospitals: 'St. Georgi' University Hospital – Plovdiv, MPHAT AD – Haskovo, 'Dr. At. Dafovski' University Hospital – Kardzhali, University Hospital – Stara Zagora.

Results

The average age of the surveyed mentors is 41.88 ± 1.04 y., and their average work experience is 18.74 ± 1.15 y.

According to their position: 1% are head nurses, 16% are nurse managers, and 83% are ordinary registered nurses (Figure 1).

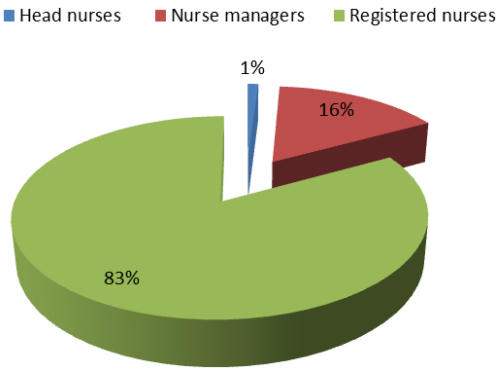

Figure 1: Distribution of the mentors according to the position held

The distribution of the mentors according to their level of education is as follows: the highest percentage is that of the holders of Specialist's degrees – 31.0%, followed by those with Bachelor's degrees – 29.0%, with Master's degrees in Healthcare Management-27.0%, and Bachelor's degrees in Healthcare management-13.0% (Figure 2).

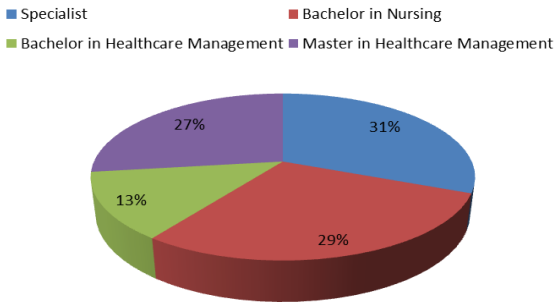

Figure 2: Distribution of the mentors according to their level of education

It is of note that only 40.0% of the mentors have Bachelor's or Master's degrees in Healthcare Management, and are therefore trained and qualified to conduct the practical training of students based on their pedagogical qualifications and aptitude.

Over half of the participants in the survey (55%) believe that pre-graduation traineeships have to be conducted under the supervision of a mentor, 16% think these traineeships have to be conducted under the supervision of a tutor, 15%-by both a tutor and a mentor, and 14% cannot decide.

During the pre-graduation traineeship in a real-life environment, students develop and consolidate a series of skills, competences and qualities which are an integral part of the modern image of the registered nurse. Logically, the mentor should be the leading factor in the pre-graduation traineeship, yet the tutor has organisational and control functions. What is important is that the traineeship has to be very well planned and organised, and this constitutes the managerial role of the tutor in the pre-graduation traineeship process.

Since the mentor is a central figure in this process, the professional and personal qualities, as well as the incentives of the mentor, are of vital importance. Therefore, we decided to identify the leading incentives for the respondents to become mentors. The analysis of the results shows that for 61.3% of the surveyed, the driving force is the opportunity to pass their personal experience to their future colleagues. Three-point eight percent consider it prestigious to be mentors. Twenty-one-point seven percent believe their work as mentors is a way of personal and professional improvement. Sixteen percent of the find their work as mentors personally rewarding and consider it an opportunity to prove their competences, 19.00 % state other incentives. The opportunity to work with students motivate Three-point eight percent of the surveyed, and the remuneration motivates only 12.4%. The total exceeds 100% because the respondents were allowed multiple answers (Figure 3).

Figure 3: Motivation of the mentors for working as such

It is immediately apparent from Figure 3 that the remuneration is not a driving factor for the majority of the mentors. According to the criterion of the position held, the findings show that head nurses and nurse managers are mainly motivated by the opportunity to pass their experience on to the students $P < 0.05$ ($\chi 2 = 5.67$). The role of a mentor invariably adds to the workload of the registered nurse, so in selecting mentors, their willingness and consent also have to be taken into consideration. This logically raises the question of the remuneration even though in some European country's mentorship is voluntary. Mentorship is considered professional recognition, honour and prestige. In those countries, nurses apply for mentorship, and their selection is based on certain criteria. The successful applicants attend regulated training before commencing their work as mentors.

Accordingly, the mentors were allowed to outline specific professional requirements that registered nurses conducting students' pre-graduation traineeships have to meet. The highest percentage of the mentors (82.10%) believe that a Bachelor's or Master's degree in Healthcare Management should be a prerequisite; 57.50% consider a certain number of years of work experience an important condition; according to 50.90% of the respondents professional competences are of vital importance; 42.55% share the opinion that the position held should be a requirement; however, only 13.44% state that nurse managers are suitable mentors.

Discussion

The analysis shows that the suggestions for a certain number of years of work experience vary widely. According to 53.00% of the respondents, mentors should have at least 5 years' experience, 39.00% believe this period should be 6-10 years,

while 8.00% of the surveyed mentors the required work experience should be over 11 years.

In the responses to this question, the following dependencies were established: in terms of “age”, respondents aged 51+ believe mentors should have over 10 years of work experience, while younger respondents aged < / = 30 believe 5 years of work experience to be sufficient $P < 0.05$ ($F = 3.32$). In terms of “position held”, most head nurses and nurse managers believe the level of education should be an important criterion in selecting mentors $P < 0.05$ ($\chi 2 = 4.37$). These results show that in developing criteria for selecting mentors, work experience and level of education should be taken into account.

The conducted survey among mentors provides grounds for the following conclusions:

1. The main factors motivating mentors to work with trainees include the opportunity to pass their personal experience on to their young future colleagues, personal satisfaction, and the opportunity for self-improvement.

2. The mentors agree that they should fulfil certain requirements.

3. Since the mentor is a key figure in pre-graduation traineeships, the following aspects have to be taken into account in developing the selection criteria for mentors: - Work experience; - Level of education of the applicant for a mentor; - A list of mentors should be compiled.

In conclusion, the performed analysis explicitly shows that in the students’ practical training in Healthcare, in addition to the required educational qualifications and work experience of the mentor, his / her personal qualities and teaching skills also play a very important role. Mentors are expected not only to ensure the safety of the patient but also to create suitable working conditions for their trainees.

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Model Control of Cupping Treatment Therapy for Patient Satisfaction at the Community Health Center in Langsa City, Indonesia

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Abstract

BACKGROUND: Traditional medicine cupping therapy is so popular for the community in Langsa city; therefore, it needs to standardise and government control.

AIM: To find out the supervision model of cupping therapy for patient satisfaction at the community health centre in Langsa City.

METHODS: The design of this study is a quasi-experiment. The design used was a non-randomized pretest-posttest control group design. The sample was chosen as many as 45 people, using a purposive sampling technique for patients visiting with inclusion and exclusion criteria. Univariate and bivariate analysis was used to see the effect of traditional cupping therapy supervision models on patient satisfaction. Then test paired t-test and One-Way ANOVA.

RESULTS: The paired t-test results showed a significant change in satisfaction with a value of $p < 0.05$. Group (SOP & supervision = 7.07) and (SOP = 3.13) each change with p-value = 0.0001. The group (control = 04) experienced a change in satisfaction with p-value = 0.02. ANOVA test results show there are differences between groups with a p-value of 0.0001. The difference in value includes SOP and supervision of the control of 7.46 with p = 0.0001. The SOP group for the control group was 3.53 with p 0.004. The SOP & supervision group towards the SOP group was 3.93 with p 0.001

CONCLUSION: The SOP group supervision and supervision model is effective in increasing satisfaction scores cupping therapy patients in the community health centre of the city of Langsa, Aceh Province, Indonesia.

Introduction

According to the World Health Organization (WHO), as many as 80% of the total population in the Asian and African continents depend on traditional medicine [1]. Traditional medicine has developed in Indonesia as a form of public health efforts. Traditional medicine is one of the seventeen kinds of health efforts organised under Law No. 36 of 2009 concerning Health [2]. Traditional medicine that is widely used by Indonesian people is Cupping Therapy. Cupping therapy is Traditional therapies used by various countries with various names such as Al-hijamah (Arabic), Pa Hou Kuan (China) or cupping (Europe and America [3], [4]. Cupping is usually used by patients with chronic diseases that can be caused by degenerative conditions, poor diet or stress [5]. The community uses cupping therapy to cure headaches, rheumatic aches, joint pain, rheumatism, colds [6], [7]. Cupping therapy is cheap alternative medicine, and it is also free from the side effects of

chemical drugs [8], [9]. Social-demographic factors, level of education, culture, and economy are the reasons for the community to switch to traditional treatment of cupping therapy [10]. Traditional treatment of cupping therapy in Indonesia is generally under the supervision of the Directorate of traditional health services. Cupping therapy in Indonesia lacks in standardisation and cupping practitioners. Monitoring model interventions need to be carried out through "Standard Operational Procedure Operational" (SOP) to assess patient satisfaction and service quality to the community.

The study aimed to analyse the effect of the supervision model of cupping therapy in increasing patient satisfaction in the work area of the Langsa Community Health Center, Aceh, Indonesia.

Methods

This research was conducted in February until April 2019 in the working area of the Langsa Community Health Center. The design of this study was a quasi-experiment. The design used was a non-randomized pretest-posttest control group design. The population in this study were all patients who received cupping therapy who visited the clinic in the Langsa City area. The sample was chosen as many as 45 people, using a purposive sampling technique for patients visiting with inclusion and exclusion criteria. The inclusion criteria in this study were: respondents who carried out cupping therapy were willing to take part in the study and sign an informed consent, aged 30 to 70 years. The exclusion criteria for this study were respondents who lived outside the Langsa city area. Respondents were grouped into intervention groups and control groups. The intervention group was given standard operating procedures (SOP) and carried out supervision while the control group was given a standard intervention. The univariate analysis was performed to see the distribution of variables, as well as the normality test. The bivariate analysis was carried out to see the effect of cupping traditional therapy supervision models on patient satisfaction using paired t-test and One-Way ANOVA.

Results

Uni-Variate Analysis

The univariate analysis was carried out to see the distribution of variables and the normality test. The following are the characteristics of respondents based on age, gender, education, and occupation in each group.

Table 1: Characteristics of respondents

Variable	Group					
	SOP and Supervision		SOUP		Control	
	Mean ± SD		Mean ± SD		Mean ± SD	
	n	%	n	%	n	%
Age	45.5 ± 7.9		43.5 ± 8.2		48.1 ± 8.2	
Gender						
Man	6	40.0	7	46.7	6	40.0
Women	9	60.0	8	53.3	9	60.0
Education						
Elementary school	4	26.7	4	26.7	3	20.0
Junior high school	2	13.3	4	26.7	3	20.0
Vocational School	3	20.0	2	13.3	3	20.0
High school	2	13.3	1	6.6	3	20.0
DIII	1	6.7	0	0	0	20.0
S1	3	20.0	4	26.7	3	20.0
Work						
Civil servants	5	33.3	4	26.7	5	33.3
Employee	1	6.7	0	0	0	0
entrepreneur	3	20.0	3	20.0	3	20.0
Retired	2	13.3	2	13.3	2	13.3
IRT	4	26.7	6	40.0	5	33.3

Based on Table 1 above, the age range of respondents is between 40 and 50 years. Respondents were dominated by women. The three groups of respondents were dominated by undergraduate education, with the SOP and Supervision group (20%), the SOP group (26%), the Control group (20%). Based on employment, the SOP and Supervision groups were dominated by civil servants (33.3%). The number of SOPs is dominated by the work of housewives (40%). The control group is dominated by civil servants and housewives (33.3%). Furthermore, the normality test on the value of the pre-test of satisfaction in all respondents. Normality analysis using a skewness test.

Table 2: Test the normality of the value of the pre satisfaction test using the skewness test

Variable	n	Skewness	p	Information
Pre satisfaction test	45	0.02	0.06	Data is normally distributed

The results of the normality test show that the p-value obtained is 0.06. This value indicates that the value of the pre satisfaction test is normally distributed.

Bi-Variate Analysis

The bivariate analysis was conducted to determine the relationship between independent variables and dependent variables. Analysis of bivariate analysis was performed by using a paired t-test and One-Way ANOVA. Paired t-test analysis is used if the same subject is measured more than once with the same instrument.

Table 3: Paired t analysis of satisfaction tests in the SOP and supervision, SOP, and control groups

Group	Satisfaction		difference	*P*
	Pretest	Test post		
	Mean ± SD	Mean ± SD		
SOP and supervision	55.20 0.67	62.27 0.6	7.07	0,0001
SOP	57.13 0.53	60.27 0.5	3.13	0,0001
Control	58.13 0.36	57.73 0.3	-0.4	0.02

The results of the analysis showed that overall satisfaction in each group showed a significant

change with a value of p < 0.05. Changes in the satisfaction of the SOP & supervision group and the SOP group indicate changes are positive. In the SOP & supervision and SOP groups, there was an increase in the value of satisfaction from the pre-test to the test post at 7.07 and 3.13. Thus, the SOP and supervision groups were shown to increase the increase in satisfaction scores. In the control group, there was a significant decrease with a p-value of 0.02. Furthermore, testing the differences in changes between groups was done by ANOVA test.

Table 4: Anova analysis of changes in the value of satisfaction in the SOP & supervision group, SOP, and control

Group	Satisfaction Mean ± SD	F	P
SOP and supervision	7.06 ± 4.45		
SOUP	3.13 ± 1.85	26.61	0,0001
Control	-0.40 ± 0.63		

The results of the analysis in Table 3 above show there are differences between groups with a p-value of 0.0001. This value shows there are differences in the value of satisfaction between treatment groups. The difference in change between the SOP group and the supervision of the control was 7.46 with p = 0.0001. The difference in change between the SOP group and the control was 3.53 with p being 0.004, while the difference in change between the SOP for the supervision of SOP was 3.93 with p 0.001. Thus, the SOP & supervision group is the dominant group in increasing satisfaction scores compared to other groups.

Discussion

Cupping is an alternative body treatment, disease prevention and treatment of disease in the community of the city of Langsa. The community uses cupping therapy to cure headaches, rheumatic aches, joint pain, rheumatism, colds [11], [7]. Many people have tried cupping to deal with complaints of health conditions, ranging from pain, hypertension to AIDS [12]. Three physiological mechanisms are affected by cupping therapy, the nervous system, system of haematology and immune system [13]. Traditional medicine cupping in Langsa dominated by patients who are well educated. The higher the level of education a person, then they tend to be more concerned about health, especially related to prevention and obtaining health information [14], [15]. Traditional treatment of cupping therapy is dominated by adult patients aged 40-60 years. Increasing age affects their health, where organ structure and function decline, so that people who older adults tend to use health services a lot compared to young age [16]. Traditional medicine cupping requires standardisation and cupping practitioners. The application of standardisation to traditional cupping treatment influences patient satisfaction. The results of this study indicate a change in satisfaction between groups given intervention. The group applied Procedure Operational Standards (SOP) and supervision experienced changes in satisfaction values, while the control group did not experience changes in satisfaction values. Changes in patient satisfaction before and after intervention can be used as a measure of the success of traditional cupping therapy services. Application of Standards Operational Procedure (SOP) and supervision is more effective in increasing patient satisfaction. Application of traditional treatment of cupping therapy in urban area community health centre needs to be supervised. Supervision can be done by increasing the training of community health centre staff and implementing standardisation. The Ministry of Health through the National Health System in 2009 has included traditional, alternative and complementary medicine as part of the health effort subsystem [17]. Traditional health care has been included in the strategic plan of the Ministry of Health 2010-2014 in the form of increasing the research, development, and utilisation of traditional medicine.

In conclusion, the paired t-test results showed that overall satisfaction in each group experienced a significant change with a value of p < 0.05. Changes in SOP & supervision and SOP group satisfaction experienced positive changes. SOP & supervision and SOP groups increased the value of satisfaction from the pre-test to the test post at 7.07 and 3.13. SOP and supervision groups were shown to increase the value of patient satisfaction.

ANOVA test results show there are differences between groups with a p-value of 0,0001. The difference in values includes the SOP group and supervision of the group control of 7.46 with p = 0,0001. SOP group for control was 3.53 with p equal to 0.004. SOP group & supervision of the SOP group of 3.93 with p 0.001. The SOP group supervision and supervision model is effective in increasing the satisfaction value of cupping therapy patients in the Langsa Community City Health Center, Aceh Province, Indonesia.

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Modelling of Risk Factors Associated with Foodborne Disease among School-Aged Children in Medan, Indonesia

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Abstract

BACKGROUND: Foodborne disease (FBD) contributes several outbreaks worsening health quality of world population. Many risk factors associated with FBD are related to its processing, preparation, and storage as well as handling practice.

AIM: The study aimed to evaluate several proposed risk factors of foodborne disease existed among school-aged children and food-handlers in the school environment.

MATERIAL AND METHODS: The descriptive cross-sectional study enrolled 124 students consisting of 64 females and 60 males in two different public schools, 064024 and 066656, Medan, Indonesia, between April and August 2018. The bacterial and parasitological examination was carried out in Microbiology and Parasitology Department. Food-handlers were assessed their appropriateness using standardised questionnaire merit to the guidelines enacted by the Ministry of Health, Indonesia (Kepmenkes RI No.942 / Menkes / SK / VII / 2003 adapted from WHO guidelines) entitled food-handlers sanitation-hygiene requirement guidelines. Data analysis was conducted using logistic regression.

RESULTS: The study obtained that there were no food-handlers performed basic principles rules producing high-risk environment and posing a threat to children. Suspected-FBD also found in 55 or 44.4% students, and it was significantly related to several risk factors such as nail hygiene, knowledge level, nail-trimming behaviour, and hand-washing behaviour among students. Data analysis revealed modeling risk factor, Y = 23.440 + 2.003 (Nail hygiene) + 1.294 (Knowledge level) + 5.025 (Nail trimming behavior) + 7.007 (Hand-washing behavior) from logistic regression equation.

CONCLUSION: Poor hygiene and sanitation of food-handlers and children per se provide a supportive environment in producing FBDs.

Introduction

Foodborne disease or FBD frequently occur in the region which its local population neglects the hygiene and sanitation of their food preparation. It was found higher in the developing country, including Indonesia, where food-handling practice does not perform adequately [1]. World Health Organization defines FBD as infectious disease produced after the ingestion of food containing pathogenic microbial or its toxin [2]. Several microorganisms can cause FBD such as bacteria, and parasites while virus and bacterial toxin commonly produce FBD symptoms with negative test results, so the results of an individual suspected for FBDs are not uncommonly conclusive. Hence, advanced laboratory examination is inevitable to establish an accurate diagnosis, spend a much longer time [3]. There are several bacterial species including *Campylobacter sp*, *Listeria monoctyogenes*, *Salmonella enterica* or nontyphoidal, Shiga toxin-producing *Escherichia coli* O157: H7, *Yersinia enterocolitica*, or Norovirus with incidence 13.82, 0.26, 15.18, and 1.15 per 100,000 people respectively contribute to the high prevalence of FBDs worldwide [4], [5].

Several outbreaks have been underreported and neglected by the local authorities relating to its

difficult diagnosis [6]. However, it is estimated that 1 in 6 Americans has one episode of FBD with 128.000 hospitalisation and 3.000 deaths, most of its sufferer are children younger than five years old or school-aged children in the United States [7]. Meanwhile, there is little evidence of FBD surveillance in Indonesia, but the Indonesia food governmental agency noted the occurrence of FBD outbreak of 39.92% among food-related disease [8].

Although it is preventable, FBDs still becoming a neglected disease that directly impedes the world communities in achieving sustainable development goals or SDGs by 2030, second (zero hunger) and third point (good health and well-being). The point includes “end hunger and ensures access by all people, in particular, the poor and people in vulnerable situations, including infants, to safe, nutritious and sufficient food all year round” in the second point which directed to zero poor health outcome of the population caused by unsafe food handling practice. Therefore, the FBDs eradication and its prevention could benefit the country in the aspect of SDGs achievement [9]. Additionally, several short- and long-term health complications consisting of mild until life-threatening condition such as bacteremia, arthritis, kidney disease, or central nervous system infection can also reduce people’s quality of life [10].

Indonesia, through the Ministry of Health, has legalised several basic principal rules No.942 / Menkes / SK/VII / 2003 entitled guidelines for food-handlers sanitation-hygiene requirement similarly adapted from WHO guidelines [11]. Nevertheless, the continuance of the food-handlers to the guidelines remains questionable. Therefore, prompt strategy to improve several factors lead to the incidence of FBDs is compulsory. It could help the authority to strategise the prevention approach [12], [13]. The study aimed to provide the analysis of FBD risk factors in the school environment by assessing food-handlers, student behaviour, and their knowledge levels related to food hygiene and sanitation.

Material and Methods

The cross-sectional study enrolled 124 students consisting of 64 females and 60 males in two different public primary schools in Medan, Indonesia (identifier 064024 and 066656) between April and August 2018. Direct observation was performed in the school environment involving students and food handlers. The study also obtained written informed consent from all participant included in the study without any coercion. Sample collection was conducted by the total sampling technique of the fifth and sixth grade in the school marked their eligibility to answer several questions. A brief explanation was also carried out before the observation. Finally, of 147 students, only 124 give their guardian approval of the study.

Proposed risk factors

There were several proposed-risk factors of foodborne disease in school-aged children evaluated during the study. The risk factor evaluated during the observation consisting of nail hygiene, nutritional status, knowledge level, nail trimming, and hand-washing behaviour. Knowledge level of students was measured using a standardised questionnaire in the previous study. It contains 15 closed questions in Indonesia language, such as the adequacy of nutritious food, food as a source of energy, carbohydrate, protein, cholesterol, starch, calcium, and healthy diet or its consumption handled in school environment particularly by food-handlers [14]. The interpretation has created three categories of levels based on the correct answer given by respondents in percentage (76-100%, high; 56-75%, moderate; and < 56%, low). Anthropology measurement (weight and height) was noted in addition to the children behaviour towards hygiene and sanitation. Food-handler behaviour was assessed using food-handlers sanitation-hygiene requirements guidelines of Ministry of Health, Republic of Indonesia (Identifier: Kepmenkes RI No.942 / Menkes / SK / VII / 2003) [11]. The rules consist of requirements in hand-washing practice, hand and cloth hygiene, several protections used while food-handling practice (apron or head-cover), equipment hygiene, behaviour, water availability, and any related illnesses of the food-handlers (cough and sneezing) (Appendix 1).

Parasitological and bacterial examination

Direct-swab of hands and their equipment was carried out to determine any pathogenic bacterial species contaminates food-handlers followed by the inoculation onto two types of agar, blood and McConkey (Oxoid, Basingstoke, United Kingdom). Any positive culture underwent further identification using gram staining, and biochemical reaction analysis for Enteric pathogen. Faecal samples of the students were examined for its positivity of any parasitic (Kato-Katz method, lugol, trichrome, and modified-acid fast staining) and bacterial manifestations (faecal culture).

Foodborne illnesses

A descriptive questionnaire consisting of closed questions such as respondent, guardian/parent's identity, the frequency of food consumption handled by food-handlers as well as FBDs symptomatology was used to determine any presence of FBDs. The assumption was created after

the evaluation revealed that there were no food-handlers abided to the guidelines of hygiene-sanitation guidelines of the Ministry of Health, Republic of Indonesia. Therefore, exposure to the high-risk environment for FBDs was evident. Consequently, an individual with gastrointestinal tract symptoms (nausea, vomiting, abdominal pain, and diarrhoea) and pathogen manifestation (parasitic and bacterial positive examination) was diagnosed with foodborne illness or food poisoning, therefore, so-called 'suspected foodborne disease' also used as the dependent variable [14].

Statistical analysis and study approval

The study used the Statistical Package for Social Sciences 21 (SPSS Inc.version 21). Each variable which had p-value < 0.25 from bivariate analysis would include in the multivariate using logistic regression for proposed risk factors. The variation inflation factor (VIF) measurement for no more than 10 aimed to avoid multicollinearity or inter-variable relationships in modelling logistic regression analysis. The study was also approved and registered by the local ethical committee for medical research, Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia (identifier: 265 / TGL / KEPK FK USU-RSUPHAM / 2018)

Results

Baseline characteristics

Food-handlers behavioural evaluation was conducted using hygiene-sanitation guidelines of Ministry of Health, Republic of Indonesia. Based on the guidelines, there were no food-handlers performed their handling practice per the rules causing the exclusion of the variable from the analysis. A total sampling of food-handlers in the school environment proved that 22 of food-handlers had taken part in FBD transmission. Equipment and hands of the food-handlers have positive culture results for *Escherichia coli, Klebsiella pneumonia,* and *Staphylococcus aureus* (skin commensal). The results of culture examination support the evidence of a high-risk environment for school-aged children.

Baseline characteristics of students enrolled in the study were depicted in Table 1. Parasitological identification found that several species consisting of soil-transmitted helminth and intestinal protozoa infected school-aged children in the environment, *Blastocystis hominis* and *Giardia lamblia* is the most prevalent infection. Besides, there was one student who positive for *Hymenolepsis nana* infection. In total, there were 24 or 19.35% of students proved its parasitic manifestation in the study. There were 55 or 44.4% of the students stated as suspected-food borne disease related to positivity of foodborne disease symptomatology and pathogen existence.

Table 1: Baseline characteristics of the students included in the study

Characteristics	Total N (%) or Mean (SD)
Gender	
Male	60 (48.39)
Female	64 (51.61)
Weight (kg)	34.9 ± 9.30
Height (cm)	140.6 ± 8.65
Nutritional status (Weight/Height)	
Obese	21 (16.93)
Normal	70 (56.45)
Wasted	27 (21.77)
Severely wasted	6 (4.83)
Knowledge level	
High	100 (80.64)
Moderate	23 (18.54)
Low	1 (0.80)
Parasitological species	
Ascaris lumbricoides	1 (4.35)
Hookworm	1 (4.35)
Trichuris trichiura	2 (8.69)
Blastocystis hominis	13 (56.52)
Giardia lamblia	6 (26.10)
Total	23 (100)

Proposed risk factors associated with suspected foodborne disease

Based on logistic regression analysis, it was found the value of 0.954 as determination coefficient (R squared). It was referred that five independent variables could explain the variable variation of risk factors related to foodborne disease among students. Then, as a result of F test was shown in p-value = 0.001, it indicated that alpha as much as 5% had been fulfilled and defined as a suitable regression model with the provided data. The formula is based on table two which shown the final analysis of logistic regression in the study, Y = 23.440 + 2.003 (Nail hygiene) + 1.294 (Knowledge level) + 5.025 (Nail trimming behavior) + 7.007 (Hand-washing behavior). The prediction of having foodborne diseases could be performed by looking into the resulted formula.

Table 2: Multiple logistic regression analysis of various risk factors associated with foodborne disease

Variables	OR	p-value	Collinearity Statistic (VIF)	95% CI
Constant	23.440	0.395		
Nail hygiene	2.003	0.038	1.203	1.085-3.398
Knowledge level	1.294	0.001	5.027	0.621-2.877
Nail trimming behavior	5.025	0.027	1.278	3.291-6.290
Hand-washing behavior	7.007	0.048	1.592	3.896-9.025

Discussion

The study has resulted in a proposed risk factor equation for FBDs among school-aged children in the two different school environments. The analysis was based on the assumption of gastrointestinal tract symptoms and pathogen manifestations of bacterial and parasitic species. Therefore, the terms 'suspected-food borne disease' was used for the study. FBDs have been neglected because of its lack

of features and self-limiting properties unless it emerges as an outbreak. There have been a few guidelines precisely defining foodborne diseases, so the negative results of several tests do not automatically exclude the patients from FBD diagnosis. The surveillance system in developed countries has provided for excellent tools shaping a prevention method for FBDs among their population. In the United State, the official has recorded the outbreak surveillance since 1973 [15], [16], [17]. In Canada, there were approximately 2.4 million people diagnosed with FBD with unknown causative agents, partly because of the diverse range of the agent, mainly toxin or food-substance producing negativity [18].

The arrangement of an outbreak surveillance system, as well as risk factors analysis, provides an accurate approach to prevent and eradicate FBDs among the susceptible population [19]. Nevertheless, the intricate diagnosis of FBDs has challenged the researcher to proposed new rules in predicting the risk factors. The risk factor analytical study usually uses the contamination of food product as dependent variables. A study involved dining hall assessment in Shah Alam, Malaysia has determined the presence of FBD risk factor, it was found that safe food source and personal hygiene were in an acceptable level while storage temperature and cross-contamination still below in standard level [20]. While a study reviewed foodborne outbreaks during 2000-2010 found that contamination factors (food safety practices) including direct-hand contact by infected food-handlers still played a pivotal role, causing school season outbreaks [13]. Our study did not divide the proposed risk factors into three parts of food processing; hence, it emphasised on the transmission among the children who perform the appropriate method of self-hygiene and sanitation.

In Japan, Michino and Otsuki conducted a study among school-aged children, it was proved that 29 incidents of contaminated food items similarly occurred as the findings of nine infected food-handlers. The study analyzed outbreaks in Japan commonly started from mass food-handling practice involving food-handlers in elementary school or nursery school [21]. Moreover, the cross-contamination of some food products was evident in Indonesia. Widjaja et al., determined that fecal contamination of drinking water (65%), dishwater (91%), and ice cubes (100%) were highly prevalent in its population. They identified no hand-washing practice, direct hand contact with foods, use of unsterilized ice cubes, gender preference of male with poor handling, and low educational background as the prominent characteristic of food-handlers enrolled in the study [22]. Food-handlers involve in a particular stage of disease transmission, and it is emphasized from literature, improper sources of food and its handling is the most common risk factors present during the outbreak [23]. Based on the World Health Organization (WHO), food handler is any person who directly involved in a food business who handles food including its packaging, food equipment, or the substance contact surfaces. The shedding of the pathogen mostly occurred during handling practice resulting in surpassing the infective dose to create symptomatology disease. The different line of toxin triggering symptoms of FBDs take place during the proliferation of any pathogen in the food product, it produces symptoms impromptu from a certain period of minutes or hours after ingestion [24]. There are some studies showed that several factors in promoting FBDs among general population besides infected food-handlers including improper storage and cooking process, poor experience staff, poor hygiene condition, cross-contamination, and raw ingredient contamination are analysed in all outbreak form [25], [26].

Based on the study, several proposed risk-factors significantly related to suspected foodborne disease among the school-aged children in the study location. Furthermore, an approach has created fundamental parts in diagnosing FBD. Albeit, it also remains uncertain problems for several decades, causing the surveillance are scarcely conducted in the developing region. Whereas food-handling practice still did not perform adequately, and higher prevalence of FBD occurs in the country without any formal notification. Therefore, establishing and initiating prompt prevention methods is mandatory to tackle FBD among the population. The study also did not escape the limitation since there are no clear guidelines to determine FBD as diagnosis, so the assumption was created to provide further analysis.

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Appendix 1

The Decision of The Ministry of Health of Republic of Indonesia (Identifier: 942/MENKES/SK/VII/2003) on the requirement of hygiene and sanitation against food handlers

Chapter II

Food Handler

Article 2

Food handlers during handling practice must meet the minimum requirement, such as (8 points):

a. Do not suffer from infectious disease, shown by the symptoms: cough, sneeze, diarrhea, and any other gastrointestinal tract symptoms
b. No skin wound without its coverage (for open wound/ carbuncle or other types of wounds)
c. To ensure hand, hair, nail and cloth hygiene
d. Using the protection (apron and headcover)
e. Hand-wash behavior before handling
f. To provide adequate equipment including utensil for food picking tools or hand mat
g. Not to smoke and directly scratch the body (ear, nose, mouth, or other parts of the human body)
h. Not to sneeze and cough in front of food products without closing mouth and nose

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Validity and Reliability of an Instrument for Assessing Self-Care Behaviours in Diabetes Mellitus Type 2 Patients in Binjai City, Indonesia

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Abstract

BACKGROUND: Self-care behaviour becomes very important for diabetic patients; good self-care behaviour will prevent complications and improving the quality of life.

AIM: The aim of this study was to provide a preliminary assessment of the validity and reliability of a new measure of self-care behaviour of Diabetes Mellitus Type 2.

METHODS: The research was a cross-sectional study. The study population was T2DM patients from Primary Health Centers (PHC) in Binjai City, Indonesia. Sample determination using a single simple formula for the hypothesis of one population with calculation is 115 patients; sampling was done by convenience sampling with inclusion and exclusion criteria. Research questionnaire Self-care behaviour of T2DM patients was forming by knowledge, attitudes, communication, family support, financing, motivation, and self-efficacy. Each of the predictors forms self-care behaviour and finally, the instrument consists of 28 questions. The data analysis techniques were Confirmatory Factor Analysis (CFA) tests with Structural Equation Models (SEM) with AMOS aids.

RESULTS: Based on the results of the confirmatory analysis proved that the instrument is valid and reliable, the measure the self-care behaviour of T2DM.

CONCLUSION: The new instrument for assessing self-care behaviour of T2DM patients is valid and reliable, besides being able to assess self-care behaviour, they can also know the components that make up self-care.

Introduction

The prevalence of diabetes in Indonesia that has been diagnosed by doctors is 1.4%; this number is expected to continue to increase [1]. The prevalence of diabetes in Indonesia based on the Indonesia Health Profile 2013 was 2.1%. This figure is higher than in 2007 (1.1%). A total of 31 provinces (93.9%) showed a significant increase in the prevalence of diabetes. The highest prevalence of diabetes at the age of > 15 years according to the doctor's diagnosis or based on symptoms was in Central Sulawesi Province (3.7%) [1]. Diabetes is a type of chronic disease that requires medical treatment and changes in the lifestyle of patients throughout life to prevent complications and affect death [2]. Changes in lifestyle are related to diabetes management for behavioural changes including physical activity, changes in diet, monitoring of blood glucose levels, and adherence to treatment to achieve a better quality of life in patients with Type 2 diabetes. This quality of life has associated with self-care behaviour. self-management education in helping patients manage their health conditions. People with diabetes desperately need the application of self-care behaviour to improve their quality of life while reducing complications related to their disease conditions [3].

Diabetes self-care is an action taken by individuals to control diabetes which includes. Treatment measures and prevention of complications [4]. Self-care is the ability of individuals, families, and

with the patient, community to prioritise health to prevent disease, deal with diseases and overcome disability using or not using health services [5]. This self-care behaviour is related to controlling blood sugar, planning a diet, physical activity of the patient, using drugs or insulin [2], [6]. There are 7 main self-care behaviors, namely: healthy eating (healthy diet), being active (adequate physical activity), monitoring (blood sugar level control), taking medicine (consumption of anti-diabetic drugs or insulin), problem solving (problem-solving), healthy coping (healthy coping) and reducing risk (reducing risk). The implementation of these seven behaviours has a positive correlation with controlled blood sugar levels, reducing complications and improving the quality of life of diabetic patients [7].

The results of a review of several studies revealed that several factors could affect the level of self-care can be categorized as 1) Factors are originating from patients, namely: knowledge, attitudes, beliefs, health literacy, low adherence, social, economic, demographic and cultural support; 2) Factors originating from the doctor, namely: effective doctor-patient communication, unpleasant doctor-patient relationship, lack of doctor's knowledge about diabetes; and 3) Factors related to health care facilities, namely: access to health services, expensive health financing, uneven distribution of health workers [8].

Some instruments already exist and are used for the assessment of self-care, but the instrument only measures without describing what components form self-care behaviour. This study will eventually produce an instrument that can assess the self-care behaviour of T2DM and also illustrates its forming factors, with this instrument, in the end, it will be able to provide input to the health services mainly primary health services to be able to provide these factors so that all patients diabetes has good self-care behaviour. The study aims to provide a preliminary assessment of the validity and reliability of a new measure of self-care behaviour of Type 2 DM and to determine factors as predictors of the self-care behaviour of Type 2 DM patients.

Methods

This research is descriptive with a cross-sectional approach.

Study Population and Sampling

The population of the study was patients with type 2 Diabetes mellitus from eight primary health centres in Binjai City. The study was conducted in the Binjai city, which is the closest small town to Medan City (the provincial capital). Based on health data in eight primary health centres, it is known that diabetes is one of the ten most diseases suffered by the people of Binjai and has an increasing prevalence every year. The determination of the sample size in this study uses a single simple formula for the hypothesis of the proportion of a population so that the sample size is 115 people.

Sampling is done by convenience sampling, which is a non-random method where samples are chosen based on the research criteria. The research criteria, namely: 1) patients who came to primary health care as routine treatment patients at the health centre; 2) patients were independent because they came for treatment without help from others; 3) had aged 40-65 years; 4) voluntarily want to be a research respondent; 5) not women who are pregnant or breastfeeding; 6) do not have complications that interfere physically, mentally and emotionally; and 7) can be invited to work together during observations or surveys.

The use of Structural Equation Model (SEM) requires a large sample size so that the results obtained have sufficient credibility (trustworthy results), Considering this, in general, the sample size needed for SEM models with significant latent variables up to 5 pieces, and 3 or more indicators explain each latent variable, it requires a sample of 100-150 data. In this study, 115 samples were considered representative of SEM analysis.

Study Tool

Based on the results of previous studies [8], self-care behaviour of Type 2 DM patients was forming by knowledge, attitudes, communication, family support, financing, motivation, and self-efficacy. Each predictor that forms self-care behaviour consists of 7 indicators given two questions in each predictor to be given to respondents so that the total becomes 28 questions. The self-care dimension categorised into two good self-care groups with poor self-care behaviour. Determination of the category is good if the total score of each self-care forming indicator is higher than the average score of each domain (normally distributed data), and the determination of the category is less if the total score of each domain is less than the average value of each domain.

Ethical Consideration

This research has received ethical approval with No: 630/TGL/KEPK FK USU-RSUP HAM/2016 from the Universitas Sumatera Utara Medical Faculty Health Research Ethics Commission, Indonesia. This research was also carried out without burdening patients or other parties. The information obtained is entirely the support of surveys or interviews for research respondents who were voluntarily involved in

this study and will be used only for research purposes.

Statistical Analysis

In this study will be carried out: 1) test the construct validity using confirmatory factor analysis and 2) perform a correlation test on each factor that measures the self-care behaviour of patients with Type 2 diabetes in Binjai City, Indonesia. The data obtained from the research are using confirmatory factor analysis (SEM). Data analysis using AMOS software.

Results

Characteristics of the Study Population

The survey results show data from research samples taken from eight primary health centres in Binjai City, described into several criteria, namely (1) age (2) sex (3) occupation (4) education (5) income (6) marital status. For more details, see Table 1 below:

Table 1: Basic Characteristics of Diabetes Mellitus Type 2 patients in Binjai City

Characteristics	Frequency (person)	Percentage (%)
Age Group		
Early adolescent (26-35 years old)	3	2.6
Late adolescent (36-45 years old)	9	7.8
Early Elderly (46-55 years old)	39	33.9
End Elderly (56-65 years old)	64	55.7
Gender		
Man	30	26.1
Woman	85	73.9
Level of education		
Illiterate	7	6.0
Primary	22	19.0
Secondary	28	24.3
High school	37	32.1
Graduate school	26	22.6
Marital Status		
Married	89	77.4
Single / Divorced	26	22.6

The Table 1 shows the majority of patients who have Type 2 diabetes are the elderly group (age 56-65 years) about 55.7%, female (74%), married (77.4%), high school graduate (32.1%), haphazard workers (50.4%), and with ethnic Jawa (44.4%).

Self-care Characteristics and Self-care Forming Dimensions of Type 2 DM patients in Binjai City

Self-care behaviour of Type 2 DM patients was forming by knowledge, attitudes, communication, family support, financing, motivation, and self-efficacy. The following is the analysis of the self-care behaviour category of Type 2 DM patients in Binjai City.

Table 2: Results of Patient Self-care Behavior Analysis

The dimension of Self-care Behavior	Good		Less Good	
	n	%	n	%
Knowledge	63	55	52	45
Attitude	67	58	48	42
Communication	58	50	57	50
Financing	62	54	53	46
Family support	78	68	37	32
Motivation	82	71	33	29
Self-Efficacy	58	50	56	49

Data in Table 2 prove that overall self-care behaviour of patients with type 2 diabetes mellitus in Binjai city is in a good category. Patients who have known themselves with diabetes and are included in Type 2 DM are beginning to realize the importance of maintaining a healthy body, maintaining the intake of food and drinks that enter the body so that they begin to routinely maintain sugar levels and fat levels in the blood and start routine many enjoyable activities for body metabolism.

Besides that, the best dimension of self-care is the patient's motivation to recover (71%). Patients are highly motivated to seek treatment and fulfil the doctor's advice in maintaining blood sugar and fat levels so that they can avoid long-term complications. The dimensions of family support are also the factors that have the most significant role in self-care behaviour (68%), meaning that families can help patients to maintain their health and support patients to change their lifestyle to be better and healthier, but the dimensions that have the least role in patient self-care behaviour Type 2 diabetes is self-efficacy and communication is 50%. It shows the patient is still trying to believe that he can heal to be able to care for himself better, and this also affects the quality of patient communication with other people so that patients will tend to be more sensitive and emotional.

Table 3: The goodness of Fit Index Cut-off Value Result of Model Evaluation Analysis

The goodness of Fit Index	Cut-off Value	Result of Analysis	Evaluation of The Model
χ2 - Chi-square	Expected small	10.620	Good Fit
Probability	≥ 0.05	0.031	Marginal Fit
RMSEA	≤ 0.08	0.066	Good fit
GFI	≥ 0.90	0.993	Good fit
AGFI	≥ 0.90	0.938	Good fit
TLI	≥ 0.90	0.840	Marginal Fit
CFI	≥ 0.90	0.977	Good fit

The data in Table 3 shows that this research model is appropriate and acceptable because it meets the specified Goodness of Fit Model Index Table criteria. Therefore, all predictors that measure self-care behaviour in this study can be accepted and assessed as a fit model, so that the construct validity of self-care behaviour tests for Type 2 DM patients in Binjai City is proven to be true in all factors that measure the patient's self-care variables. Then test the significance of each relationship between factors that measure or between predictors and the measured self-care behaviour variables. The level of significance shown in the following Table 4.

Table 4: Regression Weight on Predictor Factor Test Self-care

Variable			Estimate	P	Hypothesis
Communication	<--	SC	0.916	0.000	Valid and Reliable
Knowledge	<--	SC	0.876	0.036	Valid and Reliable
Self-Efficacy	<---	SC	0.964	0.0001	Valid and Reliable
Motivation	<---	SC	1.056	0.0001	Valid and Reliable
Attitude	<---	SC	0.813	0.0001	Valid and Reliable
Financing	<---	SC	0.330	0.041	Valid and Reliable
Family Support	<---	SC	0.320	0.003	Valid and Reliable

Discussion

All factors that are predictors that measure the self-care variables of DM Type 2 patients are known to be able to build the patient's self-care behaviour. The existence of motivation, family support, knowledge, self-efficacy, adequate communication, and financing will be able to shape self-care behaviour in patients with Type 2 DM. All of these predictors can help patients develop self-awareness to take care of themselves and their health so that they can control their blood sugar and fat levels and prevent complications that can interfere with the physical, mental, psychological and emotional condition of patients. Self-care of diabetic patients is a patient's action to treat and prevent complications [4], [9]. Self-care is the ability of individuals, families, and communities to promote health, prevent disease, maintain health, deal with diseases and disabilities with or without the help of health care providers [5], [10].

There are seven factors that play a role as a predictor of the self-care behavior of Type 2 DM patients in Binjai City, namely (1) knowledge (2) motivation (3) self-efficacy (4) communication (5) financing (6) attitude and (7) family support, proven the truth of the construct validity and statistically significant as a predictor for the self-care behavior of patients with Type 2 DM in Binjai City.

Knowledge contributes to shaping the self-care behaviour of Type 2 DM patient's Knowledge, in this case, the knowledge of people with diabetes about the disease and matters related to treatment and prevention is very decisive for the patient's independent behaviour. Research conducted in Nyatnyono Village, West Ungaran District, Semarang City Regency showed a significant relationship between the level of knowledge about diabetes and the lifestyle of diabetics [11]. Research conducted on patients in South India proves that patients who are given by education or counselling about diabetes can improve knowledge, attitudes and change the actions of patients to be more positive and have an impact on improving the quality of life of patients [12], [13]. Good knowledge and understanding of the disease will help patients accept their conditions and try to recover and live a healthy life [14].

Diabetic patients need to get minimal information given after diagnosis is established, including basic knowledge of diabetes, independent monitoring, causes of high blood glucose levels, oral hypoglycemic drugs, meal planning, care, physical activity, signs of hypoglycemia and complications. Diabetic patients who have enough knowledge about diabetes, then change their behaviour, so they will be able to control the condition of the disease and people with diabetes can live better quality. Knowledge of controlling blood sugar levels is the most important and knowledge of how to manage emotions so that patients do not become depressed because of the illness and treatment they are carrying out. Increased depression will impact on an increase in blood sugar levels, so doctors need to detect the level of depression of the patient early and give him an education in dealing with it which will help improve the patient's health and prevent complications [15].

Attitudes contribute to shaping self-care behaviour. The proper attitude of diabetic patients in Binjai as an impact from good knowledge about diabetes which results in a positive attitude. Excellent knowledge and attitude will produce good behaviour or actions because attitude can be said to be willing to act, and vice versa [16].

Based on the results of the analysis, doctor-patient communication contributes to forming self-care behaviour [17]. There is a significant relationship between the communication of health workers with self-care behaviour. The smoother the communication carried out, the better the self-care behaviour of diabetic patients [18]. Communication is an essential factor and the key to successful treatment of chronic patients such as diabetes. Excellent and effective communication between doctors/health workers with diabetic patients into positive energy, motivation and encouraging patients who will improve their health [14]. Communication training between doctors and patients in primary services shows training can improve the doctor's relationship with patients, resulting in satisfaction with both sides, the patient's willingness to be involved in medical decision making, as well as the patient commitment to the treatment plan, lifestyle changes and improvement of patient health [19]. Previous research also supported the results of this study that patients with Type 2 DM in Binjai were known to have sufficient quality of life well. The improvement of self-care behaviour is better because of an increase in physical health, social and environmental relations but not from the psychological side as an indication of communication that is attempted to run well between patients and doctors when performing patient self-care [20].

Based on the results of SEM analysis, health financing contributes to shaping diabetes patient's self-care behaviour in Binjai City. Financing is the availability of funds and the financial capacity of a diabetic patient in carrying out treatment. Availability of funds is necessary for the continuity of patient treatment. From the results of interviews conducted with diabetic patients in the city of Binjai, the most

patients already have health insurance (80%), so they get health services for diabetes such as blood sugar checks, laboratory tests and anti-diabetic medicines at the health centre or at home referral pain for free. The results showed that the dimensions of family support are one of the constituent components of diabetes patient's self-care behaviour in Binjai City.

In diabetic patients, the family support provided can help self-reliance and self-care behaviour. The usual support received from his family includes encouragement from the family to control his health to the hospital. Besides that, the family also assists patients in supporting their efforts to carry out diabetes-related care such as setting diet, regulation of taking medication and providing information related to treatment for example by using traditional plants that can reduce blood sugar levels. This kind of family support may improve the quality of life of DM patients with type 2 [21]. Family support in this study is of good value, and it can form by the pattern of community life in the city of Binjai is still very family and cooperation. They still pay attention to togetherness and help between them and of course, support each other in the family [22].

The results of the study showed that the motivation dimension was one of the constituent components of a diabetes patient's self-care behaviour in Binjai City. Motivation is an essential factor for patients with type 2 diabetes because the motivation that is present in type 2 DM patients will be able to provide a strong impetus for type 2 DM clients to conduct diabetes self-care behaviour so that optimal blood sugar control can be achieved and minimising the occurrence of complications diabetes. Self-motivation is a significant factor influencing type 2 DM patients in performing diabetes self-care, especially concerning maintaining the diet and monitoring blood sugar. Type 2 DM patients who have good motivation will perform diabetes self-care actions well as well to achieve the desired goal of controlling blood sugar so that ultimately DM complications can be minimised [17], [23].

The results showed that the dimension of self-efficacy is one of the constituent components of the diabetes patient's self-care behaviour in Binjai City. Self-efficacy is a strong predictor of DM self-management behaviour, someone who lives with DM who has a higher level of self-efficacy will participate in better DM self-management behaviour. Self-efficacy is related to specific behaviors in diabetes self-management. Low self-efficacy in each of the diabetic patient's recommended behaviors will reduce adherence to these specific behaviours [24], [25].

Limitations of the study: 1. The determinants of self-care behaviour which are ultimately formulated into forming factors of self-care behaviour, in theory, and their application in the field, are actually still many factors and indicators that are considered to shape and influence them, but in research only refers to 7 indicators. 2. This study uses a cross-sectional approach, and cross-sectional data has limitations in explaining the stability of influence between variables involved in a study from time to time. 3. The number of samples in this study only amounted to 115 patients who are considered still less representative, in future research, can be done with a more representative number of samples.

In conclusion, the new instrument for assessing self-care behaviour of T2DM patients is valid and reliable. Anyone interested in using this instrument may obtain a copy directly from the first author (there is no charge for its use). Knowledge (2) motivation (3) attitude (4) self-efficacy (5) communication (6) family support (7) financing are important things for diabetic patient. The good collaboration is important between diabetic patients, family doctors and patients' families for the formation of good self-care behaviour.

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The Lifestyle Characteristics in Non-Alcoholic Fatty Liver Disease in the PERSIAN Guilan Cohort Study

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Abstract

BACKGROUND: Non-Alcoholic Fatty Liver Disease (NAFLD) is the most common cause of chronic liver disease worldwide. Since the effect and safety of pharmacotherapy for NAFLD are unknown, the proper management of lifestyle is crucial.

AIM: The present study was conducted to determine the status of food, Physical Activity (PA), and sleep in patients with and without NAFLD.

METHODS: In this analytical- cross-sectional study, 630 clients with 36-60 years old who referred to the PERSIAN Guilan cohort study were included through simple non-random sampling. The developed questionnaire and lifestyle characteristics, including the status of nutrition, physical activity, and sleep, were completed for all samples. BMI was also calculated by determining weight and height, and fatty liver was confirmed based on abdominal ultrasound.

RESULTS: The prevalence of NAFLD in this study was by 43.7% (275 / 630). Smoking, alcohol consumption, BMI, and weight loss over the past six months, regular exercise and exercise intensity, sedentary living, speed of eating, consuming fatty food, red meat, sweets beverages, and use of saturated fatty acid (SFA), and consuming fruits and vegetables were associated with presence of NAFLD (all $p < 0.05$). However, no significant relationship was observed between the parameters of sleep duration, the interval between dinner and night sleep, consuming breakfast and snack during the day and NAFLD (All $p > 0.05$).

CONCLUSION: The onset and progression of NAFLD are associated with lifestyle. Therefore, dietary therapy solutions, physical activity, and sleep and rest situations should be paid attention for people with or at risk of NAFLD.

Introduction

Non-Alcoholic Fatty Liver Disease (NAFLD) is the most common cause of chronic liver disease in worldwide, which is characterised by the accumulation of fat in the liver of patients with no alcohol abuse, and with clinical signs of simple steatosis, steatohepatitis, advanced fibrosis, and cirrhosis [1], [2]. Fatty liver is common in industrial countries, and the prevalence of NAFLD has been grown in the Asia-Pacific Ocean region over the past two decades given the Western lifestyle and increased incidence of obesity [3]. The global prevalence of NAFLD has been reported as around 25.24%, 31%, 32%, and 27%, respectively in the adult population, southern America, Middle East, and Asia. [4], [5] Prevalence of NAFLD in the south and north of Iran has been reported as 21.5% [6] and 43.8%, [7] respectively, and it has been generally reported as 33.9% [8].

The exact NAFLD pathogenesis has remained unknown, though it seems to be multifactorial [9]. There is no consensus for the pharmacotherapy of NAFLD. Nevertheless, management of Diet and Physical Activity (PA) is an indispensable component of any therapeutic strategy for weight loss, and it may play a significant role in preventing NAFLD [10], [11]. Currently, the initial treatment involves gradual weight loss through reducing caloric consumption and enhancing physical activity to improve Liver Function Tests (LFT), Insulin Resistance (IR), fasting glucose levels, and lipid profiles. [12] Precise evaluation of extra energy consumption is required by the individual to obtain

better results in the nutritional treatment of NAFLD [13]. Generally, diets with high-calorie content, high carbohydrate, high saturated fatty acid, cholesterol, and soft drinks may increase the accumulation of fat in the liver, aggravate the clinical conditions of NAFLD, and cause its progression [9], [14]. On the other hand, restricting the calorie intake and increasing consumption of soy protein, Mono Unsaturated Fatty Acid (MUFA) supplements, omega-3 fatty acid, and probiotics are effective in preventing and treating NAFLD [9]. Also, it has been believed that PA is one of the determinant factors for controlling metabolic state. NAFLD patients are recommended to have physical activity alongside losing weight and modifying diet [15], [16]. Since the elevation of PA has a protective role against NAFLD-associated risk factors such as obesity, type II diabetes, blood pressure, and dyslipidemia, thus it seems that enhancing PA is effective in preventing NAFLD [17], [18], [19], [20].

Most studies have discussed various aspects of lifestyle such as nutrition, diet, and PA and the relationship with NAFLD, but other aspects such as sleep habits have remained understudied. Therefore, the present study was conducted to determine the relationship between lifestyle characteristics such as consumed food, physical activity, and sleep habits and NAFLD.

Methods

In this study, 630 subjects with 35-60 years old (from April 2017 to July 2017) among the clients referring to the PERSIAN Guilan cohort study (PGCS) part of the PERSIAN cohort study [21] were included in this analytical cross-sectional study through sequential sampling. The exclusion criteria included not having chronic or acute liver disease including viral hepatitis B and C, chronic and acute renal disease, cancers, and alcohol consumption (men above 20 g/d and women above 10 g/d), pregnancy, consuming medications affecting the liver such as steroids, amiodarone and tamoxifen, and patients with established hemochromatosis. Identification of the subjects was performed based on the PERSIAN cohort profile [21].

The information of this study was collected through a questionnaire which has been developed by the research group and as face-to-face. This questionnaire consisted of two sections: the first section captured demographic information (age, gender, marital status, occupation, level of education, and place of residence), while the second section involved typical lifestyle characteristics over the past six months.

These characteristics included questions such as weight loss over the past six months, alcohol consumption and smoking, dietary habits (being used to consuming breakfast, consuming snacks during the day, speed of eating, type of consumed bread, consuming fatty food, fruits and vegetables, milk and dairies, sweet beverages, type of consumed oil, type of the main consumed meat), physical activity (regular exercise, exercise intensity, daily walking, type of activity during daily living), and sleep (duration of daily sleep, interval between dinner and night sleep) of the subjects. Trovato et al. defined lifestyle according to dietary habits, exercise and physical activity, and sleep [22]. Based on Kim et al.'s study [23], the sleep duration was categorised as 5 ≥, > 5-6, > 6-7, and > 7 in this study.

The height (cm) was measured without shoes (by wall stadiometer device seca206, Hamburg, Germany) and weight (kg) was measured with light clothing and without shoes (by seca755 scale, Hamburg, Germany) by a trained person. Body mass index (BMI; kg/m2) was obtained by dividing the weight (kg) by the height squared (m2) for all subjects. BMI < 25 was considered normal, while BMI ≥ 25 was regarded as overweight [24].

To determine fatty liver, in the presence and with confirmation of two radiologists who deployed in the cohort centre, abdominal ultrasound was performed using the ultrasonic device (sonixSP series) using a 3.5-5 MHz deep probe. Echogenicity was increased in the liver parenchyma compared to kidney parenchyma or spleen in abdominal ultrasound [25] was recorded as fatty liver.

The data were expressed as frequency (percentage) and mean (standard deviation). Chi-square test, t-test, and chance ratio were calculated by univariate logistic regression to compare the variables, where $p < 0.05$ was considered significant. All analyses were performed by SPSS 18.

This study has been registered in the research and ethics committee of the Research Center of Gastroenterology and Hepatology and Guilan University of medical sciences with the number of IR.GUMS.REC.1394.499. Written informed consent form was taken from all participants to participate in the study. Further, they were free to quit this study at any stage they wished.

Results

Out of the 630 subjects, 275 (43.7%) had NAFLD, that 330 (52.4%) were male, whose age range was 35-60. The mean age was 46.99 ± 7.33 (47.10 ± 7.10 in women and 47.76 ± 7.57 in men). The demographic information of the subjects is presented in Table 1.

Table 1: Demographic characteristics of participants

Variables	Total N (%)	non- NAFLD N (%)	NAFLD* N (%)
Age (years)			
35-44	244 (38.7)	142 (58.2)	102 (41.8)
45-54	241 (38.3)	133 (55.2)	108 (44.8)
55-60	145 (23.0)	80 (55.2)	65 (44.8)
Gender			
Male	330 (52.4)	183 (55.5)	147 (44.5)
Female	300 (47.6)	172 (57.3)	128(42.7)
Marital status			
Single	20 (3.2)	12 (60)	8 (40)
Married	610 (98.8)	343 (56.2)	267 (43.8)
Job			
Farmer	66 (10.5)	39 (59.1)	27 (40.9)
Housewife	252 (40)	138 (54.8)	114 (45.2)
Employed	90 (14.3)	54 (60)	36 (40.0)
Worker	97 (15.4)	49 (50.5)	48 (49.5)
Self-employed	125 (19.8)	75 (60)	50 (40)
Education			
Illiterate	42 (6.7)	19 (45.2)	23 (54.8)
Elementary	138 (21.9)	73 (52.9)	65 (47.1)
High school	368 (58.4)	212 (57.6)	156 (42.4)
Academic	82 (13)	51 (62.2)	31 (37.8)
Residence			
City	454 (72.1)	259 (57)	195 (43)
Village	176 (27.9)	96 (54.5)	80 (45.5)

*NAFLD non-alcoholic fatty liver disease.

The results of this study indicated a significant relationship between smoking (p = 0.036, OR 95%CI = 1.47: 1.02-2.13), alcohol consumption (p = 0.030, OR 95%CI = 1.70: 1.05-2.75), and BMI ≥ 25 (p < 0.001, OR 95%CI = 7.98: 4.53-14.07) between NAFLD and non-NAFLD group. Also, weight loss was significant between the groups over the past six months (p = 0.006, OR 95%CI = 0.59: 0.41-0.86).

In this study, a significant relationship was observed between weight loss over the past six months as well as average BMI based on t-test and NAFLD (p = 0.005 and p < 0.001, respectively). However, there was no significant relationship between the duration of daily sleep (p = 0.61) and the interval between dinner and night sleep (p = 0.39) (Table 2).

Table 2: Participants in non-NAFLD and NAFLD

Variables	non-NAFLD	NAFLD	P-value*
Age (year)	47.08 ± 7.42	47.92 ± 7.23	0.15
BMI (kg/m^2)	26.56 ± 3.74	30.81 ± 4.47	< 0.001
Weight loss (Last 6 months) (kg)	1.15 ± 2.47	0.68 ± 1.75	0.005
Sleep duration(h/day)	7.22 ± 1.32	7.17 ± 1.33	0.61
Dinner-to-night sleep interval (h)	2.65 ± 1.06	2.58 ± 0.97	0.39

NAFLD non-alcoholic fatty liver disease; Body mass index (BMI); Data are presented as mean ± SD; P-value as derived by Independent sample t-test; * p < 0.05 is significant.

Similarly, according to the sleep duration categorisation, no significant relationship was observed between NAFLD and non-NAFLD group (Table 3).

Table 3: Comparison of sleep duration and physical activity between non-NAFLD and NAFLD

Variables	non-NAFLD N (%)	NAFLD N (%)	P-value*	OR (CI 95%)
Sleep duration (h/day)				
≤ 5	27 (51.9)	25 (48.1)	0.77	1.09 (0.60-1.97)
> 5-6	56 (54.4)	47 (45.6)	0.95	0.98 (0.62-1.55)
> 6-7	119 (62)	73 (38)	0.08	0.72 (0.49-1.04)
> 7	153 (54.1)	130 (45.9)	-	(ref)
Regular exercise				
Yes	97 (65.1)	52 (34.9)	0.014	0.62 (0.42-0.90)
No	258 (53.6)	223 (46.4)		(ref)
Exercise intensity				
Intense	16 (84.2)	3 (15.8)	0.007	0.15 (0.04-0.59)
Moderate	56(65.1)	30 (34.9)	0.022	0.44 (0.22-0.89)
Light	25 (45.5)	30 (54.5)	-	(ref)
Daily walking (min/day)				
≤ 30 min	180 (59.4)	123 (40.6)	0.137	0.78 (0.57-1.07)
> 30 min	175 (53.5)	152 (46.5)		(ref)
Daily Activity				
Sedentary	73 (47.4)	81 (52.6)	0.010	1.61 (1.11-2.00)
Active	282 (59.2)	194 (40.8)		(ref)

NAFLD non-alcoholic fatty liver disease; *p < 0.05 is significant.

The results of this study indicated that the chance of developing NAFLD was less in those who had regular exercise over the past six months. (p = 0.014, OR 95%CI = 0.62: 0.42-0.90). On the other hand, sedentary daily activity enhances the chance of developing NAFLD (p = 0.010, OR 95%CI = 1.61: 1.11-2.00). PA comparison between the groups is reported in Table 2.

In investigating the dietary habits of individuals with NAFLD, a significant relationship was observed with speed of eating (p = 0.03), consuming fatty food (p = 0.04), consuming fruits and vegetables (p = 0.03), daily consumption of sweet beverages (p = 0.042), use of SFA (p = 0.039), and consuming red meat (p = 0.01) (Table 4).

Table 4: Comparison of nutrient patterns between non-NAFLD and NAFLD

Variables	non-NAFLD N (%)	NAFLD N (%)	P-value*	OR (CI 95%)
Breakfast consumption				
Yes	343 (55.9)	271 (44.1)	0.13	0.42(0.13- 1.32)
No	12 (75)	4 (25)		(ref)
Snacks consumption				
Yes	312 (57)	235 (43)	0.37	0.81 (0.51- 1.28)
No	43 (51.8)	40 (48.2)		(ref)
Speed of eating				
Yes	178 (52.5)	161 (47.5)	0.036	1.40 (1.02-1.92)
No	177 (60.8)	114 (39.2)		(ref)
Bread consumption				
bran-rich	298 (56.4)	230 (43.6)	0.91	0.97 (0.63-1.49)
no bran	57 (55.9)	45 (44.1)		(ref)
Fatty food consumption				
Yes	315 (55.1)	257 (44.9)	0.04	1.81 (1.01-3.23)
No	40 (69)	18 (31)		(ref)
Meat consumption				
Red meat	32 (40.5%)	47 (59.5%)	0.01	2.42 (1.22- 4.81)
Poultry	285 (58.2%)	205 (41.8)	0.53	1.18 (0.68- 2.05)
Fish	38 (62.3%)	23 (37.7%)	-	(ref)
Fruits & vegetables				
(serv./day) 3-4	46 (67.6)	22 (32.4)	0.03	0.55 (0.32 -0.96)
2	116 (56.9)	88 (43.1)	0.49	0.88 (0.62- 1.25)
< 1	193 (53.9)	165 (46.1)	-	(ref)
Milk&dairies(glass /day)				
≥ 2	60 (55.6)	48 (44.4)	0.18	1.42(0.84-2.38)
1	208 (53.9)	178 (46.1)	0.04	1.51 (1.01 -2.27)
< 1	87 (64)	49 (36)	-	(ref)
Sweet beverages (day)				
Yes	200 (53.1)	177 (46.9)	0.042	1.400(1.01-1.93)
No	155 (61.3)	98(38.7)		(ref)
Oil consumption				
SFA	37 (45.7)	44 (54.3)	0.039	1.63 (1.02-2.61)
UFA	318 (57.9)	231 (42.1)		(ref)

NAFLD non-alcoholic fatty liver disease; SFA Saturated fatty acid; UFA Unsaturated fatty acid; *p < 0.05 is significant.

Discussion

The increase in the incidence of non-communicable worldwide diseases has changed NAFLD into a new challenge for public health. Prevalence of NAFLD in the world, Eastern countries, and Asia has been reported as 10-30% [26], 20-30%, and 15-20%, respectively [27]. In Iran, the prevalence in the north and south of the country has been reported as 43.8% [6] and 15.3% [28]. In this study, the NAFLD prevalence was 43.7%.

Lifestyle is an important factor for metabolic syndrome, and it is associated with NAFLD [29]. Further, exercising to lose weight is an indispensable

part of lifestyle interventions, and it is proposed as a useful independent item for NAFLD [22]. According to the results of the present study, PA is associated with the probability of NAFLD reduction, where individuals with less daily physical activity are at higher risk.

Hallsworth, [16] Gerber [15], and Trovato [22] stated that those with NAFLD spend more time resting and have less physical activity compared to the control group. Long-term sitting predisposes individuals further to the risk of NAFLD by increasing the fat mass or reducing the musculoskeletal mass. Thus, by reducing sitting and increasing PA, one can decrease the risk of NAFLD [25].

In this study, smoking and alcohol consumption along with high BMI were associated with NAFLD, which is consistent with the results of previous studies on the relationship between smoking, [30,31] alcohol [4], [22] and BMI [1], [6], [22], [32] and NAFLD. The onset and progression of NAFLD, apart from the quantity and quality of food and exercise, are also associated with other aspects of lifestyle, including alcohol consumption less than 20 g/day [22].

The fat quality of the diet also seems to have a significant role in the progression of NAFLD. In this regard, consuming a diet with saturated fatty acid (SFA) may cause fat accumulation in the liver. In contrast, a diet containing Poly Unsaturated Fatty Acid (PUFA) has a negative effect on the extent of accumulation of triglyceride inside the liver [33]. Our study indicated that the individuals who have SFA-containing diet are more at risk of developing NAFLD. Further, in this study, the probability of risk of NAFLD was higher for those consuming fatty food, drinking sweet beverages, consuming red meat, and eating food faster. However, the probability was lower with consuming fruits and vegetables.

Nevertheless, no relationship was observed between consuming breakfast and snack. Previous studies have been reported that skipping breakfast is associated with obesity, [34] and might be associated with the onset of NAFLD. On the other hand, the study by Imaizumi [28] did not report the relationship between skipping breakfast and NAFLD, which is in line with our study. Various studies have been reported a positive relationship between consuming carbohydrate as well as sweets and NAFLD [1], [30], [35].

When investigating dietary habits, it is important that the subjects are evaluated individually about overusing food and even proper eating behaviours. A number of nutritional patterns such as increasing food volume (eating out frequently, huge food volume per one meal, eating any kind of food), high energy diet (fast foods, outside food, and fried food), consuming suitable food and the manner of eating (being used to eating a load of food in the afternoon, eating in the evening, skipping breakfast, and eating fast) and over-consuming special nutrients have been shown to be associated with NAFLD [13]. Previous studies have considered a vegan diet and they have replaced red and fish meat with soybean and substituted refined carbohydrates with whole grains as effective for preventing fatty liver [36].

In the present study, although the subjects in the NAFLD group had shorter average sleep, no relationship was found between duration of sleep and NAFLD, which is consistent with the findings of the study by Trovato [22] and Katsagoni [29]. However, some studies have suggested that sleep duration has an inverse relationship with NAFLD, where short sleep duration is associated with increased risk of NAFLD [23], [29], [37], [38]. Sleep is an important factor for maintaining health, and short sleep duration is associated with obesity, diabetes, and fatty liver [39]. Generally, it is expected that improving lifestyle is important for preventing and treating NAFLD.

Since the onset and progression of NAFLD are associated with lifestyle, thus nutritional therapeutic solutions, PA, and sleep and rest status are required for those with or at risk of NAFLD. Increasing physical activity and a healthy diet is a therapeutic target which may prevent progression of the metabolic status and weight gain in those with NAFLD, which should be taken into account in clinical care.

Among the limitations of this study was using ultrasonography for diagnosing NAFLD. However, liver biopsy is the golden standard for fatty liver, but it is invasive and costly and is not recommended for the general population. Therefore, abdominal ultrasound was used in this study. The comments of two radiologists were used simultaneously to control this limitation. In comparison to histology, sonography is more reliable and accurate. Due to being inexpensive, safe, and available, it is the method of choice for screening fatty liver in clinical conditions and population settings [40].

On the other hand, the information related to diet, PA, and sleep of individuals was collected as self-reporting. In this method, under-reporting especially regarding diet in obese individuals might be a problem. To control it at least to some extent, attempts were made to provide sufficient explanation about the importance of correct reporting. Eventually, the cross-sectional nature of this study is a limitation which cannot determine causal relations, and the results should be confirmed in prospective studies.

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Review Article

Management of Severe Acute Pancreatitis

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Abstract

Acute pancreatitis is one of the most common causes of hospitalisation from gastrointestinal diseases. The causes of pancreatitis vary between countries. Acute pancreatitis is classified based on Revised Atlanta classification 2013 as mild, moderately severe and severe acute pancreatitis. Acute pancreatic severity can be stratified by scoring systems such as Ranson's score, BISAP score, APACHE-II score, SOFA score. In severe acute pancreatitis, to diagnose, abdominal pain raised amylase or lipase, supported imaging finding and organ failure. Organ failure can be diagnosed by using Modified Marshall Scoring System. Management is started conservatively, which are fluid resuscitation, enteral nutrition, analgesics, and antibiotics. Surgical management is indicated when infected pancreas necrosis is detected. In this review, we will discuss the current management based on recent research.

Introduction

Acute pancreatitis is one of the most common causes of hospitalisation from gastrointestinal diseases, with a global incidence ranging from 5-30 cases per 100,000 population per year. In America, pancreatitis causes more than 800,000 hospital visits and costs more than 2.6 billion dollars [1].

The causes of pancreatitis vary between countries. Alcohol is still the dominant disease in Western countries, while in Eastern countries, especially Asia, the most common cause is biliary disease (49-54%) [1], [2]. Others were caused by drug reactions, pancreatic and cystic malignancies, and hypertriglyceridemia [2].

Grading and Severity

Revised Atlanta classification 2013 are commonly used to categorise acute pancreatitis, which is mild, moderately severe and severe acute pancreatitis. Most of the acute pancreatitis were mild, in which organ failure or complications were not found. In moderately severe acute pancreatitis, Organ failure was transiently found in less than 48 hours [3], [4], [5]. Meanwhile, in severe acute pancreatitis (SAP), organ failure was seen for more than 48 hours. Moderately severe and severe acute pancreatitis manifest in systemic and local complication. The systemic complication in moderately severe acute pancreatitis was seen in chronic renal failure patient who presents with acute symptoms. Local complications usually manifest in the pancreatic and peri-pancreatic fluid collection. Those collections generally appear in the late phase of pancreatitis [4].

Acute pancreatic severity is influenced by organ failure. Revised Atlanta recommend Modified Marshall scoring system as the main tool in determining organ failure [4]. Modified Marshall scoring system (Table 1) include respiratory, cardiovascular and renal systems score. Score more than 2 of any organs indicate organ failure. Acute pancreatic severity can be stratified by scoring systems such as Ranson's score, BISAP score, APACHE-II score, SOFA score. Ranson's criteria are used within 48 hours of the onset of the attack. APACHE-II score of 9 or more is considered as

severe pancreatitis. APACHE score can be observed during the course of acute pancreatitis. The disease is assumed as severe acute pancreatitis when the score is 3 or more. BISAP score is observed during the first 24 hours of admission to predict mortality before the onset of organ failure. BISAP score of more than 3 is related to 5-20% mortality [5].

Table 1: Modified Marshall scoring system [4]

ORGAN SYSTEM	0	1	2	3	4
Respiratory PO/FiO_2 (mmHg)	> 300	226-300	151-225	76-150	≤ 75
Renal Serum creatinine (µmol/liter)	≤ 100	101-200	201-350	351-500	> 500
Hepatic Serum bilirubin (µmol/liter)	≤ 20	21-60	61-120	121-240	> 240
Cardiovascular PAR	≤ 10,0	10,1-15,0	15,1-20	20,1-30	> 30,0
Hematologic Platelet / nl	> 120	81-120	51-80	21-50	≤ 20
Neurologic Glassgow coma score	15	13-14	10-12	7-9	≤ 6

Diagnosis

Revised Atlanta classification requires the presence of abdominal pain, the increment of amylase or lipase more than 3 times upper limit of the normal range, and supported radiographic findings. The abdominal pain characterised by epigastric pain, followed by nausea and vomiting. In physical examination, rebound tenderness, abdominal distention, Cullen's sign, Grey Turner's sign can be found. In severe condition, reduced bowel sound, hypotension can also be found. Pancreatic acinar cell leakage in interstitial space and absorption into circulation cause Increment in amylase and lipase [3], [4], [5]. Contrast-enhanced CT scan (CE-CT scan) is the standard radiographic imaging in detecting acute pancreatitis [3], [4], [5]. In severe acute pancreatitis, CE-CT scan can be used to find pancreatic gland necrosis and the local complications. Pancreatic gland necrosis completely appears in 4 days after the onset of SAP. Before that time, CE-CT scan cannot precisely detect pancreatic necrosis. Other radiographic modalities that were commonly used in diagnosing acute pancreatitis were ultrasonography, MRI. Ultrasonography has a limited role in diagnosing acute pancreatitis, especially in ileus patient. Abundant air volume in the intestine in ileus patient cause difficulty in the visualization of the pancreas. MRI is considered as a good alternative in detecting pancreatic necrosis, pancreatic collection and peri-pancreatic collection. Magnetic resonance cholangiopancreatography (MRCP) can be used as an alternative to ERCP to evaluate pancreatic duct [4], [5].

Management

Fluid resuscitation

In severe acute pancreatitis, the patient could have excessive vomiting, reduced oral intake, third space extravasation, respiratory losses, and diaphoresis. Therefore, fluid resuscitation becomes the most important step in managing severe acute pancreatitis [6]. It is recommended to be done as early as possible. Crystalloid is the preferred choice in resuscitation. There are many types of research (Table 2) showed Ringer Lactate as the replacement fluid has many beneficial effects. The solution has to be given 250-500 ml per hour in the first 12-24 hours. NaCl 0.9% is not recommended since it triggered hyperchloremic metabolic acidosis when it was given in a large volume [6].

Table 2: The beneficial effect of Lactated Ringer's solution in various research

Author	Journal, Year	Method	Conclusion
De 'Madaria et al., [7]	United European Gastroenterology Journal, 2018	RCT	Lactated Ringer (LR) is associated with a reduction of CRP levels. LR has an anti-inflammatory effect in patients with acute pancreatitis
Iqbal et al. [8]	Journal of Digestive Diseases, 2018	Meta-analysis	LR has anti-inflammatory effects and is associated with decreased risk of persistent SIRS at 24h, which is a marker of severe disease in AP patients
Choosakul et al. [9]	Pancreatology, 2018	RCT	LR solution was superior to NS in SIRS reduction in acute pancreatitis only in the first 24h. But SIRS at 48h and mortality were not different between LR and NS.
Wu et al. [10]	Clinical Gastroenterology and Hepatology, 2011	RCT	Patients with acute pancreatitis who were resuscitated with LR solution had reduced systemic inflammation compared with those who received saline.

Abbreviations: RCT (Randomized control trial); CRP (C-reactive protein); SIRS (Systemic inflammatory response syndrome); LR (Lactated Ringer's); NS (Normal saline).

Few parameters can be used to predict the outcome after fluid resuscitation, which is hematocrit and BUN. Hemoconcentration, which can be seen from hematocrit, develop in the hypovolemic condition in SAP. Hematocrit < 44%-47% is a risk factor for developing necrosis in the pancreas [6] Wu et al., revealed that hemoconcentration was related to increment in mortality rate among hospital transferred patients. BUN was also recommended by Wu et al., as a predictor of pancreas necrosis. If fluid resuscitation had been done, BUN was not decreased, then the patient would have increased risk of pancreas necrosis [10].

To monitor the responsiveness of resuscitation, beside BUN and hematocrit, the physician was recommended to monitor urine output. Urine output > 0.5 ml/kg BW/hour was suggested as the target. Lactate was also mentioned as the monitoring parameter. However, there is no evidence to apply this to severe acute pancreatitis [6].

Enteral nutrition

Enteral nutrition was recommended for severe acute pancreatitis over parenteral nutrition due to many beneficial effects as shown by few meta-analysis and trial (Table 3). Enteral nutrition may maintain the function and structure of intestinal mucosa [5], [11]. Enteral nutrition was suggested to be given as early as 48 hours of admission [13], [11] Early enteral nutrition could reduce mortality, multiple

organ failure and infection in comparison with late enteral nutrition and parenteral nutrition [12]. Parenteral nutrition was previously recommended as early intervention since it reduces the stimulation of pancreas to secrete enzymes, but it can lead to intestinal atrophy and altered intestinal barrier. As consequences, microorganisms from the gut will translocate to the systemic circulation through damaged intestinal epithelial cells causing sepsis. Furthermore, toxic products and inflammatory mediators also translocate because of increased intestinal permeability in the early stage of severe acute pancreatitis [11].

Table 3: Comparison of Enteral Nutrition and Total Parenteral Nutrition

Author	Journal, Year	Method	Conclusion
Qi et al. [14]	Journal of Parenteral and Enteral Nutrition, 2018	Meta-analysis	Comparing early EN to TPN showed a significant reduction in multiple organ failure and pancreatic related infections
Vieira et al. [15]	Acta Cirugica Brasileira, 2010	RCT	More complications occurred in the parenteral group, although the difference was not statistically significant. Infectious complications were significantly more frequent in the parenteral group ($p = 0.006$)
Li et al. [16]	Journal of International Medical Research, 2018	Meta-analysis	The duration of hospitalisation was significantly shorter in the EN than TPN group. Compared with TPN, EN had a lower risk of pancreatic infection and organ failure.
Yi et al. [17]	Internal Medicine, 2012	Meta-analysis	TEN was significantly superior to TPN when considering mortality, infectious complications, organ failure
Quan et al. [18]	Clinical Gastroenterology and Hepatology, 2011	Meta-analysis	Compared with TPN, EN was associated with a significantly lower incidence of pancreatic infection complications, MOF, and mortality

Abbreviations: RCT (randomized control trial), EN(enteral nutrition), TPN(total parenteral nutrition, MOF(multiple organ failure), TEN(Total Enteral nutrition).

In a comparison of nasogastric and nasojejunal feeding, many trials and meta-analysis (Table 4) showed no significant difference in mortality, complications and length of stay [19]. Nasogastric feeding was cheaper, easy to apply and simpler. Meanwhile, Nasojejunal feeding has to be done by interventional radiologist or endoscopy operator causing a delay in feeding and increment of cost.

Table 4: Comparison of Nasogastric Feeding and Nasojejunal Feeding

Author	Journal, Year	Method	Conclusion
Zhu et al. [20]	Gastroenterology Research and Practice, 2016	RCT	There were no significant differences in the incidences of mortality, infectious complications, digestive complications, or length of hospital stay between NG and NJ nutrition groups. NG nutrition was as safe and effective as NJ nutrition in with SAP
Chang et al. [21]	Critical Care 2013	Meta-analysis	There were no significant differences in the incidences of mortality between NGT and NJT groups. NG feeding is safe and well-tolerated compared with NJ feeding
Singh et al. [22]	Pancreas 2012	RCT	Early enteral feeding through NG was not inferior to NJ in patients with SAP. Infection complications and length of hospital stay were comparable in both groups.
Kumar et al. [23]	Journal of Clinical Gastroenterology, 2006	RCT	Enteral nutrition at a slow infusion is well tolerated by both NJ and NG routes in patients with SAP. Neither NJ nor NG feeding leads to recurrence or worsening of pain in SAP
Eatock et al. [24]	American Journal of Gastroenterology, 2005	RCT	The simpler, cheaper, and more easily used NG feeding is as good as NJ feeding in patients with objectively graded severe AP

Abbreviations: RCT (randomized control trial); NGT (nasogastric tube); NJ (naso-jejunal); AP (acute pancreatitis).

Antibiotics

Many researchers conclude that antibiotics were recommended to be given in severe acute pancreatitis patients who developed sepsis, pancreatic or extrapancreatic infection, infected necrosis systemic inflammatory response [25]. Antibiotic as prophylaxis does not decrease mortality and secondary infection significantly [5], [13]. It is given as prophylaxis when infection marker, such as procalcitonin, IL-6, is detected [25]. The recommended antibiotics in treating severe acute pancreatitis that covers gram-positive (Clostridium) and gram-negative (E. coli, Klebsiella, Pseudomonas, Proteus) as well as anaerobes such as imipenem, meropenem, ciprofloxacin, clindamycin and metronidazole [13], [25]. All these antibiotics have adequate penetration and bactericidal effect in infected pancreatic necrosis. Prolong use of antibiotics have a risk of multi drugs resistance and development of fungal infection which is related to a long hospital stay and poor outcome [5], [25].

Analgesics

Pain is one of the most complained problems of acute pancreatitis patients. Therefore, pain management needs to be given in the first 24 hours to maintain the patient's quality of life. There are many choices of analgesics, such as fentanyl, meperidine, non-steroid anti-inflammatory drugs. Pain management was based on WHO analgesic ladder which consist of 4 steps (Step 1: NSAID, Step 2: low potent opioid ± NSAID ± adjuvant drugs, Step 3: High potent opioid ± NSAID ± adjuvant drugs, Step 4: interventional treatment ± high potent opioid ± NSAID ± adjuvant drugs) [27]. Opioids had been reported in the past study as a trigger of spasm of the sphincter of Oddi but in a recent Cochrane review on five RCTs with a total of 227 patients showed no difference between opioids and other analgesic options regarding the risk of complications or clinically serious adverse events [26]. A meta-analysis that was made by Stigliano et al. concluded there was no credible evidence to avoid the use of morphine in managing pain in acute pancreatitis [28].

Somatostatin and octreotide

Somatostatin and its long-acting analogue octreotide are the inhibitors of exocrine pancreatic secretion and further prevent the release and activation of enzymes. The benefit of these medications is controversial. W Uhl et al. revealed that octreotide had no benefit in the treatment of acute pancreatitis [29]. However, Paran et al., showed that in their study, complication rate was lower in treatment group than in control group (sepsis [24% vs 76%, $p < 0.0002$], ARDS [28% vs 56%, $p = 0.04$]). Therefore, they suggested that octreotide might have benefit in the treatment of severe acute pancreatitis [30].

Surgical management

Surgical interventions are indicated when infected necrosis and gallstone obstruction causing biliary pancreatitis is detected. Delayed surgical intervention was suggested because it was related to lower incidences of multi-organ failure, uncontrolled bleeding and sepsis. Therefore, the recommendation is to delay the surgical intervention until the infected necrosis process stops expanding and by the time, the necrotic tissue will liquify. After it liquefies, percutaneous or endoscopic drainage of the infected collection can be ordered. Because of open necrosectomy was associated with high morbidity and mortality, minimally invasive surgical techniques are preferred as the next step of the Step-up approach if drainage by endoscopy failed [4]. Step up approach which was started with minimal invasive drainage technique and endoscopic necrosectomy, was concluded by Rasch et al. had significant decrement of morbidity and mortality in necrotising pancreatitis compared to primarily surgical intervention [31].

Conclusion

Severe acute pancreatitis is treated conservatively by fluid resuscitation, early enteral feeding, analgesic, and antibiotic. Ringer lactate is the recommended fluid resuscitation. Enteral feeding as early as 48 hours after admission is the recommended protocol. When dealing with pain, non-opioid and opioid can be used in severe acute pancreatitis. Antibiotics are indicated when infection markers are detected. Somatostatin and it's analogue show no benefit in the treatment of severe acute pancreatitis.

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Review Article

Myeloid and Plasmacytoid Dendritic Cells and Cancer – New Insights

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Abstract

Dendritic cells (DCs) use effective mechanisms to combat antigens and to bring about adaptive immune responses through their ability to stimulate näive T cells. At present, four major cell types are categorised as DCs: Classical or conventional (cDCs), Plasmacytoid (pDCs), Langerhans cells (LCs), and monocyte-derived DCs (Mo-DCs). It was suggested that pDCs, CD1c+ DCs and CD141+ DCs in humans are equivalent to mouse pDCs, CD11b+ DCs and CD8α+ DCs, respectively. Human CD141+ DCs compared to mouse CD8α+ DCs have remarkable functional and transcriptomic similarities. Characteristic markers, transcription factors, toll-like receptors, T helpers (Th) polarisation, cytokines, etc. of DCs are discussed in this review. Major histocompatibility complex (MHC) I and II antigen presentation, cross-presentation and Th polarisation are defined, and the dual role of DCs in the tumour is discussed. Human DCs are the main immune cells that orchestrate the immune response in the tumour microenvironment.

Introduction

Since their discovery by Ralph Steinman and Zanvil Cohn in 1973, much information about dendritic cells (DCs) has accumulated in the literature [1]. In 2011 Ralph Steinman was awarded the Nobel Prize in Physiology or Medicine for his contribution in the investigation of dendritic cells and of their importance in initiating the adaptive immune response [2]. The unique function of DCs is their ability to stimulate näive T cells and to be a bridge between innate and adaptive immunity [3]. DCs have highly effective mechanisms to detect and capture antigens and to bring about of adaptive immune responses. Several recent reviews have highlighted similarities and differences in human and mouse DCs [3], [4], [5], [6].

DCs continuously interact with T cells even in the absence of infection. Moreover, DCs presenting self-antigens also interact with T cells in the steady-state [7]. Thus, DCs enforce peripheral T cell tolerance by the continuous presentation of self- or innocuous antigens to T cells in the absence of co-stimulation or activating cytokines. DCs mediate tolerance and silencing, thus preventing unwanted immune reactions to self and environmental antigens. This “tolerogenic” role of DCs is extensively studied in the light of their therapeutic application [2].

In the current review, we present the human DC subsets and some mouse DCs that were thought to be equivalent to human ones and focus on recent advances in human DC development and function.

DC development

In mice, the common myeloid progenitor or macrophage / DC progenitor (MDP) that gives rise to monocytes and macrophages on the one hand and the common DC progenitor (CDP) on the other is localised in bone marrow (BM). The CDPs in BM develop first into pre-DCs (entering blood) that in turn gives rise to classical or conventional DCs (cDCs) and into plasmacytoid DCs (pDCs) [8], [9]. Recent studies showed that cDCs are an independent haematopoietic lineage [9]. The cDC-restricted progenitor is registered in the spleen [10], and Liu et al., defined similar pre-DC populations in the BM and other tissues. In the BM CDPs change to pre-DC, that move using blood flow to peripheral lymph nodes (LN) and to non-lymphoid tissues (NLT), where they differentiate terminally into cDC subsets ($CD11b^+$ DC tissues, $CD4^+CD11b^+$ DCs and $CD8^+$ / $CD103^+$ DCs) [10]. In the periphery, cDCs live briefly and are very plastic, which allows a rapid function in response to antigens [8].

Interferon regulatory factor 8 (IRF8) is a key transcription factor important for the development of $CD8\alpha^+$ cDCs and $CD103^+$ DCs. Basic leucine zipper transcriptional factor ATF-like 3 (BATF3), Id2-GFP and the nuclear factor interleukin 3 (NFIL3) initiate the development of $CD8\alpha^+$ cDCs as well as of $CD103^+$ DCs [11], [12], [13].

In mice, pDCs develop in the BM from multiple progenitor types such as MDP, committed lymphoid (CLP) or myeloid (CMP) progenitors [14]. Pre-PDCs lose their potential to give rise to other cell types (a process called commitment) when maturing [10], [15], [16]. The earliest progenitors suck as CMPs give rise to erythrocytes, granulocytes, megakaryocytes, monocytes, macrophages, myeloid dendritic cells (mDCs) and pDCs [16]. Moreover, pDCs and cDCs developed from CDP or pre-DCs in murine BM, and express FMS-like tyrosine kinase 3-ligand cytokine receptor FLT3 (CD135) triggered by its ligand FLT3L [17], [18] as well as by several transcription factors such as PU.1 and IRF8 [8]. CDP also express macrophage colony-stimulating factor receptor (M-CSFR, CD115) and granulocyte-macrophage colony-stimulating factor receptor (GM-CSFR) [19], [20], and low levels of stem cell factor receptor (c-KIT; CD117) [14], [21], [22]. The development proceeds through immature pDCs in the BM to mature pDCs in blood [22].

In humans, the equivalents of mouse CMPs, CDPs and even pre-DCs are as yet undefined [23]. Human gene expression studies reveal that DCs form a separate cluster from monocytes and macrophages [24], a fact that has also been confirmed in mice [9]. Moreover, in inflammation monocyte-derived dendritic cells (Mo-DCs) were shown to arise from monocytes [25].

Because of their limited availability, human DC subsets have been studied mainly in culture. Commonly, the generation of interstitial DCs and LCs has been achieved in culture with $CD34^+$ hematopoietic progenitor cells (HPCs) with GM-CSF and tumour necrosis factor-alpha (TNFα) [26], [27] with or without IL4 producing Mo-DCs, and that are distinct from DCs developed from CDP [28]. Cells that are developed from whole BM *in vitro* with FTL3L have been used to study cross-presentation [29], [30]. It is established that in $CD34^+$ HPCs GM-CSF / TNF-α-driven culture system, $BDCA3^+$ expression is found on $CD14^+$-derived interstitial DCs. The addition of TGFβ enhances BDCA3 expression on $CD14^+$ DCs (manipulated differentiation towards LCs), whereas IL4 enhances BDCA-3 expression in both $CD14^+$ DCs and $CD1a^+$ DCs (interstitial DC lineage) [26], [31]. Moreover, pDCs, $CD1c^+$ DCs and $CD141^+$ DCs can be derived in vitro by culturing $CD34^+$ HPCs FTL3-L [32]. Identification of early DC precursors in human blood is difficult because all human CD34+ HPC precursors express the DCs activation marker MHC class II antigen. It is reported that human cDCs proliferate in blood or NLT [3], while the pDCs fully develop in BM and then leave it [33].

Human DCs arise from BM precursors such as granulocyte-macrophage DC (progenitors producing granulocytes, macrophages and DCs), and from macrophage-DC progenitors (producing macrophages and DCs), and MDP-derived common DC progenitors restricted to BM (producing cDCs and pDCs). Similarly, to MDPss, CDPs highly express M-CSFR and FTL3R, and low levels of c-KIT, like in mice. CDPs are the precursors of both pre-pDCs and pre-cDCs, cells that are not fully mature. The maturation of pDCs is completed in the BM, and cDCs differentiate in tissues [34], [35].

The common monocyte progenitor through GMP gives rise to blood $CD16^+$ and $CD14^+$ monocytes. The three types of DC, namely cDC1 ($CD141^+$), cDC2 ($CD1c^+$) and pDCs ($CD303^+$) develop therefrom pre-DCs [3], [36], [37]. Differentiated DC subsets and monocytes circulate in peripheral blood and can be found in lymphoid tissue as resident cells. In the skin, $CD14^+$ Mo-DCs, cDC1, cDC2, macrophages and LCs (latter both derived from fetal Yolk sac/liver progenitors) can be detected.

The *genetic control* of DC lineage achieved by distinct transcription factors, particular pattern recognition receptors that lead to the production of specialised secretory products. The development of cDC1 requires BATF3 and IRF8. The development of cDC2 is dependent on IRF4 and Kruppel-like factor 4 (KLF4). The factors ID2 BATF3, and BCL6, associated with cDC development, are expressed at low levels in CDPs. Therefore, the induction of pDC or cDC development deposed on transcription factor expression on CDPs [35].

The origin of pDCs relies on runt-related transcription factor 2 (RUNX2), classic I basic helix

loop helix (bHLH) factors, ZBTB-46, BCL11A, IRF7 and IRF8 [14], [35]. One key transcription factor for the development of pDCs is E2-2 [11]. E2-2 regulates a large pDC gene program, which in turn regulates other key transcription factors for pDC development such as IRF8 and when expressed, it unlocks pDCs differentiation. The loss of E2-2 from mature pDCs converts their phenotype and function into cDC-like phenotype [38].

LCs originate under the control of RUNX3 and ID2 and need IL34 and TGFβ for their development [23], [35].

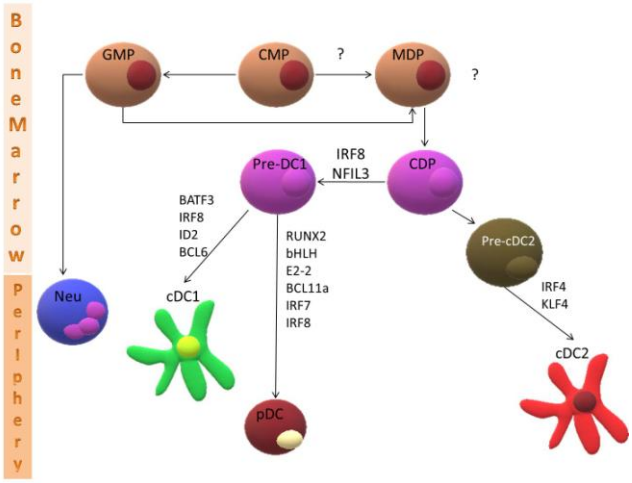

Figure 1: Development of human DCs

DC subsets

There is a great confusion in literature concerning the designation of "myeloid" or 'classical type I DCs (cDC1s)' and type II 'cDC2s'. Some investigators use CD8α$^-$ CD4$^+$ CD205 / DEC205$^-$ CX_3CR1^- CD11b$^+$) for type 1 cDCs and CD8α$^+$ CD4$^-$ CD11b$^-$ CD205 / DEC205$^+$ CX_3CR1^+ and C type lectin domain family 9 member A (CLEC9A)$^+$ for type 2 cDCs in mice [39?], [40], [41]. Moreover, CD1c / BDCA1$^+$CD11c ^{hi}CD123$^-$ are named as mDC1, and CD141/BDCA-3+CD11c^{lo} are named as mDC2 in humans [42], [43]. On the contrary, other investigators use the term of cDC1 for CD8α$^+$ or CD103$^+$ DCs and cDC2 for CD11b$^+$ DCs in mouse [44] and CD1c$^+$ (BDCA1$^+$) for cDC2 and CD141$^+$ / BDCA3$^+$ for cDC1 type in humans [37], [45].

Boltjes and van Wijk use the signature of CD1c$^+$ and CD141$^+$ DCs without determination of DCs type I or II for human DCs (3). Similarly, Collin et al., applied CD1c$^+$ (Clec7A$^+$ Clec6A$^+$) and CD141$^+$ (Clec9A$^+$ XCR_1^+) as major markers for human cDC types, and CD11b$^+$ (tissues) and CD4$^+$CD11b$^+$ endothelial cell-selective adhesion molecule (ESAM)$^+$ (lymphoid) and CD103$^+$ (tissues), CD8α$^+$ (lymphoid) Clec9A$^+$ XCR_1^+ Langerin$^+$ for mouse cDCs [37].

In their excellent review, Reynolds and Haniffa concluded [45] that mouse DCs expressing CD8α in the spleen and CD103 in NLT that are equivalent to human CD141 (thrombomodulin, BDCA-3) DCs [46], [47], [48] are type 1 cDC. The type 2 cDC phenotype in mice are LIN$^-$MHC I ^{hi} CD11c$^+$ CD11b$^+$, and this fraction also includes Mo-DCs and macrophages [25]. Therefore, mouse CD11b$^+$ DCs and human CD1c$^+$ DCs have been regarded as type cDC2 [45]. We also accept their definition of cDC1 and cDC2.

In Mice

All DC subsets in mice have corresponding human counterparts. Murine cDCs or mDCs have been traditionally categorised into two distinct subsets: the 'cDC1s' for CD8α$^+$ and CD103$^+$ (tissues) DCs and cDC2s for CD11b$^+$ DCs and CD172a$^+$ DCs that lack CD8α marker (49). The third type of murine DCs is pDC, which retains their original name [40]. Both subpopulations can be found in LT, including spleen, LNs, BM, and NLT [13], [49].

Table 1: TLR expression in mouse DCs

	cDC1	cDC2	pDC
Phenotype	NLT: CD8α$^+$, CD11c$^+$, MHC II$^+$, CD103$^+$, CLEC9A(DVGR)$^+$, XCR1$^+$, CD11b$^-$ LT: CD8α$^+$, MHC II$^+$, CLEC9A$^+$, XCR1$^+$, CD205$^-$ / DEc205$^+$, SIRPa (CD172a)$^-$, CD11b$^-$, CD4$^-$	NLT: CD11b$^+$, CD103-, MHC II+, XCR1$^+$ LT: CD11b$^+$, CD4$^+$, CD8α$^-$, SIRPa (CD172a)$^+$, CD11c$^+$	LT: CD11c$^+$, MHC II$^+$, CD317 (BST2)$^+$, Siglec H$^+$, CD45R(B220)$^+$, CD11b$^-$, Ly-6C$^+$
TF	TRF8, BATF3, ID2, Bcl6	TRF4, Notch2, Klf4	IRF8, E2-2, RUNX1, Bcl11a
Location	LT, NLT	LT, NLT	LT, NLT, BM
TLRs	TLR3 (TLR6, TLR8, TLR1, TLR2, TLR4) (TLR4 stimulates Th1 immune response of IL12)	TLR8 (TLR (7)9) (TLR2 stimulates Th2 responses)	TLR7 (4,8) (TLR1,2) TLR9
Cytokines	TGFβ (induction of Treg)		
T-cell interactions	CD8$^+$T cell responses cross presentation with MHC class I molecules; Ag and immune complexes	Th2, Th17 (allergies), (fungal) presentation of Ags with MHC class II molecules	CD4$^+$Th2 - ? and CD8$^+$Th1
Biological roles	Cross presentation - CD8$^+$T cell response	CD4$^+$T cells priming	Anti-viral response high production of IFNα / β, antiviral response

CD8α$^+$ and CD103$^+$ cDC1s. The CD8α$^+$ cDCs have been found in murine lymphoid organs. An alogous DC population exists in NLT, and these cells express CD103 integrin marker ($\alpha_\varepsilon\beta7$). CD8α$^+$ and CD103$^+$ cDC1s are the best-characterised cDC subsets, conserved through evolution, and the chemokine receptor XCR1 (important for cross-presentation) was identified first on these DCs [46]. CD8α$^+$ cDCs are highly efficient in cross-presentation of exogenous antigens on MHC-I molecules to CD8$^+$ T cells in CD1d context, a fact that is critical for immunity against viruses and bacteria [46] and in antitumor immunity [23]. Therefore, CD8α$^+$ cDCs can

activate and polarise invariant natural killer T-cells towards the production of T helper 1 (Th1) and Th2 cytokines and CD8α$^+$ cDCs secrete IL12p70 [50], [51]. Skin-derived CD103$^+$ cDCs also have cross-presentation activity using MHC class I molecules to present antigens to CD8$^+$ T lymphocytes [13].

The phenotype of CD8α$^+$ cDCs in LT has been delineated by the expression of CD11c^{hi}, MHC class II^{hi}, XCR1$^+$, CLEC9A$^+$, CD8α$^+$, CD4$^-$, CD205 (DEC205)$^+$ and that of CD103$^+$ DCs in NLT (analogous to CD8α$^+$ in peripheral tissues) – by CD11c^{hi}, MHC class II^{hi}, XCR1$^+$, CLEC9A$^+$, CD8α$^+$, CD4$^-$ CD11b$^-$ and CD103$^+$ [52].

CD11b$^+$ DCs and CD172a$^+$ DCs (cDC2s). A second and largely complementary DC subset can be distinguished in lymphoid organs by the expression of CD4 [12]. CD4$^+$ CD11b$^+$DCs cDC2 are considered to be poor cross-presenters in vivo but are more efficient while endogenous cytosolic antigens are presented on MHC class II to CD4$^+$ T cells [53]. Moreover, CD4 is not expressed on the complementary peripheral subset (in NLT), which can be instead identified by the expression of CD11b. CD11b$^+$ DCs are mostly defined by the absence of activities associated with CD8α$^+$ DCs [41]. They are inefficient at cross-presenting antigens and do not produce IL12. In contrast to CD8α$^+$ DCs, CD11b$^+$ DCs are superior in the induction of CD4$^+$ T cell immune response because of their prominent expression of MHC class II machinery [13], [53], [54].

CD11b$^+$ DCs prevail in lymphoid organs except for the thymus. They can also be detected in NLT. In the spleen DCs can be classified into three populations of resident cDCs: CD8α$^+$ DCs, endothelial cell-selective adhesion molecule (ESAM)^{hi} CD11b$^+$ cDCs and ESAM^{lo} CD11b$^+$ cDCs [23], [54]. Both CD11b+ cDCs in NLT and LT-resident DCs are occupied in the antigen presentation in MHC II-restricted manner [52], [53].

Table 2: TLR expression in human DCs

	cDC1	cDC2	pDC
Phenotype	Blood: CD141$^+$(BDCA-3)$^+$; LT: HLA-DR$^+$, CLEC9a$^+$, αCR1+, CD11c$^+$, CD11b$^-$, CD123$^-$	Blood – LT: CD1c(BDCA-1)$^+$, HLA-DR$^+$, CD11b$^+$, CD11c$^+$, SIRPa(CD172a)$^+$	Blood: HLA-DR$^+$, CD303 (BDCA-2)$^+$, CD304 (BDCA-4)$^+$, CD123 (IL-3R)$^{++}$, CD85g (IαT7)$^+$, CD11c$^-$
TF	IRF8, BATF3, ID2, Bcl6	TRF4, Klf4	IRF8, E2-2, RUNX2, Bcl11a, IRF7, bHLH
Location	Blood, LT, NLT	Blood, LT, NLT	Blood, LT
TLRs	TLR3 (TLR1, TLR2, TLR4), TLR5,6,8,10	TLR8 (TLR7,9) TLR4	TLR7, 9 (endosomes) (TLR1,6,10 low) facilitated IFN type I expression
Cytokines			IFN α (TLR7/9) IFN III
T-cell interactions	CD8$^+$T cell responses cross presentation with cellular Ags and immune complexes Th1	Th2(allergies), (Th17 fungal)	CD4$^+$Th2 CD8$^+$Th1 Granzime B secretion
Biological roles	Anti-tumor, Anti-viral	CD8$^+$Th1 (IL-12) Regulation of immune responses; Anti-parasites, anti-bacterial	Anti-viral, Anti-fungal, Anti-tumor

Conceivably, CD11b$^+$ESAM^{hi} cells are also CD11c^{hi}MHC class II^{hi}, CX$_3$CR1^{low}, CD11b$^+$ and ESAM^{hi} and are associated with CD4$^+$ DC responses, while CD11b$^+$ESAM^{lo} cDCs are CD11c^{hi}MHC class II^{hi}, CD103$^-$, CD11b$^+$ and CD24$^-$ and produce inflammatory cytokines such as IL6 [55] and IL23 [3] upon toll-like receptor (TLR) triggering [40], [54]. CD11b$^+$ DCs secrete also pro-inflammatory chemokines CCL3, CCL4 and CCL5 after TLR stimulation [56]. The two DCs types are NOTCH2-dependent [54], and the latter type is also IRF4-dependent [31].

In Humans

Historically, human DC subsets have been defined concerning some phenotypic markers and anatomic location. Human DCs express high levels of MHC class II (HLA-DR) and CD45$^+$ and lack typical lineage markers CD3 (T-cell), CD19 / 20 (B-cell) and CD56 (natural killer cell). The classical description of human DCs is HLA-DR$^+$ lineage$^-$ including myeloid and plasmacytoid subsets [37], [57].

Human DCs consist of heterogeneous with distinct functional specializations categorized into, blood and lymphoid organ-resident DCs comprising CD11c$^-$ CD123^{hi} CD303 (BDCA2)$^+$ CD304 (BDCA4)$^+$ or pDCs secreting type I IFN. The other subset in this category is mDC that has been further delineated into a CD141 (BDCA3)^{hi} CLEC9A$^+$ or the major cross-presenters to CD8 T cells [47] and CD1c (BDCA1)$^+$ population [57]. Secondly, migratory DCs also exist that reside in NLT and response to danger signals migrate to lymphoid organs. Three migratory DC subsets belonging to myeloid lineage and expressing CD11c have been described in NLT including the skin. These are epidermal LCs (langerin$^+$ CD1a^{hi} DC-SIGN$^+$), interstitial (CD1a$^+$ DC-SIGN$^-$) and CD14$^+$ (CD1a$^-$ DC-SIGN$^+$) DCs [27]. The latter subset is linked to human monocyte / macrophage populations [27], [48], [58]. Additionally, BDCA3+ CLEC9A+ DCs, capable of cross-presentation and IFNλ production have been identified in the human dermis [48]. Finally, inflammatory DCs differentiate from peripheral blood monocytes [59].

Concerning anatomic location human DCs are blood DCs (pDCs and mDCs), and mDCs have been separated into two subsets, namely BDCA3 / CD141$^+$ DCs and BDCA1 / CD1c$^+$ DCs [17]. These three DC populations are found in all lymphoid organs and are considered as resident DCs [47], [58], [60]. Conceivably, immature cDCs leave the BM, disseminate via blood to lymphoid organs and peripheral tissues, where they achieve resident and migratory phenotype [61]. The resident cDCs in lymphoid tissue stay in an immature state unless they get activation signals and become mature [62]. The migratory (mature) cDCs travel from the tissues to the LNs via afferent lymph and mature upon reaching regional LNs [63]. Migratory cDCs transport and also present peripheral self-antigens to induce T-cell tolerance [64].

In skin, liver, lung and intestine two main populations of cDCs exist, namely CD1c$^+$CD1a$^+$ and

CD141$^+$ CLEC9A$^+$ DCs [48]. These DCs migrate to draining lymph nodes and are called migratory DCs with mature phenotype [58]. In human mucosa tissues, additional DC subsets: LCs and CD14$^+$ DCs in the skin and vaginal mucosa and CD103$^-$ CD172a$^+$ DCs in the intestine [65].

Some of the used phenotypic markers for DC definition can be expressed on several cell types or can be changed upon activation [17]. For example, CD141 is also upregulated after activation on pDCs and on CD1c$^+$ DCs (except on traditional cDC1s) [42]. Intermediate levels of CD141 / BDCA-3 (thrombomodulin) are expressed on tissue CD14$^+$ DCs [66], [67]. BDCA3 is a cell surface transmembrane glycoprotein that is predominantly expressed on vascular endothelial cells and has anti-coagulant activity and anti-inflammatory function [68].

Another example is the rapid down-regulation of the classical cDC1s marker CLEC9A (restricted to CD141$^+$ DCs) during maturation [69]. Moreover, using the classical hallmark of DCs (dendritic morphology, migratory capacity and the ability to stimulate naïve T-cells) one can distinguish DCs from macrophages and monocytes [17].

Finally, transcriptomic studies are useful to confirm DC identity. Homology between human (XCR1$^+$ CD141$^+$) cDC1s and mouse (CD8α$^+$ / CD103$^+$) cDC1s is demonstrated by comparative transcriptomic, phenotype and functional analyses [45], [47], [48], [60]. It has been suggested that pDCs, CD1c$^+$ DCs and CD141$^+$ DCs represent distinct lineages and are equivalent to mouse DC subsets pDCs, CD11b$^+$ DCs and CD8α$^+$ DCs, respectively [48], [65]. The origin of CD141+ DCs is maintained by BATF3 transcription factor [70].

CD141$^+$ BDCA3$^+$

The essential role of DCs in the induction and regulation of immune responses to pathogens, self-antigens, and cancer has been well established [71]. The characterisation of human DC subsets is difficult because of their rarity, the lack of distinctive markers, and limited access to human tissues [47]. Human blood DCs comprise ~ 1% of circulating peripheral blood mononuclear cells defined as antigen-presenting leukocytes that lack other leukocyte markers such as CD3, CD14, CD15, CD19, CD20 and CD56, and express high levels of MHC class II (HLA-DR) molecules [17].

Gene -expression profiling and hierarchical clustering data have indicated that CD1c+ DCs and CD141+ DCs have a common origin and represent two different stages of a similar subset [72]. However, CD1c+ DCs and CD141+ DCs have unique gene -expression profiles differentiating them from monocytes and mo-DCs [42], [72].

Human CD141+ (thrombomodulin+) DCs comprise only ~ 0.03% of human peripheral blood mononuclear cells and 10% of human blood mDCs [42]. Moreover, CD141+ DCs are present at small numbers in tissues, and other cells also express this marker although at a low level (CD14+ DCs, CD1c+ DCs and monocytes) therefore, they are difficult to identify [47]. Their differentiation from CD1c+ DCs by flow cytometry has been made possible by the fact that CD141+ DCs express less CD11b and CD11c [37].

An efficient protocol for the isolation of highly pure CD141+ and CD1c+ mDCs from leukapheresis products from normal and healthy volunteers has been reported [73]. In an extensive and excellent study [48] isolate human DC subsets from human blood and skin (from mammoplasty), lung and liver (from peritumoral tissue), tonsil and dermatopathic LNs (from tonsillectomy and LN diagnostic excision). DCs can be isolated by fluorescence-activated cell sorting (FACS) analysis and are studied by flow cytometry. FACS-purified dermal DCs are cultured with TLR ligands, and cytokine secretion of DCs has been studied. Cross-presentation and ELISpot assay have been done. By quantitative real-time polymerase chain reaction, the following genes have been examined: *GAPDH*, *XCR1*, *TLR3*, and *CADM1*, and the results suggest that CD141^{hi} DCs are functional homologs of mouse CD103$^+$ DCs and that these are distinct from the major population of human CD1c$^+$ DCs and mouse CD11b$^+$ DCs.

The main characteristics of the human CD141$^+$ DC subset have been a high expression of TLR3 (but not of TLR4, 5, or 7) and the production of IFNβ, CXCL10 and IL12p70. Another characteristic of this subset is the induction of superior Th1 response after activation with polyinosinic–polycytidylic acid (poly I:C) and the induction of Th2 response to a lower degree, therefore, CD141$^+$ DCs are the major subset involved in the induction of CTL responses against tumours and viruses [31]. Detailed functional analyses of human CD141$^+$ DCs with impact of their role in the induction of Th1 and Th2 immune responses, TLR stimulation, TNFλ production, MHC class I and class II antigen and MHC class I cross-presentation, cytokine production and their origin in culture by CD34$^+$ HPCs has been reported [17], [23], [31], [74], [75], [75], [76], [77], [78].

It has been shown that HLA-DR$^+$ lineage$^-$ cells in human blood and tissues comprised two fractions CD14$^-$ and CD14$^+$. The CD14$^-$ fraction is further separated by CD141 and CD11c expression by flow cytometry. CD141$^+$ cells in the blood have low CD11c expression, and in tissues, they are CD141^{hi} CD11c^{lo} [48]. CD14$^+$ cells in tissues express CD141, but they correspond to CD14$^+$ “interstitial type DCs” that do not have very potent allo-stimulatory or cross-presenting capacity [27], [79]. Tissue CD141^{hi} (isolated from human skin, liver and lung) and also blood CD141$^+$ show higher expression of the cross-presentation signature *CLEC9A*, *TLR3*, *CADM1*, and *XCR1*

(whereas CD14$^+$ and CD1c$^+$ express these at much lower levels) and consequently migrate in response to XCL1. CD141^{hi} DCs migrate in response to XCL1 and show the highest expression of Flt3 and CLEC9A in blood, skin and lung whereas CD14+ DCs intensely express M-CSFR and CX3CR1, markers associated with monocyte and macrophage lineages.

CD1c$^+$ DCs show lower expression of FLT3 and CLEC9A and intermediate levels of M-CSFR and CX3CR1 than CD141^{hi} DCs [48]. Reynolds and Haniffa suppose that blood CD141$^+$ DCs are the precursors of immature CD141^{hi} DCs, before to acquire CD1a, CD1c, activation antigens, and CCR7. Moreover, migrating CD141^{hi} DCs represent the CD1a and CD1c mature fraction [45], [48].

Human CD141$^+$ DCs have been compared to mouse CD8α$^+$ DCs, and remarkable functional or transcriptomic similarities have been established [24].

Both subsets share the expression of CLEC9A [80], [81], NECL2 [71], [82] and of the chemokine receptor XCR-1 [46], and are extremely rare in blood but are present in the T cell areas of LT [82].

The recognition of pathogen-associated molecular patterns by DCs involves pattern recognition receptors and TLRs [73]. To date, 10 TLRs (TLR1-10) have been identified [83]. Human CD141$^+$ DCs have strong expression of TLR3 and do not express TLR4, -5, and -7 [47], [84]. Mouse CD8α$^+$ DCs also have high expression of TLR3 and lacked TLR7 and TLR10 [47]. However, unlike human CD141$^+$ DCs, mouse CD8α$^+$ DCs express TLR4 and 9 [85]. Human CD141$^+$ DCs express TLR10 (absent in mice) at higher levels than CD1c+ mDCs [84]. TLR10 belongs to the TLR1 subfamily like TLR1, -2, and -6 [73] and its function remains to be determined.

The effect of pathogen-TLR recognition is the induction of cytokine and chemokine production [86]. Like human CD141$^+$ DCs, mouse CD8α$^+$ DCs trough high TLR expression produce IFN-β in response to poly I:C [87].

Mouse CD8α$^+$ DCs produce IL12p70, and induce Th1 cytokines IL2 and IFNγ [47], [88]. Most importantly, human CD141$^+$ DC and mouse CD8α$^+$ DCs have the cross-presenting capacity [45]. Both subtypes express high levels of MHC class I [53] that together with TLR3 and CLEC9A (sensors of necrotic cells) regulate cross-priming [89].

Despite their similarities, there are notable differences between human CD141$^+$ DCs and mouse CD8α$^+$ DCs. Murine cDC1 differentiation requires the expression of *BATF3* and *IRF8* [47], [60] that are not selectively expressed by CD141$^+$ DC. Human DCs are isolated mainly from blood, while mouse DCs are generally isolated from spleen [45].

CD1c$^+$ mDCs

Human CD1c$^+$ DCs are the major population of mDCs in blood, peripheral tissues (NLT) and LT [37]. CD1c$^+$ DCs comprise about 1% of all mononuclear cells in blood [37]. In kidney tissue there are about four times more BDCA1$^+$ (CD1c$^+$) DCs than BDCA2$^+$ (pDCs) [90]. Human CD1c$^+$ DCs classified by 45, as cDC2 phenotype are defined as lineage$^-$ MHC class II$^+$ (HLA-DR$^+$) CD14$^-$ CD16$^-$ CD11c$^+$ CD1c$^+$, a definition, they share with *in vitro* Mo-DCs [45]. Human peripheral blood and murine cDC2 additionally express CD11b, CX3CR1 and SIRPα (CD172) [91] similarly to Mo-DCs [45].

Human CD1c$^+$ DCs are identified by the commercial antibody BDCA1 [42]. Human blood CD1a$^+$CD11c$^+$ cells express high CD1c (BDCA1) levels and can rapidly acquire an LC phenotype [92], [93]. It has been shown that blood BDCA1$^+$ DCs treated with thymic stromal lymphopoietin (TSLP) and TGFβ induce expression of high numbers of LCs (CD1a$^+$ CD207$^+$) [94].

Human tissue CD1c$^+$ DCs appeared to be more activated than blood CD1c$^+$ DCs and express co-stimulatory molecules such as CD80, CD83, CD86, and CD40, they lose homing receptors CLA and CD62L, while preserving CCR7 responsible for tissue homing [37], [93]. CD1c$^+$ DCs are also detected in T-cell areas of LNs, tonsils and spleen [47], human kidneys [57], [90], skin [94], and lung [95].

The pathogenic role of CD1c$^+$ DCs in human disease is not clear, but they have been found to accumulate in chronic kidney disease [57], atopic airway asthma and are reduced in number in non-small cell lung cancer [95] and atopic disease [96]. CD1c$^+$ DCs express a wide range of lectin receptors, TLRs (TLR2, TLR3, TLR4, TLR5, TLR7, TLR8) and other pattern recognition receptors [73], [97] that are necessary for antigen uptake, transport and presentation.

Human and mouse cDC2 share a similar cytokine production profile, which includes

IL6, IL23 and IL1β, TNFα, IL8 and IL10 [73], [79]. This cytokine profile implies a dual role of CD1c$^+$ DCs in Th1, Th2 and Th17 sensitisation.

Using a new isolation protocol Hemont et al., studied human CD141$^+$ and CD1c$^+$ mDCs concerning their TLR expression pattern and TLR ligands that determine DCs cytokine and chemokine expression. The average value is 1084 ± 580 CD1c$^+$ mDCs and 169 ± 81 CD141$^+$ mDCs / 1x10^6 peripheral blood mononuclear cells [73]. CD1c$^+$ mDCs from human peripheral blood, BM and tonsils, and LT cross-present soluble antigens and prime CTLs, and are most potent human IL12 producing DCs as compared to CD141$^+$ mDCs. CD1c$^+$ mDCs perform best upon TLR4 and TLR8 stimulation, whereas CD141$^+$ mDCs

require agonists of TLR3 and TLR8 [98]. It has been shown that the immune reaction against *Escherichia coli* and Gram-negative bacteria in humans is most efficient by Mo-DCs that phagocytised it and are the main producers of TNFα, IL8 and IL6. $CD1c^+$ DCs phagocytise *Escherichia coli* to a lesser extent than Mo-DCs and are weaker producers of cytokines in response to TLR4 ligand whereas $CD141^+$ DCs are not responsible for processing and presentation of bacterial antigens [99]. Moreover, $CD1c^+$ DCs exhibit a tolerogenic phenotype by secreting a high level IL10 and expressing CD25 and IDO molecules on their surface [99], [100], [101].

pDCs

pDCs were described first in human LNs by Frederick Siegal et al., [102] and later in mice by Asselin-Paturel et al., [111]. Recently, pDCs have been regarded as a part of the DC family [100], but cells with pDC features have been known as T-cell associated plasma cells or as plasmacytoid T cells, or as natural interferon-producing cells for several decades [101]. These cells produce 200 to 1,000 times more IFNα and IFNβ than other blood cells in response to viruses [102]. pDCs produce IFN type I after engagement of their TLRs, namely TLR7 and TLR9 of viruses or self-nucleic acids [103].

The markers usually used to identify pDCs in mice are $CD11c^+$, $B220^+$, $Ly6C^+$, bone marrow stromal antigen 2 (BST2) and sialic acid-binding immunoglobulin-like lectin H $(SIGLEC\text{-}H)^+$ [52]. Mouse pDCs also express CD8α and like human pDCs express CD4 and MHC class II. Mouse pDCs also express the chemokine receptor 9 (CCR9), Ly49Q SCA1 [18].

Human pDCs

In contrast to mouse pDCs that show intermediate levels of $CD11c^+$ expression, human pDCs are $CD11c^-$ [18]. BST2 is fairly expressed on pDCs and plasma cells in steady-state and upon activation has been found on many cell types [104]. The SIGLEC-H expression is mainly confined to pDCs [105]. In humans, the origin of pDCs is not well understood because of the lack of reliable markers, paucity of DCs in blood, and the limited access to human tissues [60]. The typical phenotype of human pDCs is LIN^- $CD11c^-$ $CD123$ $(IL\text{-}3R\alpha)^+$ [22]. It has been shown that $CD141^+$ cDCs develop after culturing $CD34^+$ HPCs with c-KIT, FLT3L, GM-CSF, and IL4 [60], and after stromal cell addition to the former cocktail, pDCs develop [106]. Importantly, DCs that arise from stromal cell cultures resembled $CD1c^+$ cDCs, $CD141^+$ cDCs and pDCs obtained from peripheral blood as determined by gene expression, surface phenotype, and cytokine production [14]. Stromal cell culture has been used to support the development of HPCs into three major human DC subsets, and into monocytes, granulocytes, NK and B cells [107].

Genome-wide analysis reveals that the gene expression profile of pDCs is closer to that of cDCs than to lymphocytes or myeloid cells [24]. After stimulation human pDCs can differentiate into cDCs [108]. PDCs are distinct from cDCs and possess features of lymphocytes [14]. The morphology of pDCs in the steady-state is that of a secretory lymphocyte [108], and their circulation/localisation is different from that cDCs resembling that of lymphocytes – pDCs matured in BM and then circulate in the blood and are seeded in the secondary lymphoid organs and thymus [33], [61]. Concerning viability pDCs are long-lived, as compared to cDCs and finally, pDCs share some molecular features of B lymphocytes (expression of CD45RA, MHC class-II molecule and BDCA-2) [109], [110].

Immature or resting pDCs are round in shape and are named pre-pDCs [108] with low expression of MHC class II and low T cell co-stimulation [63]. In peripheral tissues, pDCs are very low in number [111]. By secreting IFN I, pDCs can influence innate (e.g.NK cells) or acquired (e.g. cDCs and B cells) immunity [101]. At the site of inflammation, pDCs achieve DC morphology, proliferate and reach draining LNs (100). Mature pDCs secrete IFN, acquire a dendritic morphology and show up-regulation of MHC and T-cell co-stimulatory molecules [111]. Mature pDCs can present antigen via MHC I and MHC II to $CD8^+$ T cells and $CD4^+$ T cells, respectively [112]. It has been supposed that pDCs participated mainly in antigen presentation and immunomodulation at sites of inflammation rather than in antigen transport to draining LNs for T-cell priming [63].

DC MHC Class I and MHC Class II Antigen Presentation. Cross-presentation

Endogenous MHC I antigen presentation

MHC class I molecules are expressed on the plasma membrane of all nucleated cells and until recently were considered to present endogenous peptide-derived antigens from dead and dying cells and to preserve cognate T-cell epitopes [113]. The MHC class I molecule consist of two chains – a heavy chain and a light chain called β2-microglobulin. The genetic nucleotide polymorphisms of the heavy chain ensure plenty of peptide-binding regions for antigenic proteins [114].

Antigen presentation consists in two

processes: Firstly, capturing and processing of precursor polypeptides from the extracellular compartment, and second the resulting T-cell priming, proliferation and consequent cytokine secretion after the recognition of MHC-peptide complexes by T-cells [64].

The classical MHC I presentation pathway consist of three steps. Endogenous peptides (with errors appearing during protein synthesis) are attached with ubiquitin to be degraded in the cytosol by the proteasome, a process called antigen processing [115]. Some of these degradation products are required for MHC I attachment and are therefore delivered to the endoplasmic reticulum by the transporter associated with antigen processing, and are loaded onto newly formed MHC class I molecules [116]. In the endoplasmic reticulum longer, peptides are additionally degraded (trimmed by ERAP – endoplasmic reticulum aminopeptidases) – via endoplasmic reticulum-associated degradation and loaded onto MHC I molecules [117]. This process is called antigen loading. MHC I-peptide complexes are then exported to the cell surface through the Golgi complex (along the secretory pathway) [113]: antigen transport. T-cells use their TCR to recognise antigenic peptides presented on MHC molecules on the surface of antigen-presenting cells. MHC class I molecules are recognised by CD8 T cells [118].

MHC I cross-presentation

Cross-presentation is considered to be the presentation of acquired exogenous antigens on MHC class I molecules that prime CD8+ T cell responses [119]. Therefore, DCs have to be infected with a virus or take other exogenous antigens, to break foreign peptides through its mechanism of "endogenous antigen processing" and to present the exogenous antigens via MHC I pathway [119]. This mechanism of presentation of exogenous antigens by the machinery of endogenous antigen presentation via MHC I is termed cross-presentation [113]. Exogenous antigens are internalised through micro- or macropinocytosis, receptor-mediated endocytosis or phagocytosis. To be cross-presented, these antigens go through the same three steps that comprised the endogenous pathway i. e. processing, loading and transport [113].

Cross-presentation represents an important pathway for the initiation of immune responses against tumours and viruses. Efficient presentation of exogenous antigens is realised by some other cells such as macrophages, B lymphocytes, liver sinusoidal endothelial cells, neutrophils and DCs [120]. B lymphocytes load antigens on MHC I and MHC II molecules but are less efficient presenters than DCs [121]. Neutrophils can cross-prime CD8+ T cells by MHC I-peptide complexes and use the vacuolar pathway but die within hours [122]. Liver sinusoidal endothelial cells express low levels of MHC II molecules but are unable to activate T lymphocytes [123]. Macrophages can engulf pathogens, form phagolysosomes can initiate proliferation and differentiation of CD8+T cells, but their effectiveness is restricted because of poor migratory properties [124]. Immature DCs phagocytose antigens and upon maturation present them complexed on MHC I molecules [120]. Mature DCs have the low endocytic capacity and express high levels of co-stimulatory molecules [120]. DCs suppress lysosomal proteases and inhibit lysosomal acidification via NADPH oxidase 2-mediated alkalization of phagosomes and endosomes and thus maintain exogenous antigens for a long time ready for cross-presentation (delayed antigen degradation) [125]. Delayed antigen degradation allows antigens to move into the cytosol through channels such as SEC61 [126].

During the first step (antigen processing), two main tracks are – "available cytosolic" and "endocytic", through which the exogenous peptides are degraded and loaded [113]. In the "cytosolic" track antigens are transported from endosomes to the cytosol for processing, loading in the ER, and transport of MHC I-peptide complexes to the plasma membrane following the secretory pathway [30]. In the "endocytic" track processing (TAP-independent) and loading occur in the endosome, and MHC I molecules are recycled from the plasma membrane. The MHC I complex transported back to the plasma membrane from the endosome. TLRs induce MHC I molecules from the intracellular pool to phagosomes [30], [127].

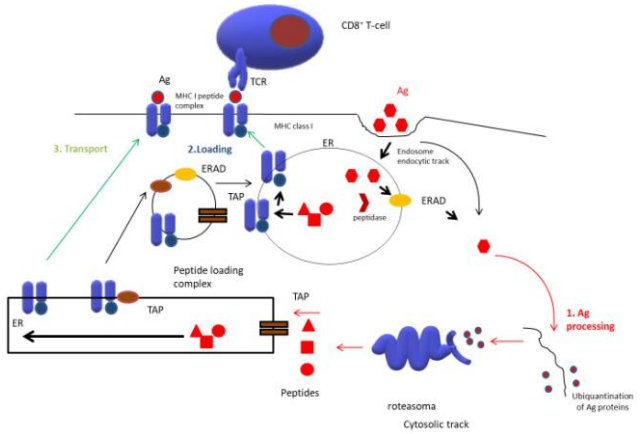

Figure 2: MHC class II antigen presentation

The new understanding of cross-presentation has been built from data obtained from experiments in culture with Mo-DCs, GM-CSF cultures (GMDCs) and FTL3L-derived DC cultures [30]. The FTL3L-derived DCs in culture express *RAB43*, a molecule necessary for cross-presentation in *in vivo* cDC1s [128] and therefore are more suitable for studying cross-presentation than GMDCs or Mo-DCs.

In mice, the CD8α$^+$ DCs (in lymphoid organs) are considered to be the dominant cross-presenting APC [129], but CD103+ DCs and inflammatory DCs are cross-presenters in certain conditions [130]. In

humans, one study shows superior cross-presentation of unstimulated CD141$^+$ cDCs compared to CD1c$^+$ cDC and pDC [46]. Others report that unstimulated CD141$^+$ cDCs are unable to cross-present [47]. Recent studies have revealed that, with the proper stimuli, all human DC subsets can cross-present in vitro [127]. However, CD141$^+$ DCs are shown to be the most efficient cross-presenting subset following poly (I: C) stimulation, when using necrotic cell-associated antigens [47], antigens delivered by Fc$_\gamma$ receptor targeting [3], or antigens delivered to endosomes / lysosomes [131]. The ability to cross-present necrotic cell-derived antigens depends on the selective expression of ClEC9A on CD141$^+$ DCs, a dead cell receptor that acts through delivery to both MHC class I and II [16], [47].

Haniffa et al., 2012 [48] tested the cross-presentation ability of human skin (tissue) CD141^{hi} DC, CD1c$^+$ DC, CD14$^+$ cells and epidermal LCs in comparison to CD141$^+$ DCs, CD1c$^+$ DCs, CD14$^+$ monocytes, and of (in vitro-derived) Mo-DCs and Mo-LCs obtained from human blood. It has been established that in blood, only CD141$^+$ DCs were able to cross-present efficiently, and require TLR3 stimulation with poly (I: C) or exposure to a maturation cocktail (containing poly (I: C), LPS, IFNγ, IL1β, TNFα and IFNα). Mature Mo-DCs and Mo-LCs are also able to cross-present upon exposure to maturation cocktail but are refractory to TLR3 stimulus. In the skin, superior cross-presenting capacity has been found in CD141^{hi} DCs in the absence of stimulation, compared to all other DC subsets, including LCs. CD1c$^+$ DCs show a little ability to cross-present [48].

In general, “resident” DCs are those in LN and other lymphoid organs and reside there during their entire life span. Moreover, the migratory DCs are that in peripheral tissues (NLT), they can capture antigen, mature, and migrate to draining LNs [33], [47]. In human axillary LNs there have been defined three subsets of blood-derived “resident” DCs namely, BDCA1$^+$ DCs, Clec9A$^+$ cDCs and BDCA4$^+$ pDCs, and three subsets of skin-derived migratory DCs, namely LCs, CD1a$^+$ DCs and CD14$^+$ CD1a$^-$ DCs [58]. It has been shown that resident DC subsets induce Th1 and Th2 polarisation and are powerful in cross-presentation [58].

In vivo cross-presentation occurs in secondary lymphoid organs, where resident CD1c$^+$ cDCs, CD141$^+$ cDCs, and pDCs display equal cross-presenting capacity [59]. Usually, antigens are captured by migratory cDC1s (CD8α+ DCs; CD141$^+$ cDC) or cDC2s (CD11b+; CD1c$^+$ cDC) at the site of infection (NLT). Migratory cDCs migrate to regional LNs, where they can prime naïve CD4$^+$ and CD8$^+$ T cells through MHC: TCR contact. Migratory cDCs transfer antigen to resident cDC1s through cross-dressing (see below). Resident cDC1s are helped through CD40 / CD40L from CD4+ T cells and can prime naïve CD8+ T cells via MHC I: TCR interaction [132].

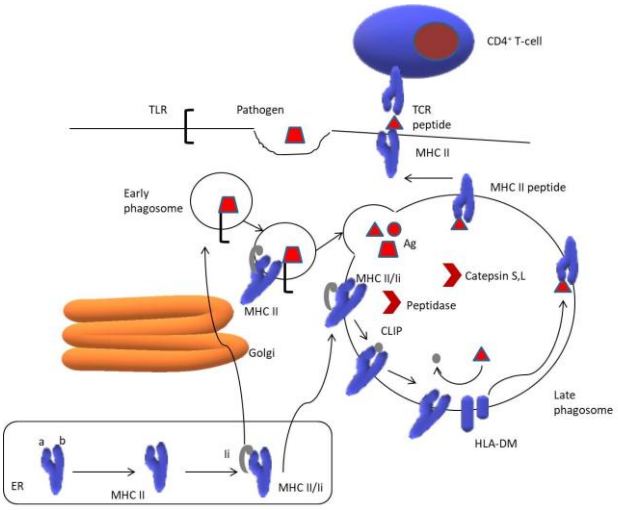

Figure 3: Encountering of antigen at the site of infection; cross-presentation occurring in secondary lymphoid organs; priming of naïve CD4+T-cells and CD8+ T-cells

MHC Class II Antigen Presentation

Professional APCs (mainly DCs and to a lesser extent, macrophages and B lymphocytes) express high levels of MHC class II molecules [16]. As opposed to macrophages, cDCs degrade the engulfed material slowly and in that way, preserve antigens for T cell recognition [50].

MHC class II molecules on DCs sample the extracellular matrix milieu and present exogenous antigens to CD4+ T-helper cells. For effective T-helper cell polarization already mature DCs express high levels of costimulatory molecules (CD80 / 86, CD40 / 40L) and secrete cytokines such as IL12p70 and IL2 (for Th1 immune responses), IL4 (for Th2 immune responses), IL23, IL6 and TGFβ (for Th17 immune responses) and TGFβ1, IL2 (for Treg responses) [125].

Similarly, to MHC class I molecules α and β chain of MHC class II molecules are synthesised in the endoplasmic reticulum and attached to the invariant chain (Ii or CD74) – (MHCII / Ii) [118]. Newly formed MHC class II molecules are delivered to the phagolysosome (late endosome) called MHCII compartment (MHCIIC). Bacterial or viral peptides, attached to TLRs are transported from the cellular surface to the late endosome via early endosome. TLR4 signalling by bacterial LPS triggers the efficient loading of MHC class II molecules with peptides [118]. In the late endosome-MHCIIC, the Ii is cleaved by the proteases (cathepsin S and L) and is replaced by the class II-associated peptide (CLIP). MHC class II molecules need a specific protein HLA-DM in humans to substitute CLIP for the antigenic peptide. Later CLIP is exchanged by the antigen, followed by vacuolar pathway transport of MHC II/antigen peptides to the plasma membrane to connect with TCR of CD4+ T cells [16], [118].

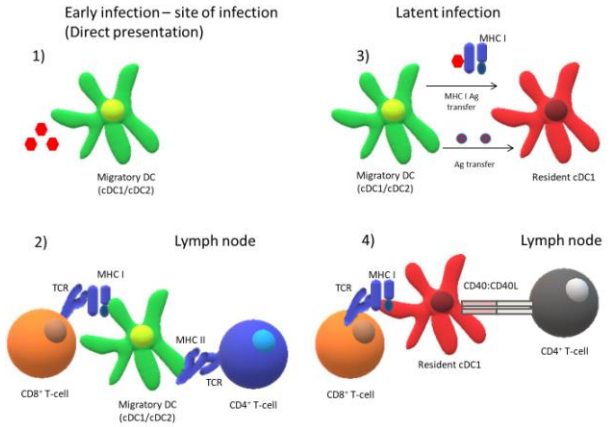

Figure 4: Th-cell polarisation

Antigen Presentation by pDCs

The antigen-presentation by pDCs is similar but with a modulated efficiency and complementary to that of cDCs. The expression of MHC class I and class II and that of T cell co-stimulatory molecules on activated pDCs is lower as compared to cDCs, and therefore pDCs are less efficient at T-cell priming than cDCs [112]. It has also been shown that pDCs like cDCs can provoke T-cell tolerance via inducing Tregs [133].

Concerning MHC class II synthesis and viability, mature cDCs lose their ability to present via MHC II (because of inactivation of the promoter III – pIII) newly encountered antigens due to silencing of MHC presentation machinery. The master transcription factor responsible for MHC synthesis CIITA is down-regulated in mature cDCs [112]. Meanwhile, pIII, responsible for MHC II activation is activated in pDCs (non-silenced upon pDC activation) [110]. Therefore, pDCs are more efficient than cDCs in the presentation of endogenous antigens via MHC II [110], but the roles of both cDCs and pDCs in that process are complementary [110].

The differences in antigen presentation between cDCs and pDCs cannot be explained only by variability in MHC expression [112]. Several studies report that pDCs efficiently present endogenous antigens, constitutively expressed, degraded and attached to MHC I or II in the cytosolic or endosomal route similarly to cDCs [110], [134]. It is known that peptides degraded in the cytosol are presented on MHC I molecules (direct presentation), while peptides processed in the endocytic route (endogenous or exogenous) are presented on MHC II molecules [134].

Nevertheless, pDCs can phagocytise exogenous antigens using specific receptors [110]. Such receptors are BDCA2, SIGLEC-H and DCIR [135] that mediate endocytosis, processing and presentation of exogenous antigens. These receptors induce a signalling cascade in pDCs inhibiting IFN I secretion [136]. Other receptors on pDCs are BST2 (CD317 or HM1.24), PDCA1 [137] and FcRII (CD32) that mediate internalisation of immunoglobulins bound to integrin [112] and the tumour antigen NY-ESO-1 [138].

Cross-presentation by pDCs

Conceivably, the most efficient cross-presenters are cDCs [119]. The cross-presenting capability of pDCs is controversial; for example, mouse pDCs do not possess cross-presenting capacity [137]. Some studies report that human pDCs can cross-present lipoproteins, cell-associated antigens or viral particles [16], [139]. The cross-presenting capacity of human pDCs may be supported by still unknown receptors that capture exogenous antigens [112].

Recently, it was shown that there is another pathway of cross-presentation of tumour-associated antigen (TAA) – peptides complexed with MHC class I molecules to $CD8^+$ CTLs, the process is called “cross-dressing” [140]. The term cross-dressing means that peptide-MHC I complexes including TAAs belonging to neighbouring tumour cells, are transferred on the membrane of DCs [140], [141]. This process has been well documented for cDCs, but only recently was it shown that pDCs are very efficient in acquiring cell membrane patches from neighbouring cancer cells, and pDCs dressed with TAA-MHC I complexes are called drag cells. Tumor-specific CTLs efficiently recognize TAA-MHC I molecule complexes on pDCs [141].

Moreover, pDCs also secrete other pro-inflammatory cytokines and chemokines, such as IL6, IL12, CXCL8, CXCL10, CCL3 and CCL4. pDCS can promote several processes: via IFNs and IL12 promote $CD8^+$ T-cells and Th1 immune responses; via TGFβ, IL6 idoleamine 2, 3-dioxygenase (IDO) and inducible co-stimulatory ligand (ICOSL) they drive Treg cell and Th17 immune responses; via IFNs, IL12 and IL18 they promote the activation of NK cells. Expressed chemokines such as CCL3, CCL4, CXCL8 and CXCL10 ensure immune cell recruitment, while the expression of MHC class I and II and of the co-stimulatory molecules CD80, CD86 and CD40 enable pDCs to cross-prime $CD8^+$ T cells as well as presenting antigen to $CD4^+$ T-cells [18]. When pDCs are either unstimulated or activated by IDO, ICOSL, OX40L, programmed death-ligand (PD-L1) or granzyme B, they promote tolerance to tumour cells, alloantigens and harmless antigens [18], [142].

Human and mouse pDCs express cell surface receptors (TLR7 or TLR9) that control the production of IFN type I in response to the appropriate ligands (nucleic acids derived from viruses, bacteria and dead cells) [143].

T-Cell Polarization

DCs have different phenotypes which alert their ability to polarise different Th subsets. Mature DCs produce cytokines, which induce differentiation of definite Th cell subsets that modify the immune response in the direction of effective or tolerogenic and immunosuppressive [144].

DC1 phenotype (secreting IL12, IL15 and IFN type I) is named so because it induces the Th1 helper subset, which is responsible for immunity to intracellular pathogens [145], autoimmunity [145] and antitumor immunity. Th1 helpers secrete IFNγ and IL2 by themselves and IL12 released by DC1s enhance antitumor immunity and activated NK cells [146].

DC2 phenotype during maturation leads to the secretion of IL1 which and they together with anti-IFNγ, favours the production of Th2 cells. IL4 (not released by DCs, but by basophils and T-cells) [147] and IL10 also induced Th2 specialisation [148]. Th2 immunity is directed against parasites and promotes allergic reactions [148]. Th2 cells secrete IL4, IL5, IL10, IL13 [125]. Two surface markers define DCs specialised in promoting Th2 cell responses. One marker is macrophage galactose-type C-type lectin 2 (MGL2 / CD301b) and the second marker is PD-L2 [149]. Th2 DCs express a specific transcription factors, IRF4 and STAT5, which regulate Th2 differentiation [150], [151].

DC17s phenotype leads in the course of their maturation secretion IL6, TGFβ (released by stromal cells, macrophages etc.), IL21 and IL23 (inhibited by IL4 or IFNγ) and drive the generation of Th17 cells via IL-6, TGF-β and STAT3 pathway [150], [152]. Th17 helper cells secrete high levels of IL-17, IL-21 and IL-22 [125]. Their role in cancer is mainly maintenance of immunosuppression [153].

DC_0 phenotype secreting IL10 and TGFβ (inhibited by IL6 and IL21) direct naïve T-cells into Treg differentiation [125].

$CD141^{hi}$ DCs are also the most active allo-stimulators of $CD4^+$ and $CD8^+$ T-cells, as compared to $CD1c^+$ and $CD14^+$ DCs. Only $CD141^{hi}$ DCs produce CXCL10 and TNFα after TLR3 stimulation but secrete little IL12 and IL23. No stimuli induce the secretion CXCL10 and TNF-α in $CD1c^+$ and $CD14^+$ DCs [48].

It has been reported that DCs activate $CD4^+$ T cells via three signals: i) stimulation of TCR through MHC class II attached to an antigen-specific peptide complex; ii) co-stimulation meaning an interaction between co-stimulatory molecules on DCs (CD80 and CD86) with their ligands on T cells (CD28) and initiation of clonal expansion of TCR-stimulated T-cells; iii) cytokines mainly provided by activated DCs that trigger polarization of naïve T-cells into effector T helper cells [148]. Upon stimulation by exogenous pathogens, allergens, or endogenous inflammatory signals, DCs produce various types of cytokines such as IL1, IL6, IL12, IL21, IL23, IL27, and TNFα functioning as signal 3 [154]. Cytokines trigger the type of STAT activation in T cells that is crucial in determining the effector lineages of T helper cells via inducing distinct transcription factors. In other words, distinct cytokines are required for the commitment of T cells to each T helper lineage.

Dendritic Cells and Cancer

DCs play a key role in the regulation of tumour-specific immune responses [155]. Different DC subsets in cancer can exert an effective anti-tumor immune response, since immature DCs capture tumor-specific antigens, while immunogenic or mature DCs up-regulate MHC and co-stimulatory molecules (CD80, CD83, CD86, CD40), secrete high levels of bioactive IL12p70, skewing and prime $CD8^+$ T cell responses (CTLs), skewing the response to Th1 and induce optimal anti-tumor immunity [156], [157].

Tolerogenic DCs have a role in maintaining immunosuppression in tumour [155]. Tumor-infiltrating DCs are immature with down-regulation of co-stimulatory molecules, secretion of IL-10 and induction of Th2 immune responses and Treg expansion in the tumour milieu [156], [158]. Tumour cells impair DCs function through the secretion of immunosuppressive molecules such as IL10, TGFβ and PGE2 [158], or VEGF, which inhibit DCs maturation and their ability to activate T cells [157]. Tumours produce elevated levels of CCL22 chemokine and ICOSL, molecules that attract DCs [159] and increase the expression of CTLA4 and PD1 / PD-L1, co-stimulatory molecules that can suppress T cell proliferation and activity in cancer [159], [160].

In human tumor tissue four DC subsets are found namely pDCs and mDCs including $CD16^+$ DCs, $BDCA1^+$ DCs, and $BDCA3^+$ DCs [155]. The tumour microenvironment inhibits immunogenic Mo-DCs through secretion of IL-10 and IL6 cytokines, and parallelly promote monocyte maturation into macrophages [161], [162]. Tumor-infiltrating DCs are shown to be immunogenic at early stages of cancer development and convert to immunosuppressive types (MHC II^{low}, $CD40^{low}$ and $PD\text{-}L1^{high}$) at advanced tumour stages. Moreover, immunostimulatory cDCs can transform into regulatory macrophage-like cells that suppress T-cell responses through nitric oxide, arginase activity and IL10 [163].

pDCs and tumour microenvironment

Immature myeloid DCs and pDCs [164] accumulate in human tumors and in peripheral blood [159] and predict poor prognosis for specific cancer types. In the tumour microenvironment, pDCs are non-activated (immature) that don't produce IFN type I

and lack the expression of co-stimulatory molecules [165], [166]. Tumor-infiltrating pDCs produce IDO that promotes Treg activation and immunosuppression in tumours [167]. Tumour pDCs express high levels of ICOS-L and in that way stimulate ICOS+ Tregs (ICOS+ FoxP3+ Tregs) [168].

Also, pDCs after activation can undergo important phenotypic changes towards mDC phenotype and induce IFN I production that amplifies the release of IL12p70 from mDCs and NK cells [169]. Interestingly, pDCs can promote Th2-like immune responses, since increased IFNα stimulate the change of pDCs into Th1-inducing pDCs, while the absence of IFNα leads to Th2-inducing pDCs [169]. It was shown that pDCs expressed on their surface CD56, PD-L1, granzyme B and the TNF-related apoptosis-inducing ligand (TRAIL) [170]. The pDCs tumour cell killing is dependent strictly on TRAIL-associated apoptosis.

DCs have the capacity of the direct cytotoxic killing of tumour cells together with $CD8^+$ CTLs, NK cells, and γ/δ T-cells) [170]. TpDC cytotoxicity was granzyme dependent [167], [171].

In conclusion, we may state that human DCs are the main immune cells that orchestrate the immune response in the tumour microenvironment and represent the most hopeful tool for future DC vaccines that are an alternative of check-point inhibitors for tumour immunotherapy.

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Review Article

Oral Ulcers Presentation in Systemic Diseases: An Update

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Abstract

BACKGROUND: Diagnosis of oral ulceration is always challenging and has been the source of difficulty because of the remarkable overlap in their clinical presentations.

AIM: The objective of this review article is to provide updated knowledge and systemic approach regarding oral ulcers diagnosis depending upon clinical picture while excluding the other causative causes.

METHODS: For this, specialised databases and search engines involving Science Direct, Medline Plus, Scopus, PubMed and authentic textbooks were used to search topics related to the keywords such as oral ulcer, oral infections, vesiculobullous lesion, traumatic ulcer, systematic disease and stomatitis. Associated articles published from 1995 to 2019 in both dental and medical journals including the case reports, case series, original articles and reviews were considered.

RESULTS: The compilation of the significant data reveals that ulcers can be classified according to (i) duration of onset, (ii) number of ulcers and (iii) etiological factors. Causation of oral ulcers varies from slight trauma to underlying systemic diseases and malignancies.

CONCLUSION: Oral manifestations must be acknowledged for precise diagnosis and appropriate treatment.

Introduction

The breach describes ulcerations in the epithelium, underlying connective tissue or both [1]. The most frequent oral mucosal lesion that comes across is oral ulceration [2], [3], [4]. Patients having ulceration of oral cavity might report primarily to a dental consultant or a general physician.

Ulcerations can be classified based on (i) duration of onset (ii) number of ulcers and (iii) etiological factors; ulcerative lesion lasts for two weeks, is considered as the chronic ulcer. Acute ulcer lasts for no longer than two weeks and is typically painful [1], [5], whereas recurrent ulcers present with a history of comparable episodes with irregular healing and chronic ulcer may last for more than two weeks [6]. The solitary ulcer is the occurrence of a single ulcerative lesion, while the term multiple explains the incidence of numerous ulcerative lesions [6].

Because of the variety of presenting features and causative factors, identification of oral ulcerative lesions may be relatively challenging. Local or systemic factors can be contributing to developing ulcers [1], [6]. Ulcers have different parts: the floor (uncovered ulcer surface), the base (ulcer rest seat), the margin (interface among the wall of ulcer and normal epithelium) and the edge (the part of the

margin and floor). The extension phase, transition phase (preparation for healing) and the healing or repair phase are the three stages that are identified throughout a simple ulcer clinical course [7], [8].

The current review article aims to introduce a systemic approach for diagnosis of oral ulcers presenting in different systemic conditions based on their updated knowledge, structure and diagnostic features while ruling out other causative factors and this will also help the dental practitioner to reach the definite diagnosis.

Discussion

It is essential to keep in mind the differential diagnosis to reach a conclusive diagnosis. The differential diagnosis should include the lesions that cannot be skipped in the beginning and to achieve the definitive diagnosis; the additional laboratory investigations are carried out. Literature research revealed that before reaching a definite diagnosis, the malignant ulcerations of the oral cavity were wrongly detected for several months as benign lesions [9], [10].

Malignant Ulcers

Most patients presenting with oral ulcerations will have symptoms for more than two weeks, reflecting the early sign of malignancy. Some of these malignancies may include epithelial neoplasms, solid tumours like lymphomas and minor salivary malignancies can also be presented as ulcers.

Within the oral cavity, the most common malignancy of epithelial origin in oral squamous cell carcinoma (OSCC) [11], [12]. Oral squamous cell carcinoma characteristically appears as a non-healing and non-tender ulcer. In the initial stages, there are various clinical presentations which can often lead to the wrong diagnosis. The commonly affected sites are ventral and lateral borders of the tongue, the floor of the mouth, and lower lip [13]. Clinically it can be presented as the white, red, red-white, exophytic or ulcerative lesion. The typical clinical presentation of OSCC ulcer is crater-like ulcer with the indurated rolled border along with the velvety base. Ulcerative lesions of OSCC are mostly solitary, but it can be presented as multiple ulcerations in a few cases [14].

The malignant tumour of the skin (hair-bearing areas) is called basal cell carcinoma. It usually arises in the sun-exposed areas of the face; from the adjacent involved skin areas, it may spread to the mucous membrane. Initially, it appears as an elevated papule, and with the disease progression, it develops into a central crusted ulcer along with smooth rolled borders [15], [16]. The clinical site of the lesion and histopathology plays a significant role in diagnosis as the OSCC can be included in the differential diagnosis [15], [17].

Ulcer's Due to Microbial Agents

Ulcers due to microbial agents (virus, bacterial and fungal infections) are frequently encircled by an erythematous halo reflecting a healthy and inflammatory response [18]. Most of these ulcers usually have a typical clinical presentation. These ulcers typically appear as vesiculobullous lesions that initially appear as intact blisters which eventually rupture leading to ulcerations. One of the most frequent viral infections presenting with oral ulcers is symptomatic herpes simplex virus (HSV) infection known as primary herpetic gingivostomatitis. More than 90%of lesions are triggered by HSV type-1, and the rest are triggered by HSV2 [19]. After 2-3 days of initial onset, the lesion in the oral cavity usually comprises of pin-headed vesicles that often rupture resulting in painful ulcerations, enclosed by yellowish pseudo-membrane. Both non-keratinized and keratinised mucosa can be affected [20]. The mild form usually presents as a small, numerous punctate superficial ulcers which are confined to the lips and gingiva, whereas the most severe form may appear as diffuse large whitish ulceration consisting of erythematous halo surrounded by a scalloped border [10]. The ulcers typically heal within 5 to 7 days without scar formation [21]. The sores of primary herpetic gingiva-stomatitis may mimic with aphthous stomatitis and acute necrotising gingivitis [10].

Other virus-induced oral ulcers are seen in shingles (Herpes zoster infections) that are caused by the reactivation of dormant varicella-zoster virus [22]. The prevalence of Herpes zoster infections increases promptly, after the age of 50 years, with a decrease in cell-mediated immunity and immunosuppressive conditions [23]. After several days of infection, unilateral clustered and painful ulcers with 1-5 mm diameter were seen on the buccal gingivae and hard palate [6]. These ulcers will frequently rupture resulting in the formation of crater-like ulcers and erosive areas. Shingles can mimic with the herpes simplex lesions, and it can be differentiated by the distinctive pattern of the distribution of the lesion [5], [6]. Within 10-14 days, the ulcers most heal and are self-limiting [20].

Epstein-Barr virus (EBV) is affiliated to the herpes virus group, and it displays tropism for B lymphocytes. The most common lesions caused by EBV are infectious mononucleosis, nasopharyngeal carcinoma and Burkitt's lymphoma [24]. Ulcers caused by Epstein–Barr virus is infrequent but might be a characteristic of infectious mononucleosis. In oral mucosa, the ulcers typically consist of small shallow ulcers [25].

Strains of Coxsackie A virus frequently causes hand foot and mouth disease [26]. It is described as mouth ulcerations and vesicular rashes involving the extremities [26], [27]. After 1 to 2 days of infection, the oral ulcers are typically restricted to the posterior part of the mouth and most commonly present on the soft palate, buccal mucosa, hard palate and tongue. Primary herpetic gingivostomatitis, recurrent aphthous stomatitis, erythema multiform, herpangina will be considered in the differential diagnosis of hand foot and mouth disease. It can be differentiated from other lesions as it involves the extremities and oral cavity at the same time. It is a self-limiting and asymptomatic disease caused by coxsackie A virus. It commonly affects children [28].

Herpangina is typically related with soreness of throat, fever, blisters and ulcers involving the posterior part of the mouth (palate and throat) [29]. As the lesion caused by the herpangina mostly involve the posterior part of the mouth and it can help differentiate it from other viral infections and aphthous ulcers [29].

Oral lesions may be the first sign of HIV infection or HIV-disease advancement [30]. Ulcers seen in the oral cavity of HIV affected patients clinically mimic with aphthous ulcerations, but in contrast, these ulcers are more constant and are most challenging to treat with steroids [31].

The bacterial infection presenting with oral ulcerations are necrotising ulcerative gingivitis (NUG), toma, tuberculosis and syphilis. Acute necrotising ulcerative gingivitis identification can be created on clinical findings alone, as there are enough clinical signs to distinguish this disease from others. The most common symptoms are interproximal necrosis along with punched out ulceration, bleeding and soreness of the affected area and are always limited to gingiva predominantly the interdental spaces. The clinical presentation of acute necrotising ulcerative gingivitis may be different as it depends on the extent and degree of severity of the lesion [32]. Scurvy, Noma, herpetic gingivostomatitis, agranulocytosis and leukaemia can be considered in the differential diagnosis [33], [34].

Primary oral infection caused by Mycobacterium tuberculosis is uncommon. It characteristically presents as solitary, necrotic and ulcerative lesions with undermined edges most commonly affecting the tongue followed by gingivae, the floor of the mouth, palate, lips, and buccal mucosa [35], [36]. At the same time, the ulcer can be irregular, indurated and more painful. Oral SCC, traumatic ulceration, the syphilitic ulcer will be considered in the differential diagnosis of the oral tuberculous ulcer [36], [37].

Primary syphilitic ulcerative lesions caused by Treponema pallidum is generally resulted because of oro-genital or oro-anal contact with an infected lesion [38]. A chancre usually develops as a solitary ulcer after one to three weeks on the lips and rarely on the other sites of the oral cavity [34], [39]. The ulceration lesion is typically deep with a brown or red-purple base and ragged rolled border along with accompanying cervical lymphadenopathy [40]. Traumatic ulceration and squamous cell carcinoma can be included in the differential diagnosis [41]. The most common oral manifestation of secondary syphilis is mucous patches characterised by irregular ulceration, covered by a grey-white necrotic membrane and surrounded by erythema. Confluent mucous patches are known as “snail tracks “ which heals in a few weeks [42]. The most common opportunistic infection of the oral cavity is “Oral candidiasis” which is caused by increased growth of Candida albicans species [43]. Candidiasis infrequently results in oral ulceration [44].

The most common characteristic of oral blastomycosis is painless, nonspecific, verrucous ulcer with indurated borders that is frequently misdiagnosed as OSCC [44]. Moreover, South American Blastomycosis may produce a larger area of ulceration in immunocompromised patients and can be suggestive of OSCC [45]. Further, the most frequent oral manifestation of mucormycosis is palatal ulceration resulting from necrosis; lips, gingivae and alveolar ridge can also be affected [46].

Ulcers Due to Hormonal Imbalance

The imbalances in the hormones are present in numerous diseases related to the endocrine system of the human body as pregnancy and puberty. They may occur during pregnancy and puberty and also by the use of oral contraceptives [34]. Many researchers have recommended a direct relation among fluctuating hormonal status and oral health [47]. Hormonal imbalances expressed as increased salivary estrogen level provoke local physical changes such as increased exfoliation of the oral epithelium causing ulcerations in oral cavity among females during the normal menstrual cycle and pregnancy [34].

Ulcers Due to Systemic Disorders

Systemic disorders may lead to disturbances in oral conditions, and one of the most common oral presentations is ulceration. The differential diagnosis of these ulcers can include chancre, ANUG, early squamous cell carcinoma, leukaemia, traumatic abscess, cyclic neutropenia [48]. Most of the time, the oral site can act as the first indication of blood born disease before other signs and symptoms appear. An abnormal decrease in the circulating red blood cells is called anemia. Pernicious anemia and iron deficiency anemia may present with superficial and small ulcer which mimic aphthous like ulcerations. The periodic decrease in circulating neutrophils due todefects in maturation of neutrophils may lead to a lethal

systemic condtion called cyclic neutropenia, with oral manifestation characterized as solitary / multiple painful ulcers with an erythematous halo that may last for 10-14 days with healing results in scarring. These ulcers may resemble with major types of the aphthous ulcer; and can be differentiated from major recurrent aphthous stomatitis (RAS) by periodontal destruction [49], [50].

Ulcers Due to Inflammatory Bowel Diseases

The most frequent inflammatory bowel disease (IBD) includes ulcerative colitis and Crohn's disease. Lesions of the oral cavity may be apparent and last for months to a year before or at the same time with the abdominal symptoms when IBD disease appear [51], [52]. Aphthous ulcerations is proved to be the most common oral manifestation of IBD during its active phase [49]. In Crohn's disease, two types of oral ulcers can occur one is characterised as deep linear ulcers, having rolled edges which frequently involve the buccal vestibules. The other type of ulcer is superficial mucosal ulceration. The differential diagnosis of such ulcers includes other granulomatous diseases like sarcoidosis [53], [54], [55]. However, the oral lesions of ulcerative colitis include oral aphthous like ulcerations, diffuse pustules, lichen planus and Pyostomatitis vegetans [56].

Ulcers Due to Immune-Mediated Disorders

One of the most common inflammatory lesions of the oral cavity is known as “Recurrent Aphthous Stomatitis” (RAS) [57]. Clinically, it is described by oral ulceration recurrent episodes in an otherwise healthy individual. non-keratinized mucosa of the oral cavity is mostly affected. It can be categorised as minor aphthous, major aphthous and herpetiform [32]. Classically the ulcers appear as a rounded, tender mucosal surface covering with fibrin slough surrounded by an erythematous border. Major aphthous ulcers may result in scarring upon healing, and these ulcers may merge to produce large ulcerative areas [58]. Aphthous ulcers are similar in appearance and site to those ulcers observed in Bechet’s disease. Though in the Bechet’s disease number of ulcerations is greater and of longer duration and is more tender in comparison to aphthous [59]. Along with the aphthous ulceration in Bechet’s syndrome, anogenital and ocular ulceration and arthralgia are helpful in diagnosis [60].

Vesiculobullous Lesions of the Oral Cavity

Various vesiculobullous immune-mediated diseases like mucous membrane pemphigoid pemphigus vulgaris, erosive lichen planus can present with chronic and multiple oral ulcerations [61], [62]. Immune-mediated vesiculobullous lesion of the oral cavity causes blisters formation followed by ulceration of oral mucosa discomfort. Lichen planus is an immune-mediated chronic disease affecting the middle age with female prediction [63]. Oral lichen planus may present in the absence of skin lesions or can occur along with skin involvement. Erosive type present with ulcer covered with pseudomembrane slough along with erythema and keratosis with the multifocal pattern of spreading, bullous like lesion combined with reticular and erosive pattern [64], [65], [66]. Hypersensitivity disease characteristically comprised of irregular erythematous vesicles along with plaques resulting in the formation of the target like or bull’s eye lesions that can be precipitated by multiple factors like drugs, viral and fungal infections. The typically affected areas are lips and buccal mucosa. The lesions are usually ulcerated having an inflammatory halo with irregular margins. The characteristic finding of the disease is severe crusting lesion involving the lips [67], [68]. Erythema multiform is often mixed up with primary herpetic gingivostomatitis but can be differentiated by the appearance and pattern of distribution of lesions of the oral cavity. Another immune-mediated vesiculobullous disease is pemphigus vulgaris described by lack of adhesion of cells resulting in the formation of blisters [69]. Oral lesions are developed in 90% of cases of pemphigus vulgaris. In 50% of cases, it is the first sign of disease. The lesion of oral cavity first appears as bulla which has a very thin roof which ruptures rapidly because of any traumatic insult, resulting in the formation of chronic painful bleeding ulcers with irregular borders which heal with difficulty whereas mucous membrane pemphigoid is characterised by immune-mediated reaction at the level of basement membrane [69]. It has a female predisposition and occurs most commonly at the age of 40. The most frequently affected sites are gingiva before it involves other mucosal sites. Lesions of mucous membrane pemphigoid are usually hemorrhagic that typically result in scar formation upon healing.

Traumatic Ulcers/ Iatrogenic/Idiopathic Ulcers

Injuries due to trauma affecting the oral cavity may characteristically result in the surface ulcerations. Traumatic ulceration is among the most common oral cavity ulcerations [70]. Sublingual ulcerations are seen in newborns and infants; in case of Riga-Fede disease and this may result because of chronic mucosal irritation because of the premature eruption of deciduous teeth (natal or neonatal teeth) and it is frequently related with breastfeeding. The traumatic ulceration in children most commonly occurs because of thermal or electrical factors and affected mostly commissure and lip areas whereas in adults the traumatic ulceration is characteristically the result of mechanical injuries like malformed or fractured teeth; ill-fitting dentures; overheated foods and radiation

injuries [71], [72]. Traumatic ulcers involving the dorsum of the tongue may mimic to the ulcerations triggered by proliferative reactive processes like traumatic ulcerative granuloma, specific infections and lymphoma and definite diagnosis are made microscopically [70]. Traumatic ulcers mostly appear as erythematous, raised edges with a yellowish-white necrotic pseudomembrane which can be easily removed. The ulcerations involving the vermillion border of the lip typically have crusted appearance. Traumatic ulcers mostly heal within ten days after the removal of injurious factors. A differential diagnosis is made from following factors: (i) the lesion's size, (ii) location, (iii) number, (iv) onset, (v) the age of the patient, (vi) association of other systems of the body and (vii) progression of the disease [15].

Conclusions

Oral ulceration diagnosis is always challenging and needs a thorough history taking and clinical examination. The fact cannot be denied that oral presentation may be a sign of some larger underlying systemic disease. Any ulcer that lasts longer than two weeks should be examined histopathologically. This newly updated review included 20 oral ulcerative lesions which are established on the number and duration of oral ulcers. This helps the dental clinicians to create a stepwise method to rule out doubtful conditions to reach a definite diagnosis.

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Review Article

Comparative Effectiveness and Functional Outcome of Open-Door versus French-Door Laminoplasty for Multilevel Cervical Myelopathy: A Meta-Analysis

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Abstract

BACKGROUND: At present, few reports are comparing these 2 major cervical posterior laminoplasty methods with Open-door and French-door Laminoplasty in terms of neurological recovery, cervical alignment, and surgical complications. Moreover, most of the research has not been well designed.

AIM: This study aims to determine comparative effectiveness and functional outcome of open-door versus french-door laminoplasty for multilevel cervical myelopathy.

METHODS: The Meta-analysis is used in this study. The study sample is a published research articles on comparative effectiveness and functional outcome of open-door versus french-door laminoplasty for multilevel cervical myelopathy on the internet through databases on PubMed and ProQuest and published between 1997 until December 2018. Weighted mean difference and pooled weighted mean difference are calculated by using the fixed-effect model or random-effect model. Data is processed by using Review Manager 5.3 (RevMan 5.3).

RESULTS: This study reviews 58 articles. There are 6 studies conducted a systematic review and continued with Meta-analysis of relevant data. The results showed significant higher postoperative Japanese Orthopaedic Association (JOA) score in open-door laminoplasty (ODL) than French-door laminoplasty (FDL) (weighted mean difference [WMD] = 0.71; 95% confidence interval [CI]: 0.35 to 1.07; $p < 0.05$). The outcome of procedures treatment of multilevel cervical myelopathy revealed the operative time, cervical range of motion, axial canal diameter postoperative, axial pain reduction and complications events in ODL and FDL there was no significant difference. But for a cervical lordotic angle in ODL and FDL, there was a significant difference; the ODL group were significantly lesser than the FDL group. The recovery rate in ODL and FDL, there was a significant difference; the ODL was shown to be significantly higher than FDL ($p < 0.05$).

CONCLUSION: This analysis suggests that neither cervical laminoplasty approach is superior, based on the postoperative radiological data and complication rate. But the open-door laminoplasty resulted in a higher functional outcome and recovery rate as compared to the French-door laminoplasty.

Introduction

Cervical myelopathy is a condition that arises when the spinal canal was narrowing and the cord becomes compressed. This is a common manifestation of chronic spinal cord compression which resulted from degenerative conditions such as spondylosis or ossification of the posterior longitudinal ligament (OPLL). In spine cervical myelopathy, it is arising from spinal cord compression due to cervical degenerative changes which are the most common cause of spinal cord dysfunction in patients older than 55 years. Vague sensory and motor symptoms involving the upper and / or lower extremities are common [1].

A combination of static compression with dynamic factors secondary to motion between the vertebral bodies, a congenitally stenotic canal, changes in the intrinsic morphology of the spinal cord, and vascular factors contributes to the development of myelopathy. A developmentally narrow spinal canal in the anteroposterior plane can help with the development of cervical myelopathy [2].

There are a variety of procedures for treating

cervical multilevel compressive myelopathy. Cervical laminoplasty is an extensile approach used to decompress the spinal cord in a patient with cervical myelopathy [3]. This new surgical technique called "expansive open-door laminoplasty" was devised by the author in 1977, which is relatively easier, safer, and better than the ordinary laminectomy from the standpoint of structural mechanics of the cervical spine in order to avoid post-laminectomy complications, such as postoperative fragility of the cervical spine to acute neck trauma, posterior spur formation at the vertebral body, and malalignment of the lateral curvature as it remained as unsolved problems [4]. Laminoplasty is a well-established procedure and is considered to be a gold standard. Even though it has resulted in favourable outcomes, the procedure has been modified because of its complications such as axial pain, loss of range of motion of the neck, postoperative C5 palsy, and late neurologic deterioration. Therefore, there is now a large variety of expansive cervical laminoplasties [3], [4].

Laminoplasty is ideal for patients with multilevel degenerative stenosis and myelopathy or ossification of the posterior longitudinal ligament. It alleviates spinal cord compression by dorsally expanding the spinal canal, thereby allowing the spinal cord to drift posteriorly away from impinging structures [3], [4].

Although there is still a loss of motion after laminoplasty, some motion was preserved in the treated segments, unlike with laminectomy and fusion. Therefore, laminoplasty may be a preferable option for younger patients without significant arthritis. If significant arthritis and/or axial neck pain is present, however, laminoplasty may not be the best choice because a fusion may provide the best chance for relieving the degenerative symptoms [2], [3].

Cervical laminoplasty is a technique for treating myelopathy and myeloradiculopathy associated with cervical stenosis of various etiologies. Variations include open-door laminoplasty, dome-shaped laminoplasty, double-door (French door) laminoplasty, the dorsolateral decompressive procedure, and others [5]. Fixation is then required to hold the lamina open at each level. There are several options available to keep open the laminoplasty, including sutures, suture anchors, bone blocks, and metal implants [2], [3]. The controversies regarding the preferred surgical treatment for cervical myelopathy is focused on anterior decompression and fusion, posterior laminectomy and fusion, or laminoplasty. As the concept of laminoplasty evolved during the 1970s and 1980s, two competing schools of thought emerged; the so-called “open door” and “French door” methods [6]. The laminoplasty allow for indirect decompression of the spinal cord by opening the lamina on one side, thereby creating a hinge joint that allows the spinal cord to float dorsally [3].

At present, few reports are comparing these 2 major cervical posterior laminoplasty methods in terms of neurological recovery, cervical alignment, and surgical complications. Moreover, most of the research has not been well designed [7].

Recently, it is unknown whether there is a significant difference between these laminoplasty methods, and some review of this comparison was limited. Thus, we compared the effectiveness of the two types of laminoplasty in this meta-analysis aiming at differences in recovery rate, clinical, surgical and radiological outcome and also the complications.

Material and Methods

Study design and research sample

This research is a quantitative research with Meta-analysis study design. Meta-Analysis is used to find out the comparative effectiveness and functional outcome of open-door versus french-door laminoplasty for multilevel cervical myelopathy. The research sample is a published research article on the internet through the database on PubMed, ProQuest and Cochrane published between 1997 and December 2017. Those data were manually scanned and reviewed with inclusion criteria: study sample is research with randomized controlled trials and non-randomized comparative, a comparative design for open-door laminoplasty versus French-door laminoplasty, patients with cervical myelopathy from cervical spondylotic myelopathy (CSM) or ossification of posterior longitudinal ligament (OPLL), studies directly comparing open-door cervical laminoplasty with French door cervical laminoplasty, and the studies reported a desirable outcome with continuous variable. Exclusion criteria were those with a cervical fracture, neoplasm, infection, or deformity; noncomparative studies, nonhuman in vivo, in vitro, and biomechanical studies were excluded and research which not available in full-text form.

Operational definitions

Variables in this study are multilevel cervical myelopathy (open-door versus french-door laminoplasty) and functional outcome (operative time, cervical lordotic angle, the global cervical range of motion, axial canal diameter postoperative, axial pain reduction recovery rate and complications events).

Research procedure

This study is conducted by collecting data through the identification of published research articles on comparative effectiveness and functional outcome of open-door versus french-door

laminoplasty for multilevel cervical myelopathy on the internet on PubMed, ProQuest, and Cochrane databases (Figure 1).

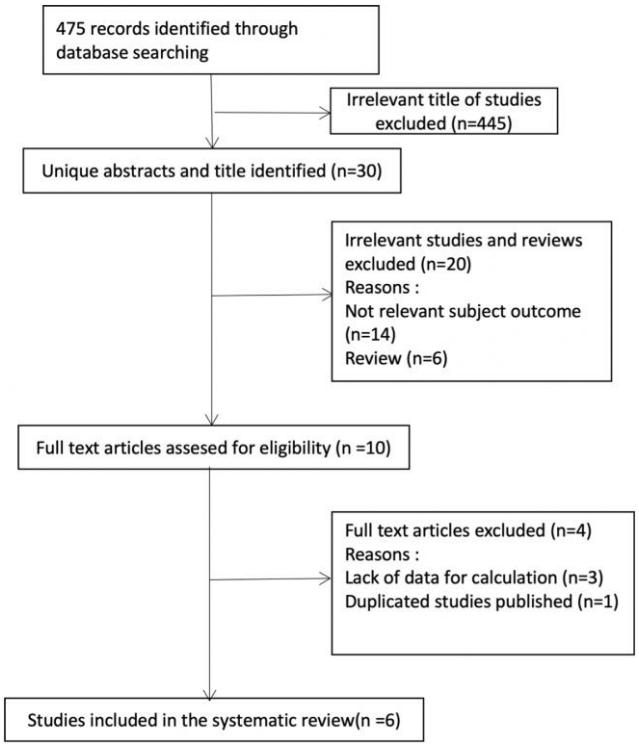

Figure 1: Flow diagram research procedure

Data collection technique

Search is limited only to English language articles. This article type is limited to journal articles. Research subjects are limited only to research subjects of a human. The time of publication is limited from 1997 to December 2018. Articles with potentially relevant titles are reviewed abstract, while irrelevant articles are excluded.

Furthermore, the article is reviewed abstract. Articles that have potentially relevant abstracts will then be reviewed in full-text while irrelevant articles are excluded. Furthermore, the article is excluded based on the research variables and the design of the study (randomised controlled trials and nonrandomized comparative).

Data analysis

The analysis held to get the value of weighted mean difference which is the combined mean difference value from the research. Data analysis by Weighted mean difference and pooled weighted mean difference method using a *fixed-effect model* or *random-effect model*. Data is analysed by using Review Manager 5.3 (RevMan 5.3).

Results

Identification of 58 articles, done by review through the title of the articles, then reviewed abstract, then reviewed in full-text form. Irrelevant articles are excluded. Selection of studies conducted to obtain 6 studies related to comparative effectiveness and functional outcome of open-door versus french-door laminoplasty for multilevel cervical myelopathy (Table 1).

Table 1. A systematic review of comparative effectiveness and functional outcome of open-door versus french-door laminoplasty for multilevel cervical myelopathy

First Author, Year	Design Study	Number of Patients (Male/ Female)		Age, Years		Follow up Months	
		ODL	FDL	ODL	FDL	ODL	FDL
Baek *et al.*, [8]	Retrospective cohort	24	10	55.5	56.2	17.6 ± 23.4	18.8 ± 36.3
Okada *et al.*, [4]	Prospective randomized trial	17 (10/7)	18 (13/5)	59.9 (31-79)	61,1 (46-79)	27.6	26.2
Park *et al.*, [9]	Retrospective trial	79 (63/16)	21 (15/6)	55.2 ± 12.7	57.6 ± 11.9	47.9 (24-68)	49.5 (25-70)
Wang *et al.*, [10]	Prospective study	24 (14/10)	25 (17/8)	59.5 (38-76)	60.4 (36-74)	21.8 ± 2.2	20.6 ± 2.0
Lee *et al.*, [11]	Retrospective trial	23 (12/11)	28 (25/3)	59.4±1.9	59.3 ± 2.0	24.6 ± 1.3	27.8 ± 1.2
Nakashima *et al.*, [6]	Prospective randomized trial	44 (29/15)	46 (28/18)	62.6 ± 9.5	63.4 ± 10.7	28.4	29.3

Based on the results of the systematic review, there are 6 studies analysed by meta-analysis. A meta-analysis of comparative effectiveness and functional outcome of open-door versus french-door laminoplasty for multilevel cervical myelopathy (Figure 2).

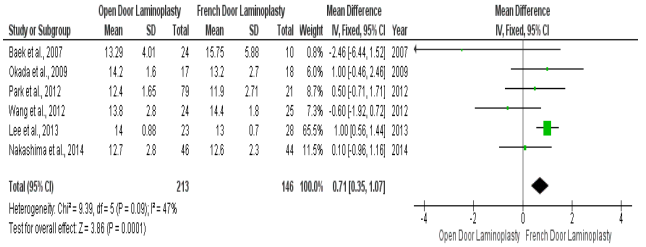

Figure 2: Forest plot showing the weighted mean difference in the Japanese Orthopaedic Association (JOA) score for Open-door laminoplasty versus French-door laminoplasty

Figure 2 the results showed significant higher postoperative Japanese Orthopaedic Association (JOA) score in open-door laminoplasty (ODL) than French-door laminoplasty (FDL) (weighted mean difference [WMD] = 0.71; 95% confidence interval [CI]: 0.35 to 1.07; $p < 0.05$) (Figure 3). The operative time in ODL and FDL there was no significant difference (WMD = -6.32; 95% CI: -17.16 to 4.53: $p = 0.25$). The cervical lordotic angle in ODL and FDL there was a significant difference, the ODL group were significantly lesser than the FDL group (WMD = -2.72; 95% CI: -3.60 to -1.84; $p < 0.05$). The global cervical range of motion in ODL and FDL there was no significant difference (WMD = -4.62; 95% CI: -13.06 to 3.82; $p = 0.28$).

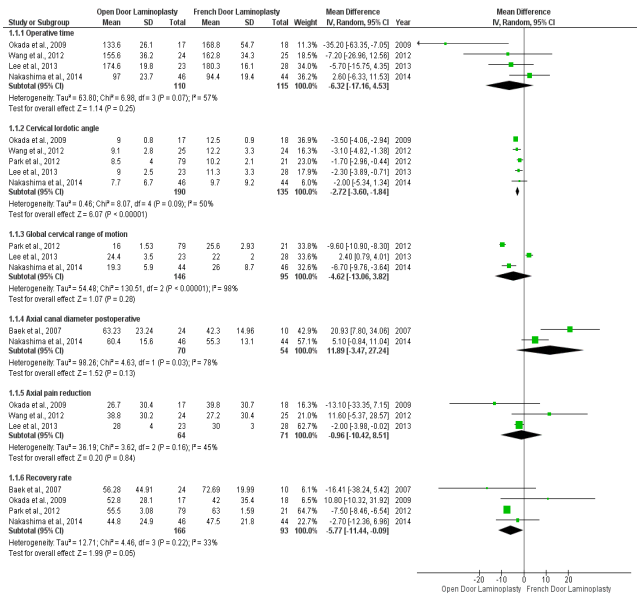

Figure 3: Forest plot showing the weighted mean difference in outcome procedures treatment of the Japanese Orthopaedic Association (JOA) score for Open-door laminoplasty versus French-door laminoplasty

The axial canal diameter postoperative in ODL and FDL there was no significant difference (WMD = 11.89; 95% CI: -3.47 to 27.24; p = 0.13). The axial pain reduction in ODL and FDL there was no significant difference (WMD = -0.96; 95% CI: -10.42 to 8.51: p = 0.84). The recovery rate in ODL and FDL there was significant difference, the ODL was shown to be significantly higher than FDL (WMD = -5.77; 95% CI: -11.44 to -0,09); p < 0.05). The complication events in ODL dan FDL (Figure 4).

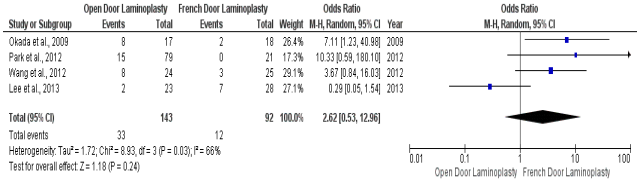

Figure 4: Forest plot showing the pooled odds ration in complication events of procedures treatment of the Japanese Orthopaedic Association (JOA) score for Open-door laminoplasty versus French-door laminoplasty

Figure 4 the complications events in ODL and FDL there was no significant difference, (OR = 2.62; 95% CI: 0.53 to 12.96: p = 0.24).

Discussion

The results showed significant higher postoperative Japanese Orthopaedic Association (JOA) score in open-door laminoplasty (ODL) than French-door laminoplasty (FDL) (weighted mean difference [WMD] = 0.71; 95% confidence interval [CI]: 0.35 to 1.07; p < 0.05). The outcome of procedures treatment of multilevel cervical myelopathy revealed cervical lordotic angle in ODL and FDL there was a significant difference; the ODL group were significantly lesser than the FDL group. The recovery rate in ODL and FDL, there was a significant difference; the ODL was shown to be significantly higher than FDL (p < 0.05).

Cervical laminoplasty has been the preferred surgical treatment for cervical compressive myelopathy cases. Its main purpose is to decompress the spinal cord by increasing the diameter of the spinal canal. The two most commonly used approaches are the open-door and French-door types, each with its pros and cons.

Hirabayashi first described a single door open laminoplasty technique in 1981, using groove creation between the lamina and articular facets of one side. This technique may cause the lamina to recover back to its original position due to failure in the attachment of the open side or the presence of fracture on the hinged side. Many researchers have published several modifications to the Hirabayashi technique to solve this problem. One is combining spinous processes grafts such as bony spacers, enhanced by titanium plates and screws [4], [11], [12].

The French-door laminoplasty, on the other hand, offers a symmetrical decompression by creating an opening in the midline of spinous processes and hinges on both laminae. Bilateral troughs are created on each lamina, thus creating a “French door” which allows the spinal cord to move posteriorly [2], [5], [12].

According to previous research over this procedure, the difference over the neurological and functional outcome is not significant [13], [14], [15]. Another research also found that the functional outcome of ODL was better than FDL [8], [10], [12]. Axial canal expansion and lordotic angle postoperative were better achieved by ODL than FDL [7], [14], [16]. The complication postoperatively was higher in ODL than FDL [15], [16].

Based on our recent analysis, the main results based on the comparison between open-door and French-door laminoplasties are that open-door laminoplasty is superior to French-door laminoplasty concerning higher recovery outcome. This reflects the efficacy of the surgical approaches in decompression of the spinal cord. But the complication rate is still higher, i.e. intraoperative blood loss and postoperative axial pain that possible from damage to the spinous process ligament-muscle complex, damage to posterior roots of C3-7 spinal roots, damage of the suture for facet joint capsule, decrease in the cervical lordotic angle and move range and long-term immobilization of neck [4], [15].

Others have suggested that French-door laminoplasty is more beneficial than open-door

laminoplasty for patients with multilevel cervical compressive myelopathy to minimise postoperative complications such as C5 palsy in patients with asymmetrical ossification of the posterior longitudinal ligament (OPLL) [7], [10], [14], [15]. This may be because French-door creates a wider spinal canal to allow more space for the spinal cord to expand.

In this analysis, open-door laminoplasty was found to be inferior to the French-door type when considering cervical lordotic angle and recovery rate. Others have suggested that French-door laminoplasty is more beneficial than open-door laminoplasty in preventing postoperative kyphosis [7].

In particular, the range of motion extension is significantly decreased in the open-door laminoplasty group. This may be due to excessive enlargement of the spinal laminae in open-door laminoplasty, which may negatively affect the results. Greater expansion of the spinal canal is easier by open-door laminoplasty than by French-door laminoplasty. Although future studies are required to ascertain the effectiveness of spinal canal expansion, according to the literature, it seems that open-door laminoplasty better enables effective spinal canal enlargement as compared with French-door laminoplasty.

These results suggest that neither cervical laminoplasty approach is superior based on the postoperative radiological data and complication rate. But open-door laminoplasty was shown to provide a higher functional outcome and recovery rate compared with French-door laminoplasty.

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Medical History

Philosophy and Hippocratic Ethic in Ancient Greek Society: Evolution of Hospital - Sanctuaries

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Abstract

The aim of this paper is to offer a new perspective of the Hippocratic thought and how it influenced the evolution of the medical art till now, highlighting the ethical aspects and hospital born from ancient temples and sanctuary. Ethics is defined as a set of values, principles, and rules that regulate human behavior and relate to how human actions can significantly affect not only their own lives but also the lives of others. The essence of a culture can be perceived by the philosophy and the means by which is placed against the illness and its treatment. In this sense, the medical anthropology of every age is an indicator of its culture and help us understand its basic dimensions such as life and death.

Introduction

Social rule systems and all the levels of human interaction are examined in order to be presented as a model for comparison between medical ethical constructions. So, the development of medical ethics was also through philosophical thought. This relationship between philosophy and medicine is very old and goes back to the ancient medical philosophers whose excellency according to the physician Galen depended on philosophy (*"the excellent physician must be a philosopher"*) [1], [2], [3].

Across the philosophical thought

Most of the work written for the medical ethics rules by ethics and especially bioethics experts refers to the Hippocratic "Oath" and some selected texts from the "Hippocratic corpus", a collection of a large amount of ancient Greek treatises regarding medical ethics and practice which is focused on the moral content. Thus the "*Oath*" testifies to the highest moral perception of the professional diligence of physicians and constitute a milestone in the ethics of the medical profession. This leads to the general conclusion that early Greek medical ethics began when Hippocrates or a group of physicians who shared his principles developed codes of professional conduct even if they

were not initially accepted by all. At that time the Greek point of view about health is a naturalistic relationship of thought. The healthy body depends on a balance between natural and society indicators elements [4], [5]. However, when an allusion is made to ancient medicine, we necessarily must take into account Hippocrates. Try to dismissed medicine from its previous supernatural and divine elements, biases and superstitions. Has been recognized not only as a pioneering physician, but first also as an outstanding philosopher. Hippocrates believe that medicine should be done wisely. He did not invent medicine but collected, filled and systematized all the knowledge that existed before him. Despite the deficient knowledge of anatomy Hippocrates, relying on the innate power of nature, which is present in human beings, laid the foundations for building its therapeutic methods. Thus, finalizing the value of medicine as science he established its entire structure. Health and diseases are the balance of the whole person (the holistic view) [6], [7], [8]. In the 6th century B.C., in the twelve colonies of Asia Minor (in the ancient region of Ionia of central coastal Anatolia in present-day Turkey), with the so-called pre-Socratic philosophers of Miletus, the transition of thought to scientific prose and the birth and development of the Greek philosophical miracle. Their contribution was influential not only in the way of the human reasoning but also in ancient medicine, whose doctrine has survived in our time as a branch of medical science based on observation and the therapy of the patient which was the ultimate goal. However, thanks to them for the first time the gradual release of medicine from its religious environment takes place. Before Socrates, it was the Pythagoreans who placed an emphasis on hygiene the well-known “Pythagorean way of living” and also the definition of health and illness. One of the most well-known representatives of the age of the Pythagoreans, who dealt with medicine, illness and healing, was Anaximander. The Pythagoreans alleged that the harmony of perfect equilibrium of the human body was the key element to its health. Health is a condition of unique balance of the four bodily fluids (blood, phlegm, yellow bile and black bile), as well as Hippocrates described and supported.

Another crucial question was the relationship between medicine and health. Aristotle’s position explains that it is not accidental but essential, and the purposes of the medicine and those who practice it is defined only from this relationship. Plato agrees with him that although those who practice medicine benefit from their art, such benefits are not essential to its purposes. Plato thinks that medicine does not benefit itself but seeks the appropriate treatment of the patient, a restriction that forbids the physician to make decisions for the advancement of medical science. Furthermore, Plato in the first book of his “Republic” (Πολιτεία), clarifies the medical reward. Tries to separate the purpose of the physician’s practice for the benefit of the patient from his intention of earning money, in fact Socrates does not say that the physician must be altruistic. On the other hand, the physician cannot look forward to his economic prosperity as an ultimate goal. In fact, he points out that each physician has two arts (téchnes), that is medicine and earning his salary. What he underlines is that the real physician should not be ignorant of the fact that he is primarily physician and the relationship with a patient must be organized around the purpose of providing the best possible medical care to him [9], [10], [11], [12]. As we have already accented, ancient people in Greece, primarily perceived medicine as art that is closely related to the natural science and practical wisdom which is a medical concept that still seems to be valid also in our time. The term art is used very often, especially in Plato, however, the ancients separated art from other intellectual disciplines. Even when they perceive art in this more limited way, they always tend to include medicine among the arts, such as shoemaking, woodworking, agriculture, rhetoric and poetry, for the reason that medicine generates health. By creating health, medicine seems both, poetic and utilitarian art because physicians use a variety of tools and methods in order to achieve health for their society. Since medicine is the most important of the arts, those who are going to follow it are required to have many spiritual and moral qualifications if they wish to serve it properly.

As we said medicine is an art. This art consists in three parts. Indeed the “Hippocratic triangle” theory it clarifies that the disease, the diseased and the physician are those essentials parts. Thus, the physician needed to gain the patient’s confidence so as the latter to reveal personal secrets about his health problems and physical imperfections. The establishment of a basic level of trust was necessary for the exercise of medicine as a feasible art. In pursuing health and balanced so that the person returns to his lifestyle, the physician inevitably deals with the human life in general and not only with the condition of the body [13], [14], [15].

Patients observation: body and soul

The promotion of health and the end of medical treatment, had to be combined with the promotion of the virtuous and prosperous life. What differentiated the Hippocratic physician from the previous healing sanctuary therapists was not mainly the change from “magic-religious” to “empirical-rational” therapeutic approach, but the focus on reading the signs of the patient’s body. The Hippocratic physician, through a methodical observation process can't be reach a reasonable patient medical evaluation. The physician age and later, is evaluated by means of his abilities and purposes which determine the nature of his profession

while morality had a minor part [16], [17], [18]. Thus, the attitude of the Hippocratic physician towards the weak person, was the vigilance that required intensive care and the ability to read and interpret a long list of signs. Bodies were considered to be in constant flow or flow conditions, depending on climate, age, idiosyncrasy, diet and activity. Thus, Hippocratic physician had to weigh the evidence that certifies the patient's condition, some of which stem from the patient's observation and decide for the appropriate treatment. Therefore, the patient's personality and idiosyncrasy may also be involved in the evaluation and the moral orientation of the Hippocratic physician was gradually developing in relation to the patient and his family. Now human's health achieves the balance between soul and body which are the two elements of our nature that play an important role into all our actions and choices. The soul not change, but the body is different for all. The Hippocratic physician must avoid doing too little or too much, he has to aid these two elements of the human nature to preserve their balance. Plato in his "Phaedrus" recognized the importance of Hippocrates method: "*If Hippocrates the Asclepiad is to be trusted, one cannot know the nature of the body, either, except in that way*". He believed that through Hippocrates way of observation of the human body, the same method could be used for the soul. If the body was capable of warding off the disease, the physician had to recognize it, take time and not intervene. If the patient was in the need of help, the physician had to act directly so as to alter the course of the illness. In addition, the conclusion of Prognostic is elucidating regarding the physician's endeavor to make the right prophesy: "*You must take into account both the good signs and the bad that occur and from them make your predictions; for in this way you will prophesy aright…*" (LCL II, 33) [19], [20], [21]. Hippocratic thought was the first not to be dictated by any central authority and priesthood, something that did not happen in another culture like the Egyptians. Must be mentioned that the independence of medicine from magical-religious constitution did not mean complete liberation from certain ethical rules. Where human physician failed, Asclepius succeeded. We have had testimonies where the patients disappointed by the "human" physician they sought alternatives to the god. Regarding the diversity of others therapists that were accepted at the time of Hippocrates in antiquity, we must mention that there was a wide variety in terms of how they were identified by the society. Some assimilated with god others laid the medical practice under the auspices of the gods and some others, like therapists-craftsmen, had healing techniques they acquired after long-term training and practice [22], [23], [24].

Discussion

For the historical course, diseases were attributed to the angry gods whose power was shown to the human race through inexplicable forces called "*odious demons*" (Daímones). On the contrary medicine was associated with gods (especially Apollo and Asclepius) and heroes (such as Amphiaraus) who were identified with health. At sixth century B.C. begins the faith to the god Asclepius as a divinity that heals the human body. The Sanctuaries devoted to the divine healer, in the so-called Asklepieions (*Ἀσκληπιεῖα*), were frequented by people who wished to pray for well-being or sought cures for various diseases [25], In the 5th century bC, Hippocratic medicine is built upon natural philosophy and make medicine a rational science and "*come to influence radical the way of healing in ancient world*". As we have mentioned the distinction between religion and Hippocratic medicine is due from the two important components of Greek society in that time: political and cultural evolution pluralism. In Asklepieions now the custom healing was to combine the art of medicine with the theurgical practice. Hippocratic holistic medicine approach to health was practiced in those sanctuaries and thus providing not only for physical and psychological care but also a social and spiritual aid. All these innovations comprised a radical re-orientation of health services. Among these, the most known were the Asclepieion of Kos, Pergamum and Epidaurus (was widely recognized as the greatest sanctuary). The Asklepieions were the first hospitals-sanctuaries and they had developed in famous healing centers visited by people from the wider contiguous areas. They were built near in a valley or on a hill (a location with the best climate condition and plentiful pure water) and they also had around theatres or / and other comforts [26], [27], When the ill person coming to the Asklepieions received many instructions in order to relax and rest. Thus, they applied baths, massages and other body exercises for therapy included theatrical and others similar recreational activities. There was a sacred place, a long building who called "*kataklintyrio*", for the presence of private rooms with bed. Patients may be participated in the sacrifice of animals and in mystic performances in honor of Asclepius and were finally relaxed and convinced that they will be certainly treated by the aid of Asclepius. In the middle of the whole infirmary there was a dome (ábaton), a special building, the main part of which was not free to everyone to visit, where the main medical treatment procedures were performed. In this place, the patient was kept under a condition of sleep or healing narcosis the so-called "incubation". Women were acting as vergers. While some priests therapist who had the art of divination looked at the flying of the birds and others examined the offal, now the Hippocratic physician, as we said, using the Hippocratic principles examined the human body by

carefully observing the fluids coming from the inside of the body (including urine or saliva) and by identifying their color, texture, smell he endeavored to determine the course of the disease and was expected to decide the therapeutic management process [28], [29], [30], Hippocratic medicine, as we mentioned, is the first who try to separate religion and healing. Despite this a patient who was has been admitted, they had to make a religious offer like a replica of the afflicted part of body thus organ or limb. Inscriptions found in marble plaques describe the treatment of some diseases and the sum of money paid for each of them. Was being treated surgical diseases, diseases of integumentary attachments, chronic pathologies of the lung, gynaecological (parturient women were not accepted) and ophthalmic, but also neurological and phycological disorders. Must be reported that the craniotomy for decompression purposes as an innovation practice studied through the Hippocratic Asklepieions and at the Hippocratic corpus where we find many references about it [31], [32], [34],

Conclusions

The way how the Greek philosophy influenced the modern rational healing and ethics in medicine is undoubtful. Thanks to the Hippocrates' philosophy, medicine became a field of research for balance between health and disease by scientific orientation. He also examined the relationship between society and morality, patient and physician and created an innovative theory regarding medicine's practice through a new ethics vision for the benefit of health sciences. The modern practice of medicine in the new hospital-sanctuaries by the Hippocratic physicians appeared to be the major advanced institution of health (far away from the therapeutic religion) which influenced the way patients face it today. However, we must observe that Hippocratic medicine on the one hand has been based to a scientific thought with the aid of the philosophy but from the other hand supernatural forces could affect the healing process (for example through astrological observations who could change the evolution of the diseases) [35], Such principles were spreaded in all countries culturally conquered by Greek people, i.e. in the so called "*Magna Grecia*", southern Italy, where Alcmaeon built up the main medical school during the 6th century B.C. [36]. Despite the evolution of the humans through all these years from the ancient society in the time of Hippocrates until our modern society, this religious influence in medicine, that is the faith in a "god-saint", who might have a role in patient's salvation, is still present in our era.

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