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Liver Protective Effects of Renin-Angiotensin System Inhibition Have No Survival Benefits in Hepatocellular Carcinoma Induced By Repetitive Administration of Diethylnitrosamine in Mice

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Abstract

BACKGROUND: Preclinical studies have demonstrated that renin-angiotensin system (RAS) signalling has strong tumour-promoting effects and RAS inhibition was associated with improvement in the overall survival in some cancer types including hepatocellular carcinoma (HCC).

OBJECTIVE: We aimed to investigate the effect of angiotensin-converting enzyme inhibitors (ACEIs) or angiotensin-II-receptor blockers (ARBs) on the survival of mice with diethylnitrosamine (DEN) induced HCC.

METHODS: HCC was induced by weekly i.p. administration of DEN. Mice were treated with sorafenib (SO) (30 mg/kg), perindopril (PE) (1 mg/kg), fosinopril (FO) (2 mg/kg), losartan (LO) (10 mg/kg), PE (1 mg/kg) + SO (30 mg/kg), FO (2 mg/kg) + SO (30 mg/kg), or LO (10 mg/kg) + SO (30 mg/kg). Survival analysis was done using the Kaplan-Meier method, and the log-rank test was used for assessing the significance of difference between groups.

RESULTS: The administration of PE, FO and LO as monotherapy or as combined with SO resulted in marked improvement in the liver histologic picture with no impact on overall survival of mice.

CONCLUSION: Interfering the RAS either through the inhibition of ACE or the blockade of angiotensin II type 1 (AT1) receptors has similar effects on the liver of DEN-induced HCC mice and is not associated with longer survival due to detrimental effects of DEN on other organs. Hence, repetitive administration of DEN in such models of HCC is not suitable for mortality assessment studies.

Introduction

Hepatocellular carcinoma (HCC), a major health problem is representing about 75% of primary liver cancers [1] [2], is considered the second most common cause of cancer-related mortality worldwide [3] and is characterised by poor prognosis. Risk factors for HCC have been described including infections with chronic viral hepatitis, non-alcoholic steatohepatitis (NASH) and alcohol consumption [4] [5] [6].

Treatment options of HCC are still limited and depend on liver function and patient condition [7]. Patients are usually diagnosed at advanced tumour stages as HCC patients are frequently asymptomatic. The oral multikinase inhibitor sorafenib, remains the only efficacious treatment, currently showing a certain degree of benefit [8] [9] and provides a modest prolongation of the median overall survival (OS) (2.8 months) [10]. Regorafenib, a closely related tyrosine kinase inhibitor demonstrated an increase in OS as a second-line (next to failure of sorafenib) [11]. None of the other agents tested showed a survival benefit versus sorafenib or placebo [12]. Therefore, a critical need exists to evaluate possible alternative strategies for the effective improvement in the survival of HCC patients.

There is emerging evidence that Angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs) have beneficial effects in patients with HCC [13]. De Paepe, Verstraeten [14] reported that angiotensin II (Ang II) type 1 (AT1) receptor may be an essential step in the development of breast cancer, also it was reported that RAS inhibition was associated with reduced risk of breast

cancer recurrence [15]. Furthermore, other studies revealed that ACEIs or ARBs might decrease the risk of esophageal [16] and keratinocyte [17] carcinoma. Their use was also associated with increased response in rectal cancer [18] and longer OS in patients with renal cell, pancreatic, brain, and lung cancer [19] [20]. However, little is known about the impact of RAS inhibition on the OS of HCC on the experimental and clinical sides.

Therefore, the present study was conducted to examine beneficial effects of RAS inhibition on liver histology and to assess the association between RAS inhibition and survival using the ACEIs, perindopril (PE) and fosinopril (FO) and the ARB, losartan (LO) by comparing their effects to sorafenib (SO) using a model of diethylnitrosamine (DEN)-induced HCC in mice.

Material and Methods

A total of 270 male CD-1 mice weighing 12 g were used in the current study. They were purchased from Schistosome Biological Supply Program at Theodor Bilharz Research Institute (SBSP-TBRI), Egypt and housed in polycarbonate cages in an animal facility certified by the association for assessment and accreditation of laboratory animal care and maintained in accordance with the National Institute of Health guide for the care and use of laboratory animals, Egypt. All experimental procedures were approved by local authorities at the SBSP-TBRI and by the Ethical Committee for Animal Handling at Zagazig University (ECAHZU), Egypt. All of the mice were fed rodent chow (23% protein and 4% fat) and received water ad libitum. They were kept under standard laboratory conditions of (21 Co, 45-55% humidity) and exposed to a 12/12 light-dark cycle. The animals were allowed to acclimate for 1 week before the experiments.

Diethylnitrosamine (DEN) was diluted (1:100 v/v) in saline. Perindopril, fosinopril and losartan were freshly prepared immediately before use by suspending in distilled water.

Mice were divided into groups as follows: Group 1: normal mice received i.p. injections of normal saline once a week (n = 6). Group 2: DEN induced HCC mice (DIHCC) received i.p. injections of DEN (Sigma-Aldrich CAS: 55-18-5, St. Louis, MO, USA) (n = 60) once a week according to the following schedule (week1 (30 mg/kg), w2 (50 mg/kg), w3 (50 mg/kg), w4 (70 mg/kg), w5 (100 mg/kg), w6→w16 (50 mg/kg)). Group 3: DIHCC mice received 30 mg/kg sorafenib (Bayer Pharmaceuticals, AG, Berlin, Germany) (n = 30). Group 4: DIHCC mice received 1 mg/kg perindopril (Servier Pharmaceutical Company, Suresnes, France) (n = 30). Group 5: DIHCC mice received 2mg/kg fosinopril (Bristol-Myers Squibb Pharmaceutical Company, New York, NY, USA) (n = 30). Group 6: DIHCC mice received 10mg/kg losartan (Merck Pharmaceutical Company, Kenilworth, NJ, USA) (n = 30). Group 7: DIHCC mice received (1mg/kg perindopril + 30 mg/kg sorafenib) (n = 30). Group 8: DIHCC mice received (2 mg/kg fosinopril + 30 mg/kg sorafenib) (n = 30). Group 9: DIHCC mice received (10 mg/kg losartan + 30 mg/kg sorafenib) (n = 30). Mice were administered suspension of drugs in distilled water by oral gavage daily starting from day 45 of experiment and sacrificed by decapitation 16 weeks post induction.

The dose of sorafenib was selected based on previous studies [21] [22] [23]. On the other hand, the equivalent mouse doses of perindopril, fosinopril, and losartan were calculated by interpolating from the corresponding lowest effective human dose using approximate dose conversion factors described by Freireich et al., [24].

Liver tissues were fixed in 10% neutral buffered formalin (pH 7.0) and embedded in paraffin. Sections (5 μm thick) from the paraffin blocks were stained with hematoxylin and eosin (H&E) for histopathological examination. HCC was graded as described by Theise, Curado [25].

Statistical analysis was performed using GraphPad Prism software version 6 (GraphPad Software Inc., La Jolla, CA, USA). For overall survival, the log-rank (Mantel-cox) test was used for assessing the significance of the difference between groups in the Kaplan-Meier analysis. P values < 0.05 were considered significant.

Results

The representative histological appearance of liver specimens from untreated normal control mice showed hepatic lobules with intact lobular architecture. Liver cells are arranged in cords of one to two cell-thick, radiating from a central vein towards the lobular periphery with blood sinusoids in-between. Hepatocytes are polyhedral with abundant granular eosinophilic cytoplasm and one spherical nucleus with dispersed chromatin. Portal tracts are of normal shape and thickness.

Sections of Liver from the DEN-treated mice showed loss of hepatic lobular architecture and enlarged hepatocytes with marked nuclear atypia, nuclear hyperchromasia, pleomorphism, increased nucleocytoplasmic ratio and appearance of tumour giant cells. Also, different sections showed HCC (grade 3) characterised by malignant hepatocytes surrounded by fibrotic stroma that were infiltrated by mononuclear inflammatory cells.

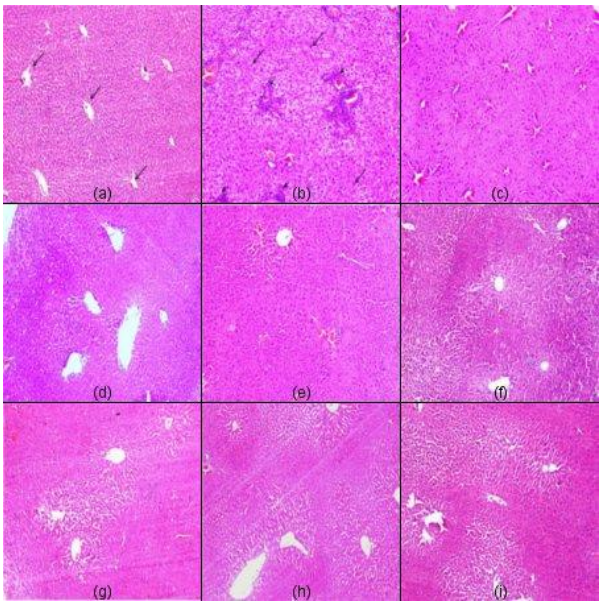

Figure 1: Representative light micrographs from: (a) normal control showing normal liver histology with radial arrangement of hepatocyte rays around central veins (arrows) that are separated by sinusoids; (b) DEN-treated mice showing HCC of grade (3) with disturbed architecture and loss of lobular pattern, hepatocellular atypia, pleomorphism, intralobular inflammatory infiltrate (arrowheads), malignant hepatocytes with moderate to marked nuclear anaplasia (arrows). Moreover, intralobular inflammatory infiltrates, fibrous tissue deposition (arrowheads) and congested blood vessels are evident. Micrographs from mice treated with sorafenib, (c), perindopril (d), fosinopril (e), losartan (f), perindopril plus sorafenib (g), fosinopril plus sorafenib (h), and losartan plus sorafenib (i) showing regression of malignant changes with almost restoration of lobular architecture and lowering of the grade of HCC to grade 1 (H&E x100)

The representative histological appearance of liver tissue specimens from DIHCC mice treated with Sorafenib showed regression of malignant changes and lowering of the grade of HCC to grade 1 with almost restoration of lobular architecture

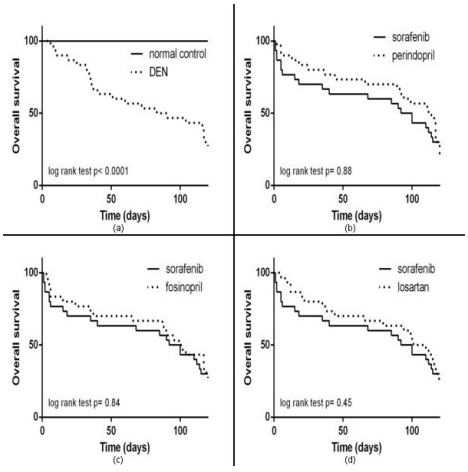

Figure 2: Kaplan-Meier survival curves of (a) DEN vs normal control; (b) perindopril vs sorafenib; (c) fosinopril vs sorafenib; (d) losartan vs sorafenib. Statistical analysis was performed using log-rank test (Mantel-cox method). P values <0.05 were considered significant

The administration of perindopril, fosinopril, or losartan either as monotherapy or combined with sorafenib improved the histological picture of liver showing moderate regression of malignant and inflammatory changes with lowering of the grade of HCC to grade 1 (Figure 1).

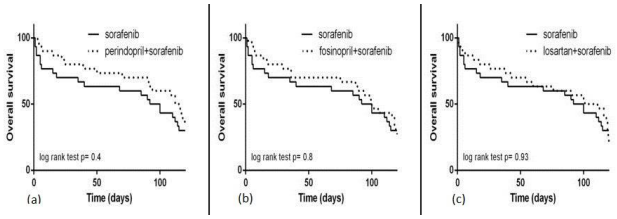

Figure 3: Kaplan-Meier survival curves of (a) (perindopril+sorafenib) vs sorafenib; (b) (fosinopril+sorafenib) vs sorafenib; (c) (losartan+sorafenib) vs sorafenib. Statistical analysis was performed using log-rank test (Mantel-cox method). P values <0.05 were considered significant

Kaplan-Meier survival curves are represented in Figure 2, 3 and 4. Normal mice had no mortality at the end of the experimental period (Figure 2a). On the other hand, DEN-treated mice had a higher mortality rate with a median survival time of 86 days (95% CI of ratio: 0.02 to 0.16) vs normal control (Figure 2a). The administration of sorafenib (Figure 2, 3 and 4), perindopril (Fig. 2b), fosinopril (Figure 2c), or losartan (Figure 2d) resulted in slight increases in the median survival time as follows: 96 days (95% CI of ratio: 0.29 to 1.4), 113 days (95% CI of ratio: 0.29 to 1.4), 100 days (95% CI of ratio: 0.41 to 1.5), and 106 days (95% CI of ratio: 0.35 to 1.46), respectively.

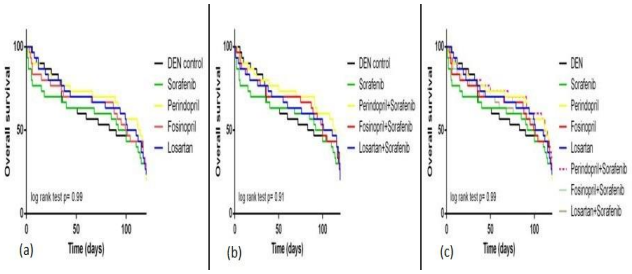

Figure 4: Kaplan-Meier survival curves of (a) monotherapy; (b) combination drug therapy; (c) monotherapy and combination therapy in DEN-treated mice. Statistical analysis was performed using log-rank test (Mantel-cox method). P values <0.05 were considered significant

Moreover, the administration of (perindopril + sorafenib, Figure 3a), (fosinopril + sorafenib, Figure 3b), or (losartan + sorafenib, Figure 3c) resulted in slight increases in the median survival time as follows: 114 days (95% CI of ratio: 0.3 to 1.38), 100 days (95% CI of ratio: 0.41 to 1.51) and 106 days (95% CI of ratio: 0.34 to 1.46). However, log-rank analysis for comparison of survival times revealed no significant differences in the lifespan of mice treated with the used drugs either as monotherapy (Figure 4a and 4c) or as adjunctive to sorafenib (Figure 4b and 4c) compared with DEN-treated mice.

Discussion

The discovery of new agents or repurposing current medications is vital to improving the survival and prognosis of HCC patients. RAS inhibition is an interesting prospective target for the chemoprevention of HCC. Therefore, we aimed to determine whether RAS inhibitors improve the overall survival of HCC in mice making a direct comparison for their effects with sorafenib which is the only efficacious drug that was found to prolong survival only for (2.8 months) [10] in HCC patients.

It is well documented that RAS plays an important role in promoting tumour growth [26] [27]. The use of ACEIs or ARBs has been found to inhibit different types of cancer including lung, breast, pancreatic, ovarian, prostatic, brain, colon, and liver cancer [28]. Furthermore, our lab demonstrated that the use of the ACEIs, perindopril and fosinopril and the ARB, losartan was found to decrease alpha-fetoprotein significantly and affect regression of malignant changes with improvement in liver histology in HCC mice through multifaceted mechanisms including the inhibition of angiogenesis, profibrotic mediators and inflammatory pathways (unpublished data)

Also, in the current study, combination therapy was evaluated to examine the potential augmenting effects of such safe agents when co-administered with sorafenib experimentally. Notably, a similar combination was recently evaluated clinically in which authors stated that patients treated with sorafenib plus RAS inhibition had a better median overall survival (19.5 mo) compared to those treated with either sorafenib (10.9 mo) or RAS inhibition (9.7 mo) alone ($p = 0.043$) [29]. Another observational study found that the use of ARBs during erlotinib treatment may prolong OS of patients with metastatic non-small cell lung cancer (NSCLC) [30].

The selection of perindopril (a high tissue affinity ACEI) and fosinopril (a low tissue affinity ACEI) [31] [32] was planned to evaluate the concept of class effect or interchangeability regarding therapeutic and side effects of ACEIs [33] [34]. Moreover, we evaluated the effect of blocking RAS signalling through antagonising Ang II via blocking its AT1 receptors using the ARB, losartan.

HCC in DEN-treated mice was evident by the appearance of malignant enlarged hepatocytes upon histologic examination. The histologic picture of HCC in mice treated with DEN was improved after treatment with the selected ACEIs, perindopril and fosinopril and the ARB, losartan either as monotherapy or when combined with sorafenib. This was manifested as a regression of HCC from grade (3) to grade (1) indicating the inhibition of malignant transformation and lower tumour production rates.

In the current study, DEN was used to induce HCC. Mice mainly develop liver tumours, but also gastrointestinal [35], skin, respiratory and haematopoietic tumours. The carcinogenic capacity of DEN is situated in its capability of alkylating DNA structures.

The DEN-induced tumorigenesis is mediated through its metabolites formed through its bioactivation by cytochrome P450 (CYP) enzymes [36]. An ethyldiazonium ion is formed and causes DNA damage by reacting with DNA-bases (nucleophiles). DEN works in a dose-dependent manner [37] to induce HCC after a period of latency. Genetically, the DEN-model verifies to be a good representation of HCC connected with poor prognosis [38]. When adult mice are exposed to a short time weekly administration of DEN, it leads to a higher incidence of a tumour in a shorter time span. For example, administration of 35 mg/kg DEN to mice weekly leads to HCC after 20-35 weeks [39].

The lifespan of DEN-treated mice was significantly decreased compared with normal mice that showed no mortality. This is consistent with DEN-induced systemic toxicity. The lifespan of mice did not significantly change by drug treatment compared with DEN-treated mice. The recent retrospective study published in 2017 by Pinter, Weinmann [29] demonstrated that the overall survival was increased in patients with HCC by the inhibition of RAS. The discrepancy between our results and the results of this study might be attributed to the repetitive administration of DEN that can have a detrimental impact on animals' survival because DEN can affect the development of aggressive metastatic lesions in the lungs [40] [41]. Also, we noticed that some animals developed solid metastatic tumours distributed all over the body particularly in the thigh.

The current study unveiled some interesting findings. First, the histologic picture of liver tissues was improved upon treatment with perindopril, fosinopril, or losartan as monotherapy or as adjunctive therapy to sorafenib and was comparable to each other and to that of sorafenib. These results demonstrate that the tissue affinity of the ACEI has no impact on its hepatoprotective effect in this model of HCC, and that is interfering the RAS either through the inhibition of ACE or the blockade of AT1 receptors has the same histologic benefit. However, perindopril seems more promising because perindopril-treated mice showed the highest survival either as monotherapy or when combined to sorafenib despite statistical insignificance. Hence, perindopril needs further evaluation with changing doses and therapy duration.

Second, despite the promising effects of perindopril, fosinopril, or losartan for managing HCC as monotherapy, they failed to produce an additive pronounced improvement from the histopathological view when administered in combination with

sorafenib. Based on the current data, it is not imaginable to explain the lack of the additive effect of the drugs used and sorafenib. Therefore, a pharmacokinetic study is needed to examine whether inhibitory effects of sorafenib on phase I enzymatic reactions might affect the metabolic activation of the prodrugs, perindopril, fosinopril and losartan.

Some studies described that sorafenib could induce mild liver dysfunction [42] [43] or severe hepatitis [44]. Metabolic dysfunction has been suggested as a mechanism for sorafenib-induced liver injury [45]. Consequently, another study is needed to examine the effect of RAS inhibitors on the sorafenib-induced liver injury, suggesting augmentation of the deleterious effects of sorafenib on the liver tissue by concomitant administration of RAS inhibitors providing an alternative explanation for the lack of more improvement upon histological examination.

In conclusion, the present results provide a potential for the therapeutic benefits of ACEIs and ARBs in liver tissues suggesting the prospective use of ACEIs or ARBs in managing patients with HCC either alone or in combination with other interventions. Furthermore, a long-term repetitive administration of DEN is not suitable to study survival in experimental animals due to detrimental effects on other organs particularly respiratory and haematopoietic systems. Alternatively, single dose or short-term administration of DEN with a longer latency time is the most suitable model for performing survival analysis in rodents.

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Basic Science

Presentation of the Molecular Subtypes of Breast Cancer Detected By Immunohistochemistry in Surgically Treated Patients

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Keywords: Subtypes of breast cancer; Luminal A; Luminal B HER-2 negative; Luminal B HER-2 positive; HER-2 enriched; Triple-negative

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Abstract

INTRODUCTION: The detection of estrogen, progesterone and HER-2 neu receptors on the surface of the tumour cell is a significant prognostic factor, alone or in combination. The presence or absence of receptors on the surface of the tumour cell is associated with the conditional gene expression in the tumour cell itself. Based on these genetically determined expressions of the tumour cell, five molecular subtypes of breast cancer have been classified on the St. Gallen International Expert Consensus in 2011 that can be immunohistochemically detected, with each subtype manifesting certain prognosis and aggression.

AIM: Analyzing the presentation of molecular subtypes of breast cancer that are immunohistochemically detected in surgically treated patients at the Clinic for Thoracic and Vascular Surgery.

MATERIAL AND METHODS: We used the international classification on molecular subtypes of breast cancer which divides them into: Luminal A (ER+ and/or PR+, HER-2 negative, Ki-67 < 14%), Luminal B with HER-2 negative (ER+ and/or PR+, HER-2 negative, Ki-67 ≥ 14%), Luminal B with HER-2 positive (ER+ and/or PR+, HER-2+, any Ki-67), HER-2 enriched (ER-, PR-, HER-2+), and basal-like (triple negative) (ER-, PR-, HER-2 negative, CK5/6+ and/or EGFR+). A total of 290 patients, surgically treated for breast cancer, were analysed during 2014.

RESULTS: In our analysis, we found that Luminal A was present in 77 (26.55%) patients, Luminal B HER-2 negative was present in 91 (31.38%) patients, Luminal B HER-2 positive was present in 70 (24.14%) patients, HER-2 enriched was present in 25 (8.62%) patients and basal-like (or triple negative) was present in 27 (9.31%) patients.

CONCLUSION: Detecting the subtype of breast cancer is important for evaluating the prognosis of the disease, but also for determining and providing an adequate therapy. Therefore, determining the subtype of breast cancer is necessary for the routine histopathological assay.

Introduction

Breast cancer is the most frequent malignant disease among women worldwide, but also in the Republic of Macedonia [1] [2] [3] [4]. Also, breast cancer is the leading cause of cancer mortality [1] [2] [3] [4]. Today, many factors are listed as most important ones for determining the prognosis of breast cancer, like tumour size, histologic subtype, tumour grade, lymphovascular invasion of tumour cells and axillary lymph node status. However, the presence of hormonal receptors (estrogen and progesterone) on the surface of the tumour cell, the presence of HER-2 neu receptor and other factors have been added to this list in the last twenty years [5]. Also, the biological

potential for proliferation and dividing was routinely examined, represented as Ki67 value. Today, every patient that is surgically treated undergoes routine examination with standard macroscopic and microscopic histological analysis, TNM staging, staging by immunohistochemical recognition of estrogen receptors (ER), progesterone receptors (PR), the presence of HER-2 neu receptors, and the prognostic value of Ki67 [6]. Each of these parameters, alone or in combination, determines the biology and aggression of the tumour, but also gives us the opportunity to treat the given type of breast cancer properly. The combination of these parameters gives us the opportunity to determine the genetic subtype of breast cancer. According to the new classification system for breast cancer subtypes presented in St. Gallen, which we use, breast cancer is divided in Luminal A, Luminal B with HER2 negative, Luminal B with HER2 positive, HER2 enriched and basal-like (triple negative) [7].

Analyzing the presence of breast cancer subtypes in our materials, and comparing the results with other studies to see if some subtypes in our materials differ from other studies published before, also, determining if the subtypes and the clinical stage are somehow correlated.

Material and Methods

A total of 290 patients, who were surgically treated for breast cancer at the University Clinic for Thoracic Surgery, Skopje, Republic of Macedonia were analysed during 2014, with complete history, using all parameters.

All cases underwent standard histological examination, including macroscopic and microscopic analysis with standard H&E staining. Immunohistochemical staining for ER, PgR, HER-2 and Ki-67 were performed on sections of formalin-fixed paraffin-embedded tissue from the primary tumours. Pathohistological tests were conducted in three accredited laboratories (two in the Institute of Pathology at the Medical Faculty in Skopje and one in a private laboratory).

Upon microwave-pretreated in citric acid (10 mM), monoclonal mouse antibody to ER, PgR, HER-2 or Ki-67 was applied for 30 min at room temperature using the following dilutions: anti-ER-1 : 100, anti-PgR-1 : 80 (DAKO laboratories, UK); pre-diluted anti-HER-2 (Hercept test, DAKO Laboratories, UK); anti-Ki-67-1 : 200 (DAKO Laboratories, UK). Upon three rinses in Tris-buffered saline (TBS) and incubation with the secondary antibody, positive brown staining was detected using standard avidin and biotinylated horseradish peroxidase (ABC) technique with 3, 3′-diaminobenzidine (DAB) as the chromogen. Slides were then counterstained in Mayer's haematoxylin for 10 seconds, dehydrated in graded alcohol, mounted and scored.

Positive and negative controls were performed with each stain, and surgical specimens from the same patient were stained on the same run.

For persistence of estrogen and progesterone receptors were included all results with +, ++ or +++ on immunohistochemical examination. For persistence of HER-2 receptors were included all patients with +++ result on immunohistochemical analysis.

In cases where ICT determined HER-2 neu positive status + or ++ patients underwent FISH analyses for defining the HER2-neu gene amplification status.

Pathohistological, grading and staging criteria for breast cancer were determined by using criteria from American Join Committee (AJC) and TNM classification according to UICC (International Union for Cancer Control) [8] [9]. According to the new classification system for breast cancer subtypes presented in St. Gallen, which we use, breast cancer is divided in Luminal A, Luminal B with HER2 negative, Luminal B with HER2 positive, HER2 enriched and basal-like (triple negative) [7].

Statistical analysis was performed with Statistica 7 by using standard descriptive analyses, χ^2 test and ANOVA test for analysing the variance.

Results

Patient's age was ranged between 18-90 years, an average of 57.6 years. The mean size of a primary tumour was 30.27 $\pm$ 18.3 mm. Axillary lymph nodes metastases were detected in 59% of the patients.

We used the new St. Gallen classification system for defining breast cancer subtypes into five groups. (Table 1) [7].

Table 1: Definition of subtypes of breast cancer- St. Gallen classification

Subtypes of breast cancer	Er and Pr	Her-2	Ki67
Luminal a	Er + and/or pr +	Her-2-	Ki67<14%
Luminal b with her-2 negative	Er+ and/or pr+	Her-2-	Ki-67≥14%
Luminal b with her-2 positive	Er + and / or pr +	Her-2 +	Any ki-67
Her-2 enriched	Er-, pr-	Her-2 +	Any ki-67
Basal-like (triple negative)	Er-, pr-	Her-2-	Ck5/6 + and/or egfr +

Subtypes are characterised based on tumour size, lymph nodes involvement, histologic subtype, the persistence of receptors, lymphovascular invasion, the presence of p53 and stage, and are presented in Tables 2-9.

Table 2: Characteristics of subtypes according to the age of the patients

	LA	LB-Her2-	LB-Her2+	HER2+	TN	Total	
Number	77	91	70	25	27	290	
(%)	(26.55%)	(31.38%)	(24.14%)	(8.62%)	(9.31%)	(100%)	
Mean age (y)	57.83	58.83	57.64	552.72	56.74	57.56	X=57.6 y

From our analysis, we found that:

- Luminal A was present in 77 (26.55%),

- Luminal B HER2 negative was present in 91 (31.38%),

- Luminal B HER2 positive was present in 70 (24.14%),

Table 3: Characteristics of subtypes according to the size of the tumour

	LA	LB-Her2-	LB-Her2+	HER2+	TN	Total	p
Tumour size							
Tis	6	2	3	2	0	13 (4.48%)	
T1a	11	11	12	1	2	37 (12.76%)	
T1b	8	3	3	1	1	16 (5.51%)	
T1c	9	17	12	7	3	48 (16.55%)	
T2	37	43	36	9	18	143 (49.31%)	
T3	3	6	2	2	2	15 (5.17%)	
T4	3	9	2	3	1	18 (6.19%)	1.0
Number	77	91	70	25	27	290	

- HER2 enriched was present in 25 (8.62%) and

- Basal-like (or triple negative) was present in 27 (9.31%) patients.

Table 4: Characteristics of subtypes according to the size of a tumour and lymph nodes involvement

	LA	LB-Her2-	LB-Her2+	HER2+	TN	Total	p
Mean tumor size (mm)	29.3	31.8	27.2	31.3	35.0	X = 30.3	
Axillary LN status							
N0	41	28	26	14	10	119 (41.03%)	
N+	36	63	44	11	17	171 (58.97%)	0.99
Number	77	91	70	25	27	290	

Discussion

Breast carcinoma is a heterogeneous disease with several clinical and histopathological presentations, which present different gene expressions in several subtypes and molecular profiles, hence giving different predictive and prognostic characteristics for the patients. Gene expressions were analysed using DNA microarrays. Due to the cost of DNA analysis, the use of immunohistochemical analysis of markers, which have been used as surrogate tools for defining subtypes of breast cancer, was generally accepted. According to the 2011 St. Gallen consensus conference, 5 subtypes of breast cancer were defined using the presence of receptors on the surface of the tumour cell, and the measuring values of Ki67 [7] [10] [11].

Table 5: Characteristics of subtypes according to histologic subtype

	LA	LB-Her2-	LB-Her2+	HER2+	TN	Total	p
Histologic subtype							
Ductal	59	76	58	20	24	237(81.72%)	
Lobular	11	6	7	2	1	27 (9.31%)	
Other	7	9	5	3	2	26 (8.97%)	0.99
Number	77	91	70	25	27	290	

Many of the prognostic factors predicting the disease are very well-known, and so is their biological mode of action and how they work to spread the disease in the body. Estrogen receptors are on the surface of the tumour cell, so once estrogen binds with the estrogen receptors, it activates many processes in the cell and stimulates growth and cell division. Hence, estrogen stimulates tumour growth. Giving drugs that block estrogen receptors or drugs that block estrogen synthesis can stop the tumour growth.

Table 6: Characteristics of subtypes according to histological grade

	LA	LB-Her2-	LB-Her2+	HER2+	TN	Total	p
Histologic grade							
1	6	3	4	0	3	16 (5.52%)	
2	58	56	49	19	20	202 (69.65%)	
3	13	32	17	6	4	72 (24.82%)	0.99
Number	77	91	70	25	27	290	

The same situation applies to the presence of HER-2 neu receptors. HER-2 is a membrane tyrosine kinase and oncogene that is overexpressed and gene amplified in about 20% of the breast cancer cases. When activated it provides the cell with potent proliferative and anti-apoptosis signals, and it is the major driver of tumour development and progression of breast cancer.

Table 7: Characteristics of subtypes according to the persistence of receptors (estrogen, progesterone and HER2 neu)

	LA	LB-Her2-	LB-Her2+	HER2+	TN	Total	p
Estrogen receptors							
Positive	72	86	57	0	0	215 (74.14%)	
Negative	5	5	13	25	27	75 (25.86%)	1.0
Number	77	91	70	25	27	290	
Progesterone receptors							
Positive	73	88	65	0	0	226 (77.93%)	
Negative	4	3	5	25	27	64 (22.07%)	1.0
Number	77	91	70	25	27	290	
Her 2 neu receptors							
Positive	0	0	70	25	0	95 (32.76%)	
Negative	77	91	0	0	27	195 (67.24%)	1.0
Number	77	91	70	25	27	290	

Overexpression will activate many pathways in the cell, resulting in uncontrolled cell growth and division, causing the tumour to grow uncontrollably. Target drug delivery, monoclonal antibody trastuzumab (Herceptin), will block these receptors, and control a tumour. Moreover, giving chemotherapeutics that interact with the rapidly

dividing cells will control a tumour. Ki67 is the factor that shows the proliferative activity of tumour cells. Ki67 correlates with the S phase of the cell cycle and with the mitotic activity. A normal breast cell has a proliferative activity of 3% (3% of the cells are in the dividing stage).

Table 8: Characteristics of subtypes according to the persistence of p53, LVI (lymphovascular invasion) and values of Ki67

	LA	LB-Her2-	LB-Her2+	HER2+	TN	Total	p
P53							
Positive	27	49	29	11	15	131(45.18%)	
Negative	50	42	41	14	12	159 (54.82%)	0.99
Number	77	91	70	25	27	290	
LVI							
Positive	22	45	27	11	12	117 (40.34%)	
Negative	55	46	43	14	15	173 (59.65%)	0.99
Number	77	91	70	25	27	290	
Ki67							
Up to 14%	77	0	36	4	9	126 (43.44%)	
More than 14%	0	91	34	21	18	164 (56.56%)	1.0
Number	77	91	70	25	27	290	

Higher Ki67 index correlates with young age, larger tumours, positive lymph nodes, negative estrogen receptors and positive HER-2 receptors [12]. An activity that is higher than 14%, at some studies large than 20%, shows aggressive tumours with poor prognosis and shorter overall survival [10] [11] [12].

Table 9: Characteristics of subtypes according to the stage of the disease

	LA	LB-Her2-	LB-Her2+	HER2+	TN	Total	p
Stage							
0	1	2	0	0	0	3 (1.03%)	
IA	16	6	11	7	3	43 (14.83%)	
IB	3	4	2	0	0	9 (3.10%)	
IIA	26	26	18	6	7	83 (28.62%)	
IIB	13	15	18	6	8	60 (20.69%)	
IIIA	7	17	13	2	4	43 (14.83%)	
IIIB	4	5	2	2	2	15 (5.17%)	
IIIC	7	16	6	2	3	34 (11.72%)	1.
Number	77	91	70	25	27	290	

Knowing the subtype:

- we can predict the biology of a tumour and its future behaviour;
- we can predict the prognosis of the disease;
- we can plan a targeted therapy for some of the subtypes.

Knowing the prevalence of subtypes in one population can help plan a general therapeutic approach [16].

Some authors define 4 subtypes: Luminal A, Luminal B, HER-2 enriched and basal cell (triple negative) (Valejos, Carey), other authors define 6 subtypes: Luminal A, Luminal B, HER2+, basal-like (triple negative), normal breast cell-like and Claudin-low (Eroles), but the most frequently used classification encompasses 5 subtypes [7] [13] [14] [17]. In practice, breast subtypes are defined by detecting the presence of estrogen, progesterone and HER-2 neu receptors on the surface of the malignant cell using immunohistochemical assays. Knowing that the presence or absence of receptors on the surface of breast cancer cell is conditioned by gene mutations and overexpression, subtypes can also be detected by assessing the gene expression. This is why the term genotype of breast cancer is cited in the literature.

The most frequent type is Luminal A which is found in 50-72% of the patients with breast cancer. Patients with this type of cancer have the best prognosis, i.e. low proliferative index, good differentiation, with the lowest risk of local recurrence and relapse [13] [15] [16] [17] [18].

However, there are different values registered in literature regarding this subtype: Italy 34%, Saudi Arabia 3.9%, China 65.3% and Japan 71% [24] [25] [26] [27].

The suggested therapy for these patients is third-generation aromatase inhibitors in postmenopausal women, selective estrogen receptor modulators (like tamoxifen) and selective estrogen receptor modulators (like fulverstone) [13] [15] [16] [17] [18].

In our examination, Luminal A type was detected only in 26.55% of the patients.

Luminal B subtype is characterised with positive estrogen receptors, with positive or negative HER2 receptors, with a higher Ki-67 value of over 14%, which in both types of Luminal B gives worse prognosis than Luminal A subtype.

Luminal B type is found in 10-20% of the patients with breast cancer, and in our examination, the HER-2 negative was detected in 31.38% and HER-2 positive in 24.14 % of the patients. Literature references are as follows: Italy 36%, Egypt 24.6% [24] [28]. This shows that most of the tumours in our group are aggressive. Luminal B subtype is much more aggressive than the Luminal A subtype and is characterised by poor differentiation, more frequent bone metastases and with a worse prognosis. Many authors suggest that patients in this subtype are younger patients with bigger tumours, with positive nodal status and higher N stage [16]. Given the presence of larger tumours and the advanced stage of the disease, Luminal B findings are more frequent in our study. Regarding patient's age, there was no difference found between Luminal A and Luminal B types; however, in our study, Luminal B prevailed in older patients [13] [15] [16] [17] [18].

The suggested treatment is with tamoxifen, but also chemotherapy in neoadjuvant and adjuvant courses [13] [15] [16] [17] [18].

HER-2 enriched subtype is found in 15-20% of the patients. In our group, it was detected in 8.62% of the patients. This subtype is characterized by high proliferative index and poor differentiation in most of

the patients, and p53 mutations are very often detected. This is a very aggressive type of a tumour, with only 12% of the patients surviving 10 years [13] [14] [16] [17] [18].

The suggested treatment requires target HER-2 therapy with monoclonal antigen trastuzumab (Herceptin) which changes the prognosis. This therapy needs to be combined with chemotherapy in the neoadjuvant and adjuvant protocol. Treatment with trastuzumab in combination with DM1 is also possible [13] [15] [16] [17] [18].

Subtype Basal-like (triple negative) is characterised with larger tumours, poor differentiation, high mitotic index and tumour necrosis. This type is found in 10-20% of the patients. A similar subgroup of this subtype is Claudin-low, where the only differentiation is the difference in EGFR and the fact that this type is found in 12-14% of the patients. Both subgroups have bad prognosis, poor differentiation and high mitotic index. Very often metastases in visceral organs, lungs and CNS are detected. This type has the worst prognosis, and very often the disease relapses in the first three years, and p53 mutations are very often detected [13] [14] [15] [16] [17] [18] [34].

In our study, the triple negative was detected in 9.31% of the cases.

The suggested therapy is chemotherapy, but also PARP inhibitors (poly ADP ribosome polymerase inhibitors) like olaparib in detected BRCA1 or BRCA2 mutations [14] [15].

The normal breast cell-like subtype that is defined by some authors is found in 5-10% of the patients. This type doesn't respond to neoadjuvant therapy, only to adjuvant chemotherapy protocol. Often, it is well-differentiated with low proliferation index and is characterised by median overall survival [14].

We registered the difference in frequencies among our subgroups and those cited in the literature. This explains the heterogeneity of breast cancer across the world. It is cited in the literature that some subtypes are more frequent in certain races (triple negative is more frequent in African-American women) [13] [19]. Knowing the prevalence of subtypes in one population, we can plan a general therapeutic approach [16].

There is no significant difference in age regarding subgroups of breast cancer, patients range between 52,72 and 58,83 years, except in Luminal B HER negative and HER enriched subgroups (P = 0.0255). Patient's age in subgroups of breast cancer is shown in Figure 1.

The mean size of tumours in different subgroups ranges between 27.18 and 35 mm. The biggest tumour diameter was found in the triple negative subgroup, but there were no significant differences in the whole group, and also between subgroups. The same situation applies to the tumour diameter defined as T stage with no difference in the position between subgroups.

High differentiation values (G3-low differentiation) of the malignant cell in Luminal B subtype of breast cancer were detected in 35.16% of the patients. In triple negative subtype, G3 was detected only in 14.8% of the patients, which is contrary to the case results reported in many studies in the literature [13] [14] [17].

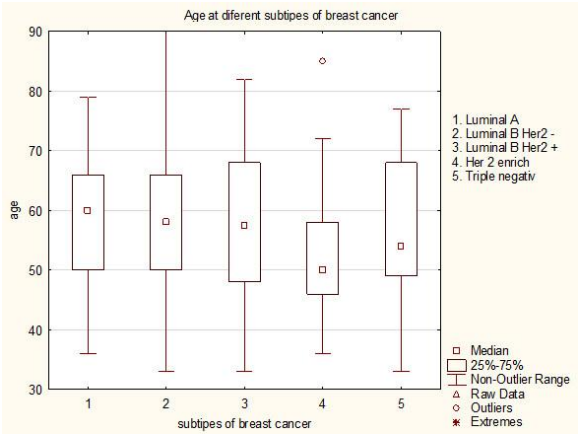

Figure 1: Patients age according to subgroups of breast cancer

Regarding lymphovascular invasion, the highest values were detected in Luminal B HER-2 negative, i.e. in 49.45% of the patients, which suggested invasive and aggressive subtype of breast cancer, with a tendency for lymphatic spread and higher frequency of axillary lymph node involvement (69.23%). The relatively low values of lymphovascular invasion in triple negative subtype are interesting to comment (44.44%), with low axillary lymph node involvement (62.96%), which correlates with the findings in the literature. Knowing the aggression of triple negative subtype of breast cancer, we can conclude that the spreading of malignant cells follows other pathways other than lymphatic [14] [17] [30] [31] [32] [33] [34] [35].

Regarding the values of Ki67, the factor that suggests the proliferative activity of the tumour, its aggression and biology, values higher than 14% were detected in Luminal B HER negative subtype (in all patients), but also in HER-2 enriched and triple-negative subtypes. On the contrary, no values higher than 14% were detected in Luminal A subtype, which suggests that this subtype is less aggressive [13] [14] [15] [16] [17] [18].

The patterns of distribution of cells positive for estrogen, progesterone and HER-2 neu receptors were significantly different in subgroups, which is normal because we know that these receptors are main factors for defining the different subtypes of breast cancer [7] [14].

Knowing the subtype of breast cancer, in addition to the histological type and TNM stage, can suggest further prognosis of the disease, detect the spreading and find where metastases can appear later, but can also suggest further therapeutic approach [15].

Also knowing that all factors that determine breast cancer subtypes can be evaluated from core biopsy materials before starting treatment, some subtypes (the more aggressive ones) can be treated with adequate drugs in neoadjuvant protocol [14].

In conclusion, detecting the subtype of breast cancer is important for disease prognosis, but also for determining and providing an adequate therapy. Hence, the molecular subtype of breast cancer needs to be determined in a routine histopathological assay.

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Basic Science

Colonic Stem Cells Expression of Lgr5 and CD133 Proteins as Predictive Markers in Colorectal Cancer among Egyptian Patients

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Abstract

AIM: Colorectal cancer is the fourth common tumour in Egypt after lymphoid, breast and urinary tumours. The study aims to assess the expression of Lgr5 and CD133 in pre-malignant (adenomatous polyps and IBD), malignant colorectal lesions and normal colonic mucosa by immunohistochemical staining.

MATERIAL AND METHODS: This prospective study was done on 100 patients presenting with colonic symptoms, patients were divided into four groups; group I including 20 patients in the control group, group II including 20 ulcerative colitis (U.C) patients, group III including 20 patients with adenomatous polyps and group IV including 40 patients with colorectal cancer (CRC).

RESULTS: Lgr5 and CD133 expression was significantly higher in carcinoma than in adenomas, IBD and normal mucosa ($P < 0.001$). Lrg5 and CD133 was positively correlated with histological grade ($P = 0.001$), depth of invasion ($P = 0.001$), lymph node metastasis ($P < 0.001$), distant metastasis ($P < 0.004$) and TNM stage ($P < 0.001$).

CONCLUSION: Role of Lgr5 and CD133 as stem cell marker was expressed and presented with different expression in the normal colonic mucosa, adenoma and CRC and showed increased expression in an advanced stage of CRC. This may suggest its possible involvement in colorectal tumorigenesis and invasion.

Introduction

Worldwide, colorectal cancer is the third most commonly diagnosed cancer in males and the second in females [1]. Traditional models of tumorigenesis suggest that every cell within the tumour population is capable of tumour initiation and propagation. The newly discussed cancer stem cell (CSC) model, however, proposes that only a small fraction of cells possess tumour propagation abilities. This hypothesis raises questions regarding the efficiency of current diagnostic and therapeutic measures, suggesting that CSCs are a rational target for the development of robust diagnostic, therapeutic, and follow-up strategies [2]. Many pieces of evidence suggest that cancer is a disease of stem cells [3] [4]**.** The cancer stem cell model was described for hematologic malignancies in 1997, and since then evidence has emerged to support it for many solid tumours as well, including colon cancer, this model proposes that certain cells within the tumour mass are pluripotent and capable of self-renewal and have an enhanced ability to initiate distant metastasis. Becker et al., [5] in their study suggest that (1) Lgr5 is a potential marker of intestinal stem cells in humans and (2) loss of restriction to the stem cell niche is an early event in the premalignant transformation of stem cells and may play a role in carcinogenesis. Femia et al., [6] found overexpression of Lgr5 in precancerous lesions and tumours, they support Lgr5 as putative neoplastic stem cell marker, and they identified Lgr5-positive cells are co-expressing nuclear β-catenin (NBC) which could be a subpopulation with the highest stem cell

features. CD133 (Prominin-1), a transmembrane glycoprotein which was first identified as a potential cancer stem cell (CSC) marker for brain tumours [7]. Both O'Brien et al., [8] and Ricci-Vitiani et al., [9] found that the tumours formed from CD133 positive cells injected into severe combined immunodeficiency (SCID) mice resembled a tumour from which they were taken and formed tumours of differentiated cell types that were mostly CD133 negative. The CD133 negative cells from these tumours did not form metastases in mice even when injected at much higher numbers than CD133 positive cells.

This aim of the study is designed to investigate the distribution and expression of immunostaining of Lgr5 and CD 133 proteins in stem cells of human colon in malignant and premalignant colonic lesions in Egyptian patients.

Patients and Methods

This prospective case-control study was conducted on 100 subjects who attended the Gastrointestinal Endoscopy unit in Nasser Institute for research and treatment Hospital, and in collaboration with Theodor Bilharz Research Institute, during the period from March 2015 to March 2017. Patients were categorised into four groups:

Group I: included 20 patients had a normal colonoscopy and served as a control group.

Group II: included 20 patients with IBD (ulcerative colitis) diagnosed endoscopically and pathologically.

Group III: included 20 patients who were found to have colorectal polyps endoscopically and proved to be adenomatous pathologically and served as adenoma group.

Group IV: included 40 patients with colorectal cancer diagnosed endoscopically and pathologically.

Inclusion criteria: Adult patients >18 years old and have eligible indications for colonoscopy as follow:

1. Patient with lower GIT symptoms including chronic diarrhoea, chronic constipation, alternating bowel habits and bleeding per rectum.

2. Cases with relevant alarming symptoms and signs for CRC, e.g. significant unexplained weight loss, unexplained anaemia.

3. Patients with remote metastases proved to be adenocarcinoma and were suspected to have CRC.

4. Patients underwent screening for CRC.

5. Patients who have a family or personal history of CRC, genetic CRC syndrome or adenomas.

6. Patients with inflammatory bowel diseases were diagnosed for more than 8 years.

7. Patients are known to have FAP, family history of FAP or history of previous adenomatous polyps.

Exclusion criteria: Patients previously received chemotherapy for colorectal malignancy.

Patients subjected to the following: Informed written consent, Full history taking and thorough clinical examination (pallor, abdominal masses). Laboratory investigations including Stool analysis, Complete blood count (CBC), erythrocyte sedimentation rate (ESR) and Carcino Embryonic Antigen (CEA). Abdominal ultrasound for scanning masses, metastases and lymphadenopathy. Full colonoscopic examination by using Pentax colonoscope by model number (*EC 3840 L*).

Tissue Samples (biopsy specimens)

Biopsies were taken from the inspected lesions and the nearby normal colonic mucosa. Biopsies were taken using a standard cold biopsy forceps (CFB-2.5- 230-S, Wilson Cook medical®). In the *Control Group* multiple biopsies were taken from the colonic mucosa in all cases while in *IBD Group* multiple biopsies were taken from the inflamed mucosa and multiple biopsies were taken from the nearby grossly normal colonic mucosa while in *Adenomas Group* polyps will be removed using a polypectomy snare and multiple biopsies were taken from the nearby mucosa and in *CRC Group* multiple biopsies were taken from cancer and multiple biopsies from the nearby mucosa.

Specimens were fixed in 10% buffered formalin. Paraffin blocks were prepared. Histopathologic sections were cut at 4 μm thick. All slides were treated with 3-aminopropyl-triethoxysilane (3APTES/SIGMA-A-3648). These slides were used instead of the ordinary albumenised slides to minimise staining artefacts and for better fixation of sections on the slides.

Immunohistochemical reaction was performed using avidin-biotin complex (ABC) immunoperoxidase technique. Sections were de-waxed in xylene and hydrated in descending grades of ethanol. Endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide and then in 100% methanol for 20 minutes.

Antigen retrieval was performed by microwaving the sections in citrate buffer (PH 6.0) for 15 minutes at 700 W. Sections were incubated overnight at 40C with the anti-human primary monoclonal antibodies against Lgr5 and CD133 (Santa Cruz Biotechnology Inc.; Santa Cruz, USA)

and diluted at 1:100 and 1:150 respectively. Next day, sections were washed in PBS then incubated with streptavidin-biotin-peroxidase complex and substantiated using a peroxidase/DAB (diaminobenzidine) enzymatic reaction for Lgr5 and CD133. Staining was completed by 5 to 10 minutes incubation with 3, 3'-diaminobenzidine (DAB)+substrate-chromogen which resulted in a brown-coloured precipitate at the antigen sites of Lrg5 and CD133 (cytoplasmic stain). Slides were washed in PBS for five minutes then placed in 70%, 95% and 100% alcohol for five minutes each. The nuclei were counterstained with Mayer's hematoxylin. Coverslips were mounted using Dpx. Positive and negative control slides for each marker were included in each session. As a negative control, liver tissue section was processed in the sequences mentioned above but with the omission of the primary antibodies.

All immunostained slides were analysed using Zeis microscope with high resolution (Axio Scope, Germany) in ten successive high-power fields (HPFs). Both Lgr5 and CD133 antigens were expressed as brown cytoplasmic staining. Two features of immuno-reactions were assessed separately on a semi-quantitative basis (H score) as follows: 1) the extent of staining was assessed as the percentage of positively stained cells in 10 HPFs in the highest expression (hot spot) areas in each case. Then, means of percentages were calculated. 2) The intensity of staining of the positive cells was relatively designated as + (mild or weak), ++ (moderate), and +++ (strong) according to Itoi et al., [10]. Then data were converted to immune-histochemical score (ranged from 1 to 6) by multiplying intensity and extent scores. An immunohistochemical score of 5-6 was considered strong immunoreactivity and was given score 3, 3-4 was considered moderate and was given score 2, and 1-2 was considered weak and was given score 1.

The data were analysed using Microsoft Excel 2010 and Statistical Package for Social Science (SPSS version 22.0) for Windows (SPSS IBM., Chicago, IL). Results were expressed as mean ± SD with 95% confidence interval using mean for quantitative variables, frequencies and percentages for qualitative ones. P<0.05 was considered statistically significant. Quantitative data were analysed by applying the one-way analysis of variance (ANOVA) test for comparison of the mean of more than two groups, while independent-samples t-test was used for comparison of the means of two groups. Chi-square test was used to compare proportions between two qualitative parameters.

Results

The demographic features of the whole studied patients and each group are summarised in (Table 1).

Table 1: Age and sex of the studied groups

Descriptive parameters		Control group N = 20	IBD group N = 20	Adenomas group N = 20	Malignant group N = 40	Total patients N = 100	P. Value
Age	Mean±SD	55.3±15.02	30.7±7.7	40.2±12.7	53.7±15.12	44.975±18.17	0.001**
Sex	Female	7 (20.0%)	8 (22.9%)	12 (10.9%)	16 (45.7%)	43 (43%)	0.9
	Male	13 (23.6%)	12 (21.8%)	8 (11.4%)	24 (43.6%)	57 (57%)	

Bleeding per rectum was the most common presentation in the studied patients; presenting 35% of the total patients, malignant group (37.5%), and adenoma group (50%). Weight loss was statistically highly significant (P = 0.03) in patients of the malignant group (80%).

Table 2: Clinical presentation in the studied groups

Clinical presentation	Control group N = 20	IBD group N = 20	Adenomas group N = 20	Malignant group N = 40	Total patients N = 100	P. Value
Abdominal pain	8 (40.0%)	5 (25.0%)	2 (10.0%)	5 (12.5%)	20 (20.0%)	0.04*
Bleeding per rectum	0 (0.0%)	10 (50.0%)	10 (50.0%)	15 (37.5%)	35 (35.0%)	0.001**
Constipation	6 (30.0%)	0 (0.0%)	0 (0.0%)	3 (7.5%)	9 (9.0%)	0.003**
Diarrhea	6 (30.0%)	0 (0.0%)	5 (25.0%)	2 (5.0%)	13 (13.0%)	0.001**
Anemia	0 (0.0%)	2 (10.0%)	1 (5.0%)	7 (17.5%)	10 (10.0%)	0.1
Weight loss	0 (0.0%)	0 (0.0%)	2 (10.0%)	8 (20.0%)	10 (10.0%)	0.03*
Mucorrhea	0 (0.0%)	3 (15.0%)	0 (0.0%)	0 (10.0%)	3 (3.0%)	0.006**

Mucorrhea was statistically highly significant (P = 0.006) in IBD group. Constipation and diarrhoea were statistically significant in control patients (Table 2; Figure 1A-F).

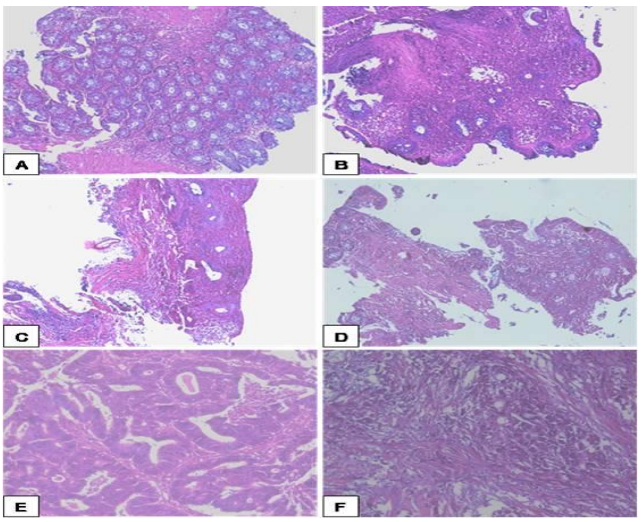

Figure 1: A) Control case of mild colitis (H & E, x 100); B) UC case, (H & E, x 100); C) UC case with mild dysplasia, (H & E, x 100); D) A case of CD (H & E x 100); E) A cases of well-differentiated adenocarcinoma (GII); F) A cases of poorly differentiated (H & E x 400)

Fungating mass was the most the most common endoscopic finding of CRC group (62.5%) while ulceration was present 60% of IBD group and the polypoid lesion was present 100% of adenoma group (Table 3).

CEA was highly elevated in CRC group (P = 0.001) with the high significant difference compared with other studied groups (370.4 mg/L vs 6.8, 6.9 and 5.8 mg/L, respectively) (Table 4).

Table 3: Endoscopic findings in the studied groups

		Control group N = 20	IBD group N = 20	Adenomas group N = 20	Malignant group N = 40	Total patients N = 100	P-Value
Site	Rectum	4 (20%)	6 (30%)	5 (25%)	10 (25 %)	25 (20%)	0.7
	Sigmoid	0 (0.0%)	0 (0.0%)	5 (25.0%)	8 (20%)	13 (13%)	0.01*
	Recto-sigmoid	10 (50%)	8 (40%)	4 (20%)	9 (22.5%)	31 (31%)	0.08
	Hepatic flexure	6 (30%)	0 (0.0%)	3 (15.0%)	9 (22.5%)	18 (18%)	0.07
	Caecum	0 (0.0%)	0 (0.0%)	3 (15.0%)	4 (10.0%)	7 (7%)	0.1
	Whole colon	0 (0.0%)	6 (30.0%)	0 (0.0%)	0 (0.0%)	6 (6%)	0.001**
Endoscopic finding	Mass	0 (0.0%)	0 (0.0%)	0 (0.0%)	25 (62.5%)	25 (20%)	0.001**
	Angio-dysplasia	2 (10.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (20%)	0.04*
	Polyp	0 (0.0%)	0 (0.0%)	20 (100.0%)	0 (0.0%)	20 (20%)	0.001**
	Hyperemia	3 (15.0%)	6 (30.0%)	0 (0.0%)	0 (0.0%)	9 (20%)	0.001**
	Ulceration	0 (0.0%)	12 (60.0%)	0 (0.0%)	5 (12.5%)	17 (20%)	0.001**
	Stricture	0 (0.0%)	2 (10.0%)	0 (0.0%)	10 (25%)	12 (20%)	0.008**
	Normal	15 (75.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	15 (20%)	0.001**

Lgr5 was positive in 37 patients (92.5 %), 12 patients (60%), 10 patients (50%), 3 patients (15%) in malignant, IBD, adenoma, and normal mucosa respectively, high statistically significance difference between groups at P < 0.001 (Table 5; Figure 2 A-E).

Table 4: Laboratory investigations of the studied groups

		Control N = 20 Mean ± SD	IBD N = 20 Mean ± SD	Adenomas N = 20 Mean ± SD	Malignant N = 40 Mean ± SD	Total patients N = 100 Mean ± SD	P. Value
Lab. Investigation	HB	13.3 ± 1.07	9.9 ± 1.01	10.9 ± .0.59	8.4 ± 16.7	11.4 ± 12.9	0.01*
	TLC	7.13 ± 2.7	8.79 ± 2.7	8.34 ± 2.4	7.94 ± 2.1	8.1 ± 12.3	0.1
	PLT	257.1 ± 111.3	215.6 ± 46.2	247.9 ± 132.9	214.15 ± 87.9	238.7 ± 92.7	0.1
	ESR	43.45 ± 21.8	61.4 ± 25.96	50.0 ± 26.9	66.7 ± 27.9	55.4 ± 24.3	0.02*
	CEA	6.8 ± 3.05	6.9 ± 2.05	5.8 ± 2.09	370.4 ± 395.6	97.5 ± 86.9	0.001**
Stool Finding	Blood	6 (30.0%)	4 (20.0%)	6 (30.0%)	7 (17.5%)	23 (23%)	0.5
	Mucous	4 (20.0%)	6 (30.0%)	4 (20.0%)	7 (17.5%)	21 (21%)	0.7
	EH	5 (25.0%)	6 (30.0%)	4 (20.0%)	9 (22.5%)	24 (24%)	0.8
	Giardia	4 (20.0%)	4 (20.0%)	4 (20.0%)	11 (27.5%)	23 (23%)	0.8
	Pus	1 (5.0%)	0 (0.0%)	2 (10.0%)	6 (15.0%)	9 (9%)	0.2

Four cases out of 5 (80%) of GI showed positive Lgr5 expression while 16 cases out of 18 (88.8%) of GII showed positive Lgr5 and 17 cases out of 17 (100%) of GIII showed positive Lgr5 expression. There is a significant difference between GIII compared to GII and GI at P < 0.01 and P < 0.05 respectively (Table 6; Figure 2 A-E).

Table 5: Immunohistochemical staining of Lgr5 (intensity & extent) and CD133 of the studied groups

		Control N = 20	IBD N = 20	Adenomas N = 20	Malignant N = 40	Total patients N = 100	P. Value
Lgr5 intensity	Negative	17 (85.0%)	10 (50.0%)	8 (40.0%)	3 (7.5%)	38 (38.0%)	0.001**
	Weak	3 (15.0%)	4 (20.0%)	4 (20.0%)	2 (5.0%)	13 (13.0%)	0.2
	Moderate	0 (0.0%)	6 (30.0%)	8 (40.0%)	8 (20.0%)	22 (22.0%)	0.01*
	Strong	0 (0.0%)	0 (0.0%)	0 (0.0%)	27 (67.5%)	27 (27.0%)	0.001**
LRG5 Extent	Negative	17 (85.0%)	10 (50.0%)	8 (40.0%)	3 (7.5%)	38 (38.0%)	0.001**
	Less5	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (5.0%)	2 (2.0%)	0.3
	5-25	3 (15.0%)	2 (10.0%)	4 (20.0%)	0 (0.0%)	9 (9.0%)	0.05*
	25-50	0 (0.0%)	2 (10.0%)	0 (0.0%)	0 (0.0%)	2 (2.0%)	0.04*
	50-75	0 (0.0%)	6 (30.0%)	8 (40.0%)	8 (20.0%)	22 (22.0%)	0.01*
	75-100	0 (0.0%)	0 (0.0%)	0 (0.0%)	27 (67.5%)	27 (27.0%)	0.001**
CD133	Negative	17 (85.0%)	6 (30.0%)	8 (40.0%)	0 (0.0%)	31 (31.0%)	0.001**
	Mild	3 (15.0%)	8 (40.0%)	6 (30.0%)	3 (7.5%)	20 (20.0%)	0.01*
	Moderate	0 (0.0%)	6 (30.0%)	6 (30.0%)	11 (27.5%)	23 (23.0%)	0.05*
	Strong	0 (0.0%)	0 (0.0%)	0 (0.0%)	26 (65.0%)	26 (26.0%)	0.001**

CD133 was expressed in 40 patients (100%), 12 patients (60%), 14 patients (70%) and 3 patients (15%) in malignant, adenoma, IBD and control patients respectively, with high statistically significance between different groups at P < 0.001. A very significant difference in CD133 expression was found between colorectal carcinoma and normal mucosa (P < 0.001) (Table 5; Figure 3 A-F).

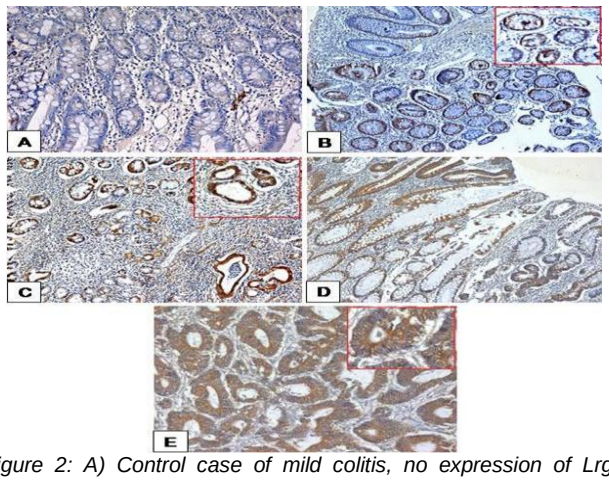

Figure 2: A) Control case of mild colitis, no expression of Lrg5 immunostain in the cytoplasm of cells lining the colonic glands (IHC, DAB, x200), B) Ulcerative Colitis case, with moderate expression of the Lrg5 protein in the cytoplasm of cells lining the colonic gland. (IHC, DAB. x40), C) A case of Crohns Disease, with moderate expression of CD Lrg5 protein in the cytoplasm of cells lining the gland and crypt epithelium (red arrow)(IHC, DAB. x40), D) A cases of tubular adenoma, showing moderate expression of CD Lrg5 protein in the cytoplasm of cells lining the gland(red arrow) and crypt epithelium (black arrow) (IHC, DAB. x100), E) A cases of moderately differentiated adenocarcinoma (GII), showing marked expression of CD Lrg5 protein in the cytoplasm of cells lining the gland(red arrow) and crypt epithelium (black arrow) (IHC, DAB. x100)

As regards this correlation between immunoexpression of CD133 and histopathological grade, the intensity of CD133 was strong in GII (55.7%) and GIII (88.3%). There is a significant difference between GIII compared to GII and GI at P<0.01 and P < 0.05 respectively (Table 7; Fig. 3 A-F).

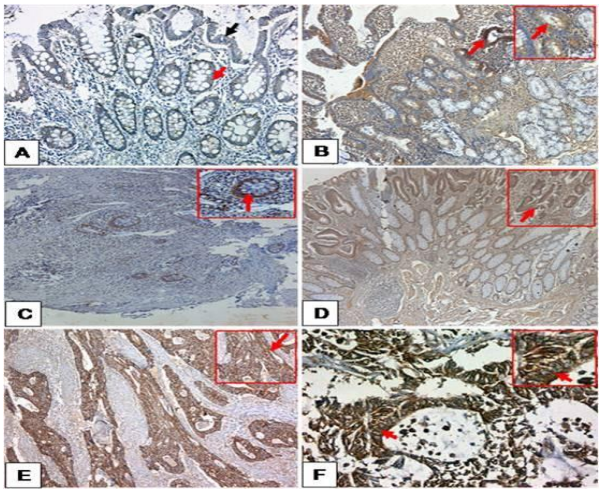

Figure 3: A) Control case of mild colitis, showing mild expression of CD133 immunostain in the cytoplasm of cells lining the crypt epithelium(black arrow) and glands (red arrow)(IHC, DAB, x200), B) Ulcerative Colitis case, showing mild to moderate expression of CD133 immunostain in the cytoplasm of cells lining the colonic glands (red arrow) (IHC, DAB, x100), C) A case of Crohns Disease, showing moderate expression of CD133 immunostain in the cytoplasm of cells lining the colonic glands (red arrow) IHC,DAB, x100), D) A cases of tubulovillous adenoma, showing moderate expression of CD133 immunostain in the cytoplasm of cells lining the colonic glands (red arrow) (IHC,DAB, x40), E)&F A cases of moderately differentiated adenocarcinoma, showing marked expression of CD133 immunostain in the cytoplasm of cells lining the colonic glands (red arrow) (IHC, DAB, x200, x400)

Four cases out of 5 of Dukes' A (80%) were positive for Lgr5, 13 cases out of 15 of Dukes' B (86.6%) and 20 cases out of 20 of Dukes' C (100%) were positive for Lgr5. There is significant difference between Dukes' B and C comparing to Dukes' A at $P < 0.01$ and $P < 0.05$ respectively (Table 8).

Table 6: Correlation of Lgr5 score and histopathological grades in the malignant group

Histopathological grade	Lrg5 score staining +, Mild (Score 1) No %	++,Moderate (score 2) No %	+++, Strong (Score 3) No %
Well differentiated (GI) N=5	1 (25%)	3 (75 %)	0(0%)
Moderately differentiated (GII) N=18	1 (16,6%)**	6 (33.4%)**	9 (50%)**
Poorly differentiated (GIII) N=17	0 (0%)	3 (5.9%)**,^	14(94.1%)**,^

Cross tables, Pearson Chi-Square; $P < 0.05$; *p < 0.01 compared to GI group; ^p < 0.05 compared to GII group.

Three cases out of 5 of Dukes' A (60%) were moderately positive for CD133, 10 cases out of 15 of Dukes' B (66.7%) showed strong expression of CD133 and 18 cases out of 20 of Dukes' C (90%) were strongly positive for CD133 (Table 9).

Table 7: Correlation of CD133 immunoexpression and histopathological grades in the malignant group

Histopathological grade	Immunoexpression of CD133 staining +, Mild No %	++,Moderate No %	+++, Strong No %
Well differentiated, (GI) N=5	3 (60%)	2 (40%)	0 (0%)
Moderately differentiated, (GII)N=18	0 (0%)	2 (11.7%)**,^	15(88.3%)**,^
Poorly differentiated, (GIII) N=17	0 (0%)	2 (11.7%)**,^	15(88.3%)**,^

Cross tables, Pearson Chi-Square; $P < 0.05$; * $P < 0.05$ compared to GI group; **$P < 0.01$ compared to GI group; ^$P < 0.05$ compared to GII group.

Discussion

Worldwide, colorectal cancer is the third most commonly diagnosed cancer in males and the second in females. Colorectal cancer (CRC) annually affects more than one million men and women and causes more than half a million deaths [1]. CRC is presented as a multistep genetic disorder characterised by specific mutations in signal transduction pathways. The development and progression from adenoma to cancer and metastatic disease require the simultaneous failure of protective mechanisms [11].

Table 8: Correlation of Lgr5 score and histopathological stages in malignant group

Histopathological Stages	Lrg5 score immunoexpression +, Mild (Score 1) No %	++,Moderate Score (2) No %	+++, Strong (Score 3) No %
Dukes' A N=5	0(0%)	4 (100%)	0(0%)
Dukes' B N=15	0 (0%)	3 (23.1%)*	10(76,9%)*
Dukes' C N=20	0(0%)	2 (10%)*,^	18 (90%)*,^

Cross tables, Pearson Chi-Square; $P < 0.05$; *$P < 0.01$ compared to Duck A group; ^$P < 0.05$ compared to Duck B group.

Colorectal cancer develops from a dysplastic precursor lesion, regardless of whether it arises sporadically, in the setting of high-risk hereditary conditions, or in the context of chronic inflammation like inflammatory bowel disease (IBD).

Table 9: Correlation of CD133 immunoexpression and histopathological stages malignant group

Histopathological Stages	Immunoexpression of CD133 staining +, Mild No %	++,Moderate No %	+++, Strong No %
Duke A N = 5	2 (40%)	3 (60%)	0 (0%)
Duke B N = 15	2 (13.3%)	3 20%)*	10 (66,7%)*
Duke C N = 20	0 (0%)	2 (10%)*,^	18 (90%)*,^

Cross tables, Pearson Chi-Square; $P < 0.05$; *$P < 0.01$ compared to Dukes' A group; ^$P < 0.05$ compared to Dukes' B group.

In IBD, however, dysplasia can be polypoid or flat. In fact, the rather unusual macroscopic appearance and biologic behaviour of dysplasia in IBD have stimulated a good deal of research into the natural history and molecular pathogenesis of CRC in patients with IBD [12]. Cancer stem cell theory in CRC has been investigated, and it is based on evidence that only a small subset of cells, the CSCs, within the tumour population, can initiate and sustain tumour growth and several stem cell markers have been studied [13].

Metastasis is responsible for approximately 90% of cancer-associated mortality and can be divided into translocation and colonisation. As the CSC population represents the only cells that propagate tumours, it can be extrapolated that these cells are responsible for metastasis formation. It is, therefore, a prerequisite that these cells must be able to detach from a primary tumour, invade, access, and survive at the circulation, disseminate at distant sites, transmigrate across the endothelial lining of the target tissue, and form secondary tumours [14] [15].

Several markers have been identified as solid cancer stem cell markers. CD133 is a transmembrane pentaspan protein which was initially described as a surface antigen specific for human hematopoietic stem cells. Indeed, CD133 alone or in combination with other markers is currently used for the isolation of stem cells from numerous tissues, such as bone marrow, brain, kidney, prostate, liver, pancreas, and skin. Furthermore, investigators have used monoclonal antibodies to CD133 for the identification and isolation of a putative cancer stem cell population from malignant tumours of brain, prostate, liver, pancreas, lung, and colon [16].

On the other hand protein glycoprotein coupled receptors (GCRs) have been investigated to be closely associated with tumorigenesis [17]. Lgr5 which is one of (GCRs) members proved to be a stem cell marker [18].

This prospective study was conducted on 100 patients presenting with colonic symptoms, attending to Gastrointestinal Endoscopy unit in Nasser Institute for research and treatment Hospital. This study was aiming to assess the expression of CD133 and Lgr5 in pre-malignant (adenomatous polyps and IBD),

malignant colorectal lesions and normal colonic mucosa by immunohistochemical staining.

According to the immunohistochemical results, lgr5 was detected as brown cytoplasmic granules with different expression pattern in the normal colonic mucosa, adenoma and carcinoma. Our results showed that Lgr5 protein was strongly positively (score 3) in 67.7% of cases (27/40), moderately (score 2) in 20% of cases (8/40) and mild (score 1) in 5% of cases (2/40) of colorectal carcinomas respectively, with high statistically significance difference between groups at P < 0.001. Statistically speaking a significant correlation was found between a score of lgr5 expression and the type of the lesion as the score increases with progression of the lesion from adenoma to carcinoma (P-value < 0.01). This was in agreement with Fan et al**.,** [18] who stated that 54% of colorectal carcinoma cases showed score group (3) of lgr5 expression. This means that lgr5 expression might be involved in colorectal carcinogenesis.

In the current study, as regards the tumour grading, Lgr5 immunoexpression in CRC group. Three cases (80%) of GI, 16 cases (88.8%) of GII and 17 cases (100%) of GIII with the highly statistically significant difference between GIII compared to GII and GI at P < 0.01 and P < 0.05 respectively. These results were in disagreement with Fan et al., [18] who found that no correlation was found between lgr5 score and the grade of CRC as 34% of grade II showed score 1, 14.9% of grade II showed score 2 and 4.5% showed score 3 . In grade III 86.6% of cases showed score (1) and 13.4% of cases showed score (2) and no cases showed score (3). This current study was in disagreement with Takeda et al., [19] that found lgr5 expression was not correlated to the degree of differentiation of CRC cases.

These results were in contrast with Simon et al., [20] that found lgr5 was correlated significantly with tumour grade in gastric adenocarcinoma (P-value < 0.01).

Concerning the expression of CD133 in CRC cases, the present study revealed a significant difference between colorectal carcinoma and normal mucosa (P < 0.001). CD133 was positive in 15% of cases (3/20) of normal mucosa (control cases). CD133 protein was strongly positively in intensity in 65% of cases (26/40), moderately 27.5% of cases (11/40) and mild intensity of CD133 in 7.5% of cases (3/40) of the colorectal carcinomas respectively, with high statistically significance (P < 0.001). These findings are in agreement with Takahashi et al., [21] and by Yang et al., [22]. However; our results are in contrary to those of Hongo et al., [23]**,** Choi et al., [24]**,** Horst et al., [25] and Kojima et al., [26]. This can be explained by the difference in the sample size; the studies which disagreed with our results had a larger sample size.

These findings are in agreement with those detected by Wang et al., [27], found that high CD133 expression was significantly associated with moderately and poorly differentiated CRC. In contrast Coco et al., [28], stated that no significant relation between CD133 expression and histologic grade and also with that found by Hongo et al., [23], Takahashi et al., [21], Choi et al., [24], Horst et al., [25] and Saigusa et al., [29].

As regard Dukes' staging and Lgr5 expression our results showed that four cases out of 5 of Dukes' A (60 %) were positive for Lrgr5, 13 cases out of 15 of Dukes' B (86.6%) showed immunoreactivity for Lgr5, and 20 cases out of 20 of Dukes' C (100 %) were positive for Lgr5 with statistically significance difference (P < 0.01). There was a significant correlation between a score of lrg5 and the stage of CRC (P-value < 0.05). These results suggest that lrg5 expression perhaps play a role not only in tumour initiation but also in the progression of a tumour. These results in agreement with Merlos et al., [30], Takahashi et al., [21], and Uchida et al., [31] found increased Lgr5 expression in advanced stages of CRC cases.

Concerning the expression of CD133 in CRC cases in correlation to Dukes' stage, there are three cases out of 5 of Dukes' A (60%) were moderately positive for CD133, 1 cases out of 15 of Dukes' B (66.7%) showed strong expression of CD133, and 18 cases out of 20 of Dukes' C (90%) were strongly positive for CD133. This is in contrast with results of Wang et al., [27], Takahashi et al., [21], Choi et al., [24], Horst et al., [25] and Kojima et al., [26] found no significant relation between CD133 expression and modified Dukes. Horst et al., [25] reported significant relation with N and M stage. The studies had different results from ours are much larger in the number of patients and also Coco et al., [28] studies have different cut-off used to discriminate low and high expression.

In conclusion, as stem cell marker of cells with intestinal differentiation, Lgr5 and CD133 were presented with significantly increased expression with progression from normal colon towards CRC. Lgr5 expression and CD133 was positively correlated with stage of CRC suggesting its possible involvement in colorectal tumorigenesis progression and patient's outcome.

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Basic Science

Usefulness of Sunlight and Artificial UV Radiation Versus Chlorine for the Inactivation of *Cryptosporidium* Oocysts: An in Vivo Animal Study

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Keywords: Cryptosporidium; Sunlight; Ultraviolet; Chlorine; Inactivation

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Abstract

BACKGROUND: *Cryptosporidium* is an important waterborne protozoan.

AIM: The aim of this study was to investigate the effect of sunlight being the natural source of UV and artificial UV irradiation on *Cryptosporidium* oocysts versus the effect of chlorination, being the traditional method of water disinfection and to provide an insight into the viability and degree of infectivity of *Cryptosporidium* oocysts, using an animal model.

METHODS: An experimental study including 300 neonatal mice was carried out to investigate the effect of artificial ultraviolet (UV) irradiation and sunlight being the natural source of UV irradiation versus chlorine, the traditionally used water disinfectant on the infectivity of *Cryptosporidium* oocysts present in water. For each item, nine different exposure times were investigated. Parasitological assessment (Modified Ziehl Neelsen stained stool smears) and histopathological assessment of the excised segments of the small intestine (stained by both Haematoxylin & Eosin and ZN stain) of mice were used to verify the inactivation of oocysts.

RESULTS: *Cryptosporidium* oocysts failed to induce any noticeable infection after 4 hours of artificial UV exposure that provided a UV dose of 10mJ/cm2 and after an 8 hours exposure to sunlight, whereas they showed resistance to disinfection by chlorine.

CONCLUSION: The results of the study demonstrate the important role of an 8 hours sunlight exposure of potable water in plastic bottles in achieving complete inactivation of any contaminating *Cryptosporidium* oocysts, thus offering an applicable, economical and convenient method for the control of cryptosporidiosis especially in developing countries.

Introduction

Cryptosporidium is a protozoan that may lead to fatal illness in immunocompromised persons [1]. It inhabited intestines of humans and animals and distributed through bowel motions into the environment [2]. During the last 20 years, waterborne diseases were frequently caused by *Cryptosporidium.* Its presence in surface, ground and potable water supplies is of particular concern as it disseminates rapidly in addition to its resistance to chlorine and other commonly used chemical disinfectants [2]. The questionable efficacy of chlorine, its safety, and potential health hazards, had led to consideration of other alternative disinfectants [3]. A recent addition to the panel of water disinfectants for protozoan parasites is UV irradiation [4]. Studies concerning such an issue may lead to improve water quality and help in designing water and wastewater treatment processes offering safe drinking water and protection of public health [5].

This study aimed to investigate the effect of sunlight being the natural source of UV and artificial UV irradiation on *Cryptosporidium* oocysts versus the effect of chlorination, being the traditional method of water disinfection and to provide an insight into

the viability and degree of infectivity of *Cryptosporidium* oocysts, using an animal model.

Methods

The study was conducted according to the national guidelines for animal research; 300 neonatal albino mice were divided into 4 groups, one receiving untreated *Cryptosporidium* oocysts to be used as a control group (30 mice), and 3 groups infected with *Cryptosporidium* oocysts previously exposed to different disinfectants, in terms of chlorine (the traditional water disinfectant), sunlight (containing UVA and UVB) and artificial UV irradiation (representing UVC). Each of these 3 test groups was further subdivided into 9 subgroups (10 mice/ subgroup). Each of the 3 tested groups of mice were infected after exposure of *Cryptosporidium* oocysts to the tested disinfectants for variable periods of time, starting from 15 minutes (min), 30 minutes, one hour (h), 2 hours, 4 hours, 8 hours, then for 1 day(d), 2 day and 4 days (Table 1).

Table 1: Categorization of different groups and subgroups of mice included in the present experimental study

Groups	No. of mice	Subgroups								
Control group	30	-								
Mice received chlorine treated oocysts	90	9 different exposure time (10 mice/each) = 90								
Mice received artificial UV rays exposed oocysts	90	15 min	30 min	1 hour	2 hours	4 hours	8 h hours	1 day	2 days	4 days
Mice received sunlight exposed oocysts	90									
Total number of mice	300									

Cryptosporidium oocysts were isolated from diarrheic calves, mixed with 10 ml of distilled water and then filtered through sterile gauze. The homogenate was successively passed through two metal sieves of pore size 125µm & 75µm, followed by centrifugation at 2500 x g for 15 min. The supernatant fluid was discarded, and the sediment was then washed twice with 1ml of phosphate buffer saline (PBS) (pH was adjusted to 7.2-7.6) with centrifugation at 13,000 x g for 15 minutes each. After repeated washing, faecal debris was eliminated and thus purified. *Cryptosporidium* oocysts were obtained [6]. The oocysts were then preserved by mixing with an equal volume of 2.5% potassium dichromate ($K_2Cr_2O_7$) and stored at 4ºC until being used for animal inoculation. Just before use, the oocysts were washed for at least three times in distilled water to remove the potassium dichromate and centrifuged at 1500 x g for 15 min until it became clear [7]. For counting oocysts, a smear was prepared on 3 slides formed of about 50 mg from each sample and stained with modified Ziehl-Neelsen stain (ZN). Oocysts were counted in each slide, and the mean number of the oocysts was calculated to get the mean number/mg of the sample [8].

Purified oocysts were added to 0.5 ml of distilled water in ordinary transparent polyethene terephthalate bottles and then exposed to sunlight for variable durations as previously mentioned. Eight hours exposure was chosen from 8 am to 4 pm (maximum intensity of solar irradiation). For one day exposure, the previously mentioned inoculum was exposed to sunlight from 8 am to 4 pm, and then was covered with aluminium foil until next day 8 am, and then mice inoculation was done to investigate the possibility of the occurrence of dark repair [9]. The same procedure was followed for 2 days and 4 days exposure. The temperature inside the bottles was measured each hour with a thermometer [9]. For artificial UV irradiation, 10ml of PBS containing the purified suspension of oocysts was placed in a petri dish (56mm) and then placed under a 5-W low-pressure mercury lamp (QCGL5W-1497D; Iwasaki Electronic Co. Ltd., Tokyo, Japan). Using UV dose rate meter, the intensity of the UV light at a wavelength of 253.7 nm was measured. Exposure was completed throughout the mentioned durations under regular mixing using an electromagnetic stirrer [10]. Chlorine was used as Sodium hypochlorite solution (NaOCl), 4 ppm at 25ºC and applied for the same variable durations and as described by Carpenter et al., [11]. The inoculum dose was prepared to be 2.5×10^4 oocysts/mouse [9].

Neonatal mice were orally infected with a dose of 2.5×10^4 *Cryptosporidium* oocysts/mouse [9] using oesophagal tube [12]. According to Rossi et al., [13], all mice were sacrificed at 14 days post infection after giving intra-peritoneal anaesthesia. The terminal ileum was removed, fixed and prepared for histopathological examination.

Faecal samples were collected daily from infected mice until 14 days post infection. Parasitological examination was done using Modified ZN stain (cold method) for detection and grading of the intensity [14]. Haematoxylin & Eosin and modified ZN stained tissue sections were examined [15] to detect endogenous oocysts, assess inflammatory intensity as represented by inflammatory cells and activity represented by neutrophils [16]. While intensity of infection was determined by counting the parasite in 10 villous crypt units according to Healey et al., [17], then the mean oocysts number/single unit/mouse in each group was calculated.

All data were analysed using Statistical Package for the Social Sciences (SPSS) version 16 for Windows (SPSS Inc., Chicago, IL, USA). Data were reported as mean values ± SD for the quantitative variables. To compare the mean numbers of oocysts obtained after different time exposures in each group, we used Paired t test. To compare the mean numbers of oocysts between the three tested groups we used Analysis of variance (ANOVA) followed by pair wise analysis (Bonferroni test). P values ≤ 0.05 were considered statistically significant.

Results

Concerning the control group, shedding of *Cryptosporidium* oocysts was first observed on the fourth-day post infection. The mean number of oocysts was 2 ± 1.5/mg stool, and then gradually increased to report mean of 7.65 ± 0.4/ mg stool on day 8 to day 11. The maximum shedding of oocysts was reported from day 12 up to the end of the experiment with a mean value of 17.8 ± 2.3/mg stools. This difference was statistically significant ($p < 0.05$). Therefore, the mean oocysts count in the control group from day 12 to day 14 was used to compare the results of the other tested groups. As regards the temperature inside the bottle exposed to sunlight, a peak of 42°C was recorded. The mean number of oocysts in collected stool samples was 13.2, 12.9, 6.6, and 4.9 oocysts/mg stools when *Cryptosporidium* isolates were exposed to sunlight before mice inoculation for 15 min, 30 min, 1 h, and 2 h respectively. On exposure for 4 h, 9 mice out of 10 became infected in this subgroup whereas one mouse was found free of infection with a mean count 2.1 oocysts/mg stool. No *Cryptosporidium* oocysts were observed in the stool of any of the mice inoculated by oocysts exposed to sunlight 8 hours or longer, achieving 100% reduction rate in infectivity. Artificial UV radiation exposed oocysts showed mean counts of 11.12, 8.1, 2.8 and 1.4 when *Cryptosporidium* isolates were exposed to UV rays for 15 min, 30 min, 1 h, and 2 h respectively before mice inoculation. In 2 hours artificial UV exposure subgroup, the stool of 7 out of 10 mice showed *Cryptosporidium* oocysts while stool of 3 mice proved to be negative with a mean count 1.4 of oocysts/mg stool. Four hours artificial UV exposure for *Cryptosporidium* isolates subgroup showed 100% inactivation. Negative stool samples for *Cryptosporidium* were encountered in mice inoculated with *Cryptosporidium* isolates that were exposed to UV radiation for 4 hours or longer. Regarding results of chlorine-treated oocysts group, all stool samples of mice in all subgroups were positive for *Cryptosporidium* oocysts.

Table 2: Effect of exposure of *Cryptosporidium* oocysts to sunlight, artificial UV rays and chlorine on their infectivity to mice

Time of exposure	Group Sunlight oocysts/ mg stool Mean ± S.D	Reduction rate (%)	Group of artificial UV rays oocysts/ mg stool Mean ± S.D	Reduction rate (%)	Group chlorine oocysts /mg stool Mean ± S.D	Reduction rate (%)	ANOVA	P value
15 min	13.20 ± 1.68	25.84	11.12 ± 1.31	37.52	11.20 ± 1.31	37.07	6.33	0.006*
30 min	12.90 ± 1.19	27.52	8.1 ± 0.73	54.49	11.80 ± 1.31	33.70	51.11	0.000*
1 hour	6.60 ± 1.07	62.92	2.8 ± 0.78	84.26	11.10 ± 1.19	37.64	161.28	0.000*
2 hours	4.90 ± 0.87	72.47	1.4 ± 0.51	92.13	10.70 ± 1.33	39.88	234.53	0.000*
4 hours	2.1 ± 0.73	88.2	0	100	10.20 ± 0.91	42.69	626.61	0.000*
8 hours	0	100	0	100	9.60 ± 0.96	46.06	987.42	0.000*
1 day	0	100	0	100	9.90 ± 0.99	44.38	991.11	0.000*
2 days	0	100	0	100	10.00 ± 1.05	43.82	900.00	0.000*
4 days	0	100	0	100	7.40 ± 0.84	58.42	770.06	0.000*

*Significant p-value.

Even after 4 days of chlorine treatment of *Cryptosporidium* oocysts, the reduction rate of infectivity was only 58.42%. The mean no. of oocysts/ mg stool for different exposure times and reduction rates of infectivity in different groups are presented in Table 2 and Figure 1.

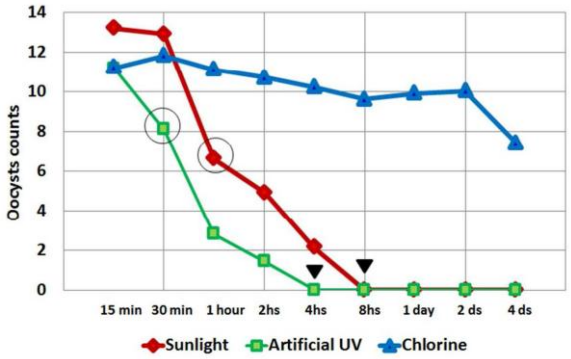

Figure 1: Line chart showing mean Cryptosporidium oocysts count/mg of stool in each subgroup of mice infected with oocysts previously exposed for various durations of time to sunlight, artificial UV irradiation and chlorine treatment. Circles denote the start of the significant differences and arrowheads denote full inactivation of artificial UV rays at 4 hours while absolute sunlight inactivation occurs at 8 hours

Using Paired t-test to compare between subgroups, the mean number of oocysts/mg stool at different exposure times within each group showed that, for the sunlight group, there was no statistical difference between the mean number in the 15 min and 30 min while the significant statistical difference was begun between 15 min and 60 min and extended to all other durations that showed significantly declining values. While for artificial UV rays group, the difference was statistically significant from the start between the 15 min and 30 min exposure and onward. As for the chlorine group, there was no statistical difference between 15 min and 60 min, 1 hour, 2 hours, 4 hours and onward. Comparing the results of the three groups, ANOVA test showed a significant difference between the mean numbers of oocysts among the three tested groups in all-time exposures. Bonferroni test showed a significant difference between the 3 individual groups except at 15 min where there was no significant difference between groups of artificial UV radiation and chlorine, and at 30 min there was no significant difference between sunlight and chlorine groups. Moreover, there was no statistical difference between sunlight and artificial UV radiation groups starting from 8 hours and onwards.

The ability of treated oocysts to induce infection in mice was determined by counting the endogenous stages in histopathological preparations, then calculating the mean number per single villous crypt unit for each animal and each group of animals, thus determining the intensity of infection (Figure 2). The mean number of endogenous stages was calculated in the control group to be 26 ± 5.3. All mice in the control group showed severe to moderate inflammatory intensity, while the inflammatory activity was rated as moderate. A significant difference between the mean numbers of oocysts among the

3 tested groups at all durations was recorded ($P \leq 0.05$). Bonferroni test showed a significant difference between the endogenous stages counts in the 3 groups. However, there was no statistical difference between subgroups sunlight and artificial UV radiation starting from 8 hours and onwards. In the chlorine-treated group, all histopathological samples showed *Cryptosporidium* oocysts regardless of variation in exposure time.

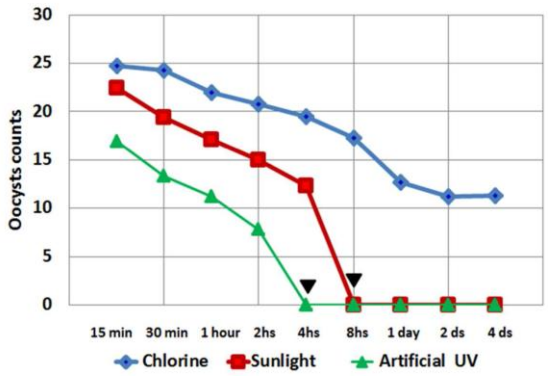

Figure 2: Line chart showing mean Cryptosporidium oocysts/villus crypt unit in each subgroup of mice infected with oocysts previously exposed for various durations of time to sunlight, artificial UV irradiation and chlorine treatment. Arrowheads denote complete inactivation caused by artificial UV rays at 4 hours while full sunlight inactivation appears at 8 hours

Severe inflammatory reaction with moderate neutrophils infiltration was reported in intestinal sections of mice infected by samples exposed to chlorine for 2 and 4 days. No oocysts were detected in the subgroups exposed to sunlight for 8 hours, 2 days and 4 days. The moderate inflammatory reaction was observed up to the subgroup exposed to sunlight for 8 hours with slight neutrophil infiltration indicating a mild inflammatory activity.

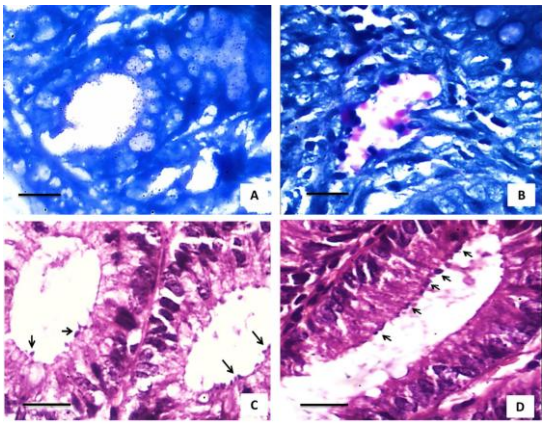

Figure 3: Photomicrographs for different histopathological sections prepared from mice intestines. A) Histopathological section stained with Modified ZN stain shows negative result for Cryptosporidium infection; B) Histopathological section showing pink spherical structures about 2-5 µm attached to microvillus surface of epithelium, confirming Cryptosporidium intestinal infection (scale = 25 µm); C), D) Histopathological cross sections for the intestine stained with Haematoxylin & Eosin show many Cryptosporidium oocysts (black arrows) attached to microvillus surface of epithelium (Scale = 50 µm)

The slight inflammatory reaction, though without neutrophils was observed in the subgroup exposed for 2 days to sunlight. Histopathological sections of subgroups exposed to artificial UV rays for 4 hours or more proved to be free from *Cryptosporidium*. Moderate inflammatory reaction and moderate inflammatory activity were reported in the subgroups exposed for one hour or two hours while severe inflammatory reaction with moderate neutrophil infiltration was recorded with the subgroups exposed to artificial UV irradiation for 15 min and 30 min. Those exposed for 4 hours reported only slight inflammatory reaction with minor neutrophil activity.

Discussion

Concerning exposure to sunlight, the results of the present study showed that at least 8 hours were needed to achieve infection inhibitory effect with zero *Cryptosporidium* oocysts in the stool samples of the infected mice. This nil effect was achieved as well in case of one day, 2 days and 4 days exposure to direct sunlight. This may be due to the helping effect of the sunlight which reached 42°C. Gomez-Couso *et al*., [9] recorded slightly higher temperature 45°C to achieve significant reduction in their murine model. Using a batch-process solar disinfection system (SODIS), exposure times of more than 10 hours was recorded to simulate solar irradiation rendering *Cryptosporidium* oocysts non infective [18]. On the other hand, the effect of sunlight on the survival of *Cryptosporidium* oocysts in a waste stabilization pond system in the northwestern of Spain was studied [19], reporting only a 40% reduction after 4 days exposure. However, these dissimilar results may be related to the difference in the nature of water used. In addition, most of the previously cited studies were performed in Europe, where the sun rays may be masked by a lot of clouds and the temperature is usually lower than that recorded in this experiment. In fact, the portion of sun rays that reaches us is made up of two types of rays: long wave ultraviolet A (UVA) and short wave ultraviolet B (UVB) and basically, there is no UVC [20]. The shorter bands of UVC, as well as the more energetic UV radiation produced by the sun, are absorbed by oxygen and generate the ozone in the ozone layer [21]. The fraction of UVB which remains in UV radiation after passing through the atmosphere is heavily dependent on cloud cover and atmospheric conditions. Thick clouds block UVB effectively; but in "partly cloudy" days, patches of blue sky showing between clouds are also sources of (scattered) UVA and UVB, which are produced. The underlying mechanism by which sunlight irradiation inactivates microorganisms is possibly through damaging their DNA. Dark repair can occur after exposure of these

inactivated microorganisms to darkness. Dark repair is a phenomenon in which damaged DNA of these inactivated microorganisms can be repaired and regain its activity in the absence of light. Thus, the resulted inactivation might not be permanent inactivation [9]. For this reason, in the present study, the complete inactivation for *Cryptosporidium* oocysts that is occurred in case of one day sunlight subgroup which is the same effect obtained on 8 hours sunlight subgroup denoted that no resuming of infectivity had occurred for these oocysts upon absence of light, resulting in permanent inactivation.

Concerning chlorine as a disinfectant, it was extensively studied using different formulas, concentrations and in different conditions. Therefore, it is difficult to find identical results among variable scientific works. In this study, chlorine was used in a solution form (Sodium hypochlorite) in a concentration of 4 ppm at 25^{o}C. Regardless the variable reduction rate reported in the current study which ranged from 33.07 to 58.42, even after 4 days of treatment, chlorine failed to inhibit *Cryptosporidium* infection. Instead, the small intestine of infected animals showed signs of inflammation. This inflammation was severe in animals infected with sampled exposed to 2 and 4 days exposure, indicating the irritant effect of such chemical disinfectant with time, which was not only dose- dependent but also time dependent. Using sodium hypochlorite as well, Fayer *et al.,* [22] tested its effect with different concentration (5.25, 2.63 and 1.31%). Using an in vivo animal model they reported lower oocysts counts in histopathological examination of the infected small intestine, whereas the number of parasitic stages identified in the animals' stool samples was more or less similar to our results, confirming the reported data. Relatively higher reduction rate was recorded by Barbee et al., [23] who showed a decrease in infectivity of *Cryptosporidium* by 90% after exposure to Clorox (NaOCl, 5.25 %). While, Weir *et al.,* [24] described a poor effect of sodium hypochlorite (6%) up to an exposure time of 33 min which was undoubtedly an insufficient duration compared to the other recorded data including our study.

Furthermore, Venczel *et al*., [3], using another formula of chlorine found that 5 mg/ litre dose of free chlorine at 25°C produced no measurable inactivation of *Cryptosporidium* oocysts after 4 or 24 hours. Two years earlier, Finch *et al*., [25] did not find as well any effect of free chlorine in inactivation of *Cryptosporidium* oocysts. Despite the previously mentioned negative reports about chlorine, Delling *et al*., [26] used different substances; one of them was sodium hypochlorite (NaOCl) at different concentrations for several exposure times. Unexpectedly, their results showed an inactivation over 99 % by using 3 and 6 % NaOCl after 12 hours exposure.

Concerning the effect of artificial UV irradiation to inactivate *Cryptosporidium* oocysts, in the current study an exposure to low pressure (LP) UV lamp for 4 hours at 25°C that was equivalent to 10 mJ/cm^2, proved to be effective in aborting *Cryptosporidium* infection, resulting in a 100% reduction rate. Nearly similar, Clancy *et al*., [4] noticed that low doses of UV radiation from either low or medium pressure lamps were effective in achieving an inactivation level of greater than three logs (99.9%) for *Cryptosporidium* oocysts in waste water effluent as measured by cell culture. UV collimated-beam apparatus was used to focus parallel rays and expose suspensions of purified *Cryptosporidium* oocysts in phosphate-buffered saline at 25°C to various doses of monochromatic LP UV rays [10]. The former workers using in vitro cell culture assay reported *Cryptosporidium parvum* infectivity reductions at a dose of 3 mJ/cm^2 (530 J/m^2). These results indicate that *Cryptosporidium* oocysts are very sensitive to inactivation by low doses of monochromatic LP UV radiation [10] [27]. In the present study, the effect of temperature on artificial UV irradiation had not been investigated because UV ray is temperature independent according to Craik *et al*., [28]. Also, the previous authors [28] found that the conventional low-pressure mercury arc lamp and the medium-pressure lamp were equally effective at inactivation of *Cryptosporidium* oocysts so that there was no apparent biocidal benefit arising from the broad emission spectrum or higher irradiance of the medium-pressure UV lamp. The choice of the lamp to be used for a given application should, therefore, be based on economic considerations. Low-pressure lamps have about twice the germicidal efficiency (UV dose delivery per watt of input power) compared to medium-pressure lamps, and thus low-pressure lamps will be the choice for smaller systems, so we used LP UV lamps in the experimental setting. Certainly, there is a very beneficial point favouring the use of UV over the other disinfectant methods [10] [27]. It is a physical process that does not rely on the use of chemical additions, it has been shown to be highly effective in the inactivation of protozoa, it requires relatively short contact times, and no UV disinfection by-products have been currently identified [29].

Finally, another important result in this study is that exposing plastic bottled water for 8 hours to sunlight, the chosen time to be that of maximum intensity of UV radiation, completely aborts the ability of *Cryptosporidium* oocysts to produce infection, thus an excellent method for *Cryptosporidium* inactivation in summer months. Still, there is an urgent need for different systematical investigations for the solar inactivation mechanisms for *Cryptosporidium* oocysts under a wide range of environmental conditions. Such studies will offer an economic and convenient method for *Cryptosporidium* oocysts inactivation especially in developing countries even in household applications. Furthermore, the possibility of synergistic effects

from mixtures of disinfectants is recommended to be investigated in further studies.

Ethical approval

All applicable international, national, and institutional guidelines for the care and use of animals were followed. All ethical standards applied in the animal house of Kasr-Alainy school of Medicine, Cairo University was followed.

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Basic Science

Isothermal PCR for Feasible Molecular Diagnosis of Primary Toxoplasmosis in Women Recently Experienced Spontaneous Abortion

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Abstract

AIM: The current study aimed to assess the practicability of a simple loop-mediated isothermal amplification (LAMP) about real-time quantitative PCR to diagnose primary toxoplasmosis among high-risk pregnant women.

METHODS: Cloned Toxoplasma samples were used to calculate the analytical sensitivity while specificity was assessed using pooled DNA samples extracted from other parasitic stages.

RESULTS: Both techniques showed 100% sensitivity and specificity and then applied to detect recent Toxoplasma infection in peripheral blood of 77 IgG negative women out of a total 139 women lately experienced spontaneous abortion. The 2 techniques obtained positive results in 8 samples confirming primary toxoplasmosis.

CONCLUSION: Generally, LAMP assay is a simple, cost-effective molecular technique can be completed in less than half an hour to diagnose primary Toxoplasma infection. The technique can be applied in a minimally equipped laboratory by ordinary workers to screen the vulnerable groups. Further analysis using larger samples with the quantitative approach is recommended to confirm the sensitivity of this emergent molecular technique.

Introduction

Toxoplasma gondii (T. gondii) is a worldwide zoonotic protozoal parasite that causes harmless infection in a huge number of human population, yet it causes serious illness in merely immunocompromised hosts. Also, *Toxoplasma* trophozoites may cross the placental barriers, causing variable degrees of damage to fetuses resulting in stillbirth or spontaneous abortion [1]. Critical ocular and neurological signs plus other congenital manifestations may also arise as a result of congenital infection. These dangerous sequels typically depend on gestational age, virulence of the organism and immune status of the mother [2].

Association of *Toxoplasma* infection with spontaneous abortion is frequently studied with variable approaches. *Toxoplasma* infection among Mexican was recorded in women suffered from spontaneous abortion by detecting either specific antibodies or circulating parasitic DNA among 55% of their study group [3]. The authors recommended further studies to ascertain diagnostic policies to be trailed, to prevent congenital toxoplasmosis in the populations at risk. Toxoplasmosis was reported in 15.2% women with spontaneous abortion as well, using serological methods [4]. Based on the genomic detection of circulating *Toxoplasma* DNA

up to 14.7% positive results were recorded regarding abortion in association with *Toxoplasma gondii* infection [5].

Based on serological methods**,** Saki et al., [6] detected IgM antibody in women who had an abortion and advised not to neglect the role of *Toxoplasma gondii* in the occurrence of abortion as a result of primary infection. However, the result of serological assays should be interpreted with caution, IgM antibodies may persist up to 1 year after *Toxoplasma* infection, so it is not appropriate to diagnose recent infection. Hence, to improve the diagnosis of primary infection with *T. gondii* during pregnancy, other techniques are suggested as anti-*Toxoplasma* IgG avidity and molecular detection by the nucleic acid polymerisation techniques to discriminate between recent and prior infection [7].

In fact, *Toxoplasma* can circulate at low concentrations, or intermittently, thus might be detected in earlier phases of infection as circulating genomic materials which need a sensitive and specific technique to confirm infection in such critical time. Molecular methods are reported to be effective enough to detect low concentrations of circulating genomic materials as low as 10 tachyzoites/ml which was reported in about 40% of cases in a study applied in France [8].

Real-time visualisation in addition to the precise digital quantitation of the amplified DNA products without risk of contamination, greatly direct scientists to replace the traditional PCR multi-instruments by a single quantitative PCR machine. However, the relatively high cost and the need for highly equipped laboratories still limit the wide use of such advanced technologies for diagnostic purposes of some infectious agents including *Toxoplasma* parasite [7]. Consequently, it is appropriate to develop simple, rapid, sensitive, cost-effective and less time- consuming diagnostic molecular method to complement the limitations of conventional and real-time PCR to diagnose such infection, especially in vulnerable groups [9].

At present, variable isothermal approaches targeting nucleic acid augmentation have been established, possibly terminating the need of expensive, sophisticated devices and complex laboratories especially in developing countries where inappropriate infrastructure is still the usual situation. One of the most verified isothermal DNA amplification methods is a LAMP (Loop-mediated isothermal amplification) which was originally described by Notomi et al., [10].

In the present study, a trial was made to apply LAMP technique in comparison to quantitative real-time PCR (qPCR) to detect primary toxoplasmosis in women suffered from spontaneous abortion for more feasible molecular implementation in our relatively low equipped laboratory.

Patients and Methods

Concerning ethical considerations that were approved by the local ethical committee, Faculty of Medicine, Cairo University, this cross- sectional study was conducted from January 2014 to December 2016. Women included in the study were from the outpatient's clinic of Obstetrics and Gynecology Department, Kasr Alainy Hospital and suffered from spontaneous abortion during the first trimester of the first pregnancy. The total enrolled number was 139 cases; their age range was within 21 to 34 with no major demographic variability. They were almost of the same educational and socioeconomic level being attending governmental, free of charge hospital. Diagnosis of spontaneous abortion was performed following history taking and clinical examination plus findings of ultrasonography to exclude medical causes of abortion, women with Rh-incompatibility, threatened abortion, incompetent cervix, fibroid or any uterine abnormalities. Blood samples were collected from included cases immediately after abortion and sera were separated and were tested for anti-*Toxoplasma* IgG and IgM antibodies using a commercial ELISA Kit (Cal biotech Inc., CA). Serological tests were performed according to the manufacturer's instructions. Samples showed positive IgG results were excluded from the study. Cases were advised to return in follow up visits for normal checking and to inform them about their results. Positive cases were managed according to the currently approved protocols in the Obstetrics and Gynecology Department, Faculty of Medicine, Cairo University. The women who did not show any positive results were at risk of primary *Toxoplasma* infection at any time and were instructed to take care of their next pregnancy to avoid such serious infection.

Genomic DNA from peripheral blood samples was extracted using the DNeasy® Blood and Tissue Kits (Qiagen, CA, USA). Quality control and computing procedure were applied to rule out false positives in molecular techniques.

Four *Toxoplasma*-specific primer sets were used for LAMP technique to detect B1 gene of 35-fold repeats; B3: ACGTGACAGTGAAGAGAGGA, F3: CAGATGTGCTAAAGGCGTCA, BIP: TGTTCGCTGTCTGTCTAGGGCAGGTGGTCGACTT CATGGGA and FIP AGGCGGAACCAACGGAAATCCTTGCTGTTCTGTC CTATCGC. Twenty five µl LAMP mixture was prepared containing 2 µl of the extracted nucleic acid template, 40 pmol for each of FIP and BIP primers, 5 pmol for the other primers B3 and F3, 1 µl of *Bst* DNA polymerase (NEB) in 2.5 µl of buffer [20 mM Tris-HCl (pH 8.8), 8 mM $MgSO_4$, 10 mM $(NH4)_2SO_4$, 0.1% Tween 20], 10 mM KCl, 0.8 M betaine, in addition to 1.4 mM (dNTP). Using a heater block, the reaction of the isothermal technique LAMP was applied for 30 min at 65°C and then was

inactivated for 2 min at 80°C. The positive amplification results were visually detected by adding 2µL of 1:10 diluted 10,000 x concentration fluorescent dye SYBR Green I (Invitrogen) to the reaction tubes. Green fluorescence was observed in a successful LAMP reaction, whereas it remained the original pinkish-orange in the negative one [11].

Light Cycler ® instrument (Roche Diagnostics, Hoffmann-La Roche Ltd, USA) was used, applying SYBR Green technology. Primers from bases (5´-CCG TTGGTT CCG CCT CCT TC- 3´) and (5´-GCAAAA CAG CGG CAG CGT CT-3´) were used to amplify *Toxoplasma* B1 gene. The resulting PCR fragment of *T. gondii* was analysed using the Light Cycler® Red 640 (detected in channel 640). The reaction mixture (20 µl; Master SYBR Green kit; Roche Diagnostic) contained 0.5 µM of each primer, 5 mM $MgCl_2$ and 5 µl template DNA. Amplification was performed for 50 cycles: 5s denaturation at 95°C, 10s annealing at 61°C and 15s extensions at 72°C, with an overall ramp rate of 20°C/s [12].

Positive and negative control: To assess the analytical sensitivity of the molecular techniques, cloned and purified *Toxoplasma* genomic materials (Roche Diagnostics), with serial dilutions were used as control positive samples. Also, the standard curve created by these cloned DNA samples were used to allow the absolute quantification of the unknown samples. The concentrations of the standard samples were prepared to be from 10^1 to 10^6 genomic equivalents/reaction of *T. gondii* DNA. Data analysis was done as described in the Light Cycler® instrument operator's manual. Specificity was approved by defining their melting curves and melting temperatures (*T*m) (95°C, 4.40°C/s ramp rate; 40°C, 2.20°C/s ramp rate; 65°C, 4.40°C/s ramp rate; 95°C, 0.02°C/s ramp rate continuous measurement). In addition to 10 pooled DNA samples extracted from other parasitic stages; *Giardia, Cryptosporidium, Blastocystis, Leishmania spp. And Plasmodium spp.* To confirm the specificity of the molecular techniques, a negative control was applied by replacing the template DNA with ultrapure water. The standard row originated from variable *Toxoplasma* genomic concentrations showed Crossing Points (CPs) were automatically recorded.

Results

To confirm primary recent *Toxoplasma* infection, detection of *Toxoplasma* B1 gene in the peripheral blood samples was done by both qPCRs applying SYBR Green technology and LAMP. Initial serological screening demonstrated positive IgG results in 62 out of the 139 cases (44.6%). Therefore, the subsequent molecular investigation was done for the remaining 77 samples. IgM was detected in 4 serum samples out of the 77 cases. Concerning the sensitivity of LAMP technique and qPCR, both techniques equally succeeded to polymerise all positive control samples denoting equal analytic sensitivity for the 2 molecular techniques. Whereas, control negative samples did not show any amplification signs. Turbidities resulted by the positive reaction due to the accumulation of magnesium pyrophosphate as a by-product of the LAMP reactions were easily observed visually. Positive reactions in LAMP turned green on the addition of SYBR Green I to the PCR tubes. A standard curve was generated by qPCR using the cloned positive samples to enable absolute quantitation of the possible positive samples within the 77 samples related to the immunologically negative IgG women.

To diagnose recent *Toxoplasma* infection in the 77 samples cases included in the present work, DNA was extracted from the collected samples and tested using both LAMP and Quantitative real-time PCR (qPCR), applying SYBR® Green detection protocol and different primer sets which targeted *T. gondii* B1 gene. Green fluorescence was observed in 8 samples out of the 77 samples. The same samples generated fluorescence signals (positive results) when analysed by SYBR® Green qPCR. For qPCR, as the amount of accumulated DNA in the PCR reactions increases, the amount of fluorescence from the dye increases consistently. Quantitative genomic estimation of these 8 positive samples in qPCR ranged from 1.2×10^1 to 6×10^7 genomic equivalents. Crossing points (Cps) showed different values ranging from 16.94 to 31.02 reflecting the different DNA quantities in the 8 samples (Figure 1). To confirm specificity and true positivity of these 8 samples, gene scanning option and high resolution melting curve analysis were applied using the software of the programmed Light Cycler® 1.x/2.0/480 instrument. Positive samples showed almost the same values of *T*m (84.2 ± 2.43) confirming their specific amplification. The mean value of melting temperature related to the positive control was 85.55 ± 0.53 SD. No significant difference was found between the 8 positive samples and the control positive samples. Moreover, no significant difference was reported between the 4 IgM positive samples and the other 4 samples which proved positive only by molecular techniques, putting in mind that all the 8 samples did not suffer from any manifestations related to acute toxoplasmosis. Nucleic acid amplification and visualization of the amplicons were completed within less than ½ an hour in the LAMP and in one hour using the real-time PCR assays, in addition to extra ½ an hour to confirm specificity by high resolution melting curve analysis. Clinically, these 8 women did

not suffer from any manifestations of acute toxoplasmosis.

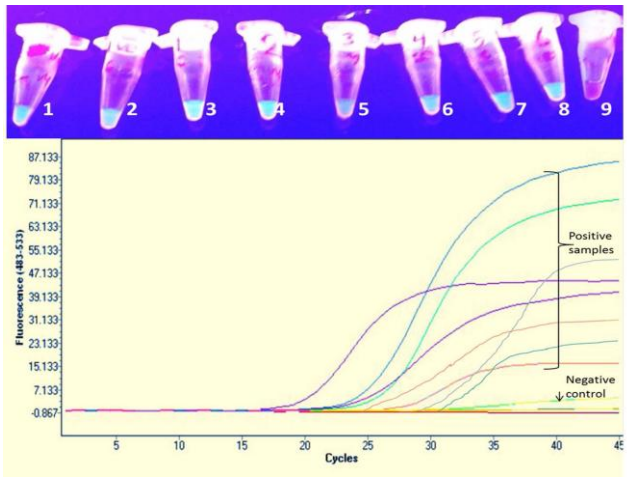

Figure 1: Samples in the upper photo show positive isothermal amplification for 8 samples (from 1 to 8) the sample number 9 is the negative control. The lower photo represents the real-time quantitative curves for the 8 positive samples with different crossing point at different cycles according to their genomic equivalent

Discussion

Diagnosis of primary toxoplasmosis in pregnancy is vital for the early management of this serious infection either by medical treatment or additional intervention to inhibit congenital transmission to the fetuses or unnecessary termination of the pregnancy [13]. The main target of the present study was to assess the usefulness of isothermal molecular technique that can be practised simply in our relatively low equipped laboratory. Also, to spotlight on primary toxoplasmosis about spontaneous abortion among our study group. In general, the risk of silent maternal *Toxoplasma* infection which may be transmitted to the fetuses is usually high [14]. And the risk of morbidity and mortality of the developing fetuses increases when primary *Toxoplasma* infection happens during the first trimester. While late transmission (last trimester), can cause ocular or neurological signs during the first 2 decades of life [15].

Commonly, diagnosis of acute primary infection is extremely difficult. A residual IgM antibody limits its use to detect early acute infection. The IgG avidity assay is now used to distinguish between acute and chronic toxoplasmosis, yet necessitates the presence of IgG antibodies. Low avidity means that the infection was acquired within the last 3 months [16]. However, the previous author criticized the method being not suitable for immunocompromised patients, plus the vital need of another assay as IgM ELISA to confirm the exact phases of infection. This certainly will raise the cost of the investigations. Specific *Toxoplasma* IgM typically develops in the early phase of the primary infection, within 1 to 2 weeks and reaches the highest level from 4 to 8 weeks after infection. The factors affecting immune response and antibody production depends on variable influences including the virulence of the organisms, inoculum volume, infective form plus the analytical sensitivity of the diagnostic method used to confirm the infection [17]. This may explain the negative results of IgM ELISA among 4 out of the 8 cases proved positive by molecular assays. These 8 cases possibly got the infection recently after pregnancy and not in the prenatal period. Possibly, the circulating parasitic stages that affected the placenta were enough to cause the evidenced damage and resulting in termination of the pregnancy.

Concerning the molecular techniques, using a simple heater, LAMP technique was completed within less than half an hour and successfully obtained the same results achieved by qPCR that was performed on a highly advanced instrument. Both obtained positive results for the same 8 samples, in addition to the equal analytical sensitivity which was proved following screening for serially purified cloned specific genomic materials (from 10^1 to 10^6). Such sensitivity of LAMP may be explained by the nature of the technique which is based on using a larger number of primers, (4 primers in this study) instead of 2 in qPCR to target different internal regions on the same target DNA [18]. A higher sensitivity of isothermal amplification technique than the ordinary multi-thermal PCR assays was also reported with other parasitic infections as *Babesia* spp., *Trypanosoma* spp. and *Theileria* spp [19] [20] [21]. The amplified products can be easily visualised in LAMP technique using simple UV light or even by direct turbidity inspection of the end product in the tubes. Hence, these give the technique an additional benefit and eliminate the necessity for gel electrophoresis and minimise the hazard of contamination. However, further work should be continued to apply a simple quantitative method for the amplified products.

Blood samples were used in the present study to detect the circulating *Toxoplasma* parasites in the peripheral blood. Molecular diagnosis of ocular toxoplasmosis was done using blood samples as well and produced the same results obtained with aqueous humor samples [22]. The authors recommended the use of blood samples to avoid invasive techniques. Thus, in this study, we evaluate the clinical sensitivities and specificities of a LAMP assay based on blood samples and compare the results with those of real-time qPCR. The specificity of the molecular technique in this study was confirmed by the highly advanced melting curve analysis, resulting in 100% specificity for both of them.

Moreover, no positive results were initiated from the 10 pooled samples extracted from other protozoal infections. Indeed to comment on the sensitivity, more studies are recommended using larger standardized samples, better to be performed on experimentally infected animals with different intensities of infection. Hence, the apparently 100% sensitivity of LAMP achieved in the present study in relation to (qPCR) should be further assessed to calculate the definite sensitivity. In other studies, sensitivity ranged between 80 to 87.5% was reported using LAMP to detect *Toxoplasma* infection in blood samples positive for IgM [11]. However, such reported sensitivity perhaps was undervalued by the previous authors due to its direct connection with the results of IgM. It seems unfair to link the results of a molecular technique necessitated the presence of circulating DNA to diagnose recent *Toxoplasma* infection to IgM antibodies which may be residual for years after infection. In fact, spontaneous abortion was not the target group in the previous study.

On the other hand, the quantitative real-time PCR assay has been successfully used to detect and accurately quantify *Toxoplasma* DNA in human body fluids including blood. With the use of B1 gene, the technique is considered as the best-performing technique for the diagnosis of congenital toxoplasmosis, compared with conventional PCR and nested-PCR [23]. The real-time PCR is also used to evaluate toxoplasmosis progression and treatment efficacy since it can estimate the intensity of *T. gondii* infection [24]. For this reason, qPCR was used in a comparative approach in this study to assess sensitivity and specificity of LAMP assay for simple, cost-effective diagnosis of primary toxoplasmosis.

In conclusion, molecular assays seem to be able to diagnose initial phases of *Toxoplasma* infection in earlier stages of gestation when the fetus is not yet immunocompetent. LAMP assay is a simple, cost- effective molecular technique with a detection limit of 10 specific genomic equivalents and can be completed in less than half an hour to diagnose primary *Toxoplasma* infection using peripheral blood samples. The ability to apply the technique in a minimally equipped laboratory by ordinary workers gives the technique great potential to be used in developing countries where toxoplasmosis is endemic, specifically for high-risk groups.

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Anatomic Variations of Pancreaticobiliary Union

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Abstract

AIM: This study was designated to evaluate the frequency of anatomic variations of the pancreaticobiliary union.

MATERIALS AND METHODS: Our research was observational, comparative and analytical. The investigation was conducted from January 2016-May 2017. This study included 63 patients from Clinic of Gastroenterology and Hepatology – Prishtina, assessed pancreaticobiliary union with Magnetic Resonance cholangiopancreatography.

RESULTS: Union of the common bile duct and the major pancreas was biliary-pancreatic type The angle between common bile duct and the major pancreas duct had different sizes average 35.6°. We did not distinguish significant statistical significance in the size of the pancreaticobiliary angle. In men, the union angle was from the average 36.9°, while in females was average 34.3°. No correlation between the age and size of the angle between common bile duct and the major pancreas duct.

CONCLUSIONS: The union of the common bile duct and the major pancreas duct was in most cases B-P Type. The common channel and angle between common bile duct and the major pancreas duct were normal in most cases.

Introduction

Normally, the major pancreatic duct and the common bile duct open into the second part of the duodenum alone or after joining as a common channel. The length of the common channel ranges from 1-12 mm normally, with a mean of about 4-5 mm [1].

The junction of the common bile duct and pancreatic duct is crucial for sphincteric control of bile and pancreatic juice drainage, with bidirectional regurgitation occurring if the union is above Oddi's sphincter. An abnormal pancreaticobiliary junction is a junction of the common bile duct and the main pancreatic duct outside the wall of the duodenum that forms a long common channel (< 8 mm) [2].

According to the type of biliary duct and main pancreatic duct joining the duodenal wall, the joining was classified into three categories: V type, B-P type and P-B type. The V type is the pancreatic duct and biliary duct joining the duodenal wall without a common channel; the B-P type is the biliary duct draining into the pancreatic duct and forming a common channel, and the P-B type is the pancreatic duct draining into the biliary duct and forming a common channel. Studies have revealed that the P-B type was equal to an acute angle and the B-P type was equal to a right angle [3] [4].

In 2015, the Committee on Diagnostic Criteria of the Japanese Study Group on Pancreaticobiliary Maljunction (PBM) proposed a classification of PBM into four types: (A) stenotic type, (B) non-stenotic type, (C) dilated channel type, and (D) complex type [5].

According to Kimura, the mode of the abnormal pancreaticobiliary junction can be classified into 2 types: type I in which the main pancreatic duct enters the common bile duct and type II in which the common bile duct enters the pancreatic duct [6].

The frequency of anomalous arrangement of the pancreaticobiliary duct (AUPBD) varies from 1.5-3.2% [7].

The anatomy of the distal ends of the common bile duct and the main pancreatic duct has received attention because of its importance in pancreaticobiliary diseases. The two ducts open in the duodenum either separately or via a common channel [8].

The junction of the common bile duct and pancreatic duct is crucial for sphincteric control of bile and pancreatic juice drainage, with bidirectional regurgitation occurring if the union is above Oddi's sphincter. An abnormal pancreaticobiliary junction is a junction of the common bile duct and the main pancreatic duct outside the wall of the duodenum that forms a long common channel (< 8 mm) [2].

Magnetic resonance cholangiopancreato-graphy (MRCP), which is widely used in the clinic, is a noninvasive and multidirectional imaging technology that can also clearly display the anatomical structure of the pancreaticobiliary duct system [9].

This study aimed to evaluate the frequency of anatomic variations of pancreaticobiliary union presented by the magnetic resonance cholangiopancreatography (MRCP). The most specific purpose was to assess the variations by tips, angle and relations with gender and age groups.

Material and Methods

Our research was observational, comparative and analytical. Regarding time, it was retrospective research. The investigation was conducted in January 2016-May 2017. This study included hospitalised and outpatient patients in Clinic of Gastroenterology and Hepatology, University Clinical Center of Kosovo. The examinations were carried out using the Siemens Type of MRCP in patients at Private Hospital “Aloka” in Prishtina and Clinic of Radiology, University Clinical Center of Kosovo. In this study, 63 (32 males and 31 females) were included. Patients were assessed pancreaticobiliary Union with MRCP (Magnetic Resonance cholangiopancreatography). The data was presented with tables.

Mann-Whitney, One Way ANOVA and Kruskal-Wallis tests were used.

Results

The union of the common bile duct and the major pancreas duct in 31.7% of the cases was B-P Type (biliary pancreatic). The most common women were P-B Type with 12.9% of cases compared with 3.1% males (Table 1).

Table 1: The Union of the common bile duct and the major pancreas duct by sex

	M		F		Total	
Types of PB Union	N	%	N	%	N	%
Separated or "duodenal" Type	10	31.3	8	25.8	18	28.6
B-P (biliarypancreatic) Type	11	34.4	9	29.0	20	31.7
P-B (pancreaticobiliary) Type	1	3.1	4	12.9	5	7.9
Pathology that deform PB Union	9	28.1	10	32.3	19	30.2
Artefacts	1	3.1	-	-	1	1.6
Total	32	100.0	31	100.0	63	100.0

At age 20-29 in 100.0% of cases, union common bile duct and the major pancreas was "duodenal type" (separated), while age over 70 years in 50.0% of cases was B-P type. The angle between common bile duct and the major pancreas duct had different sizes from 4° to 90° average 35.6° (SD ± 21.1°).

Table 2: The union common bile duct and the major pancreas duct by age

	Age by years												Total	
	20-29		30-39		40-49		50-59		60-69		70+			
Types of PB Union	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Separated (V) or "duodenal" Type	4	100.0	1	12.5	3	27.3	4	26.7	3	33.3	3	18.8	18	28.6
B-P (biliarypancreatic) Type	-	-	2	25.0	3	27.3	5	33.3	2	22.2	8	50.0	20	31.7
P-B (pancreaticobiliary) Type	-	-	-	-	2	18.2	1	6.7	1	11.1	1	6.3	5	7.9
Pathology that deform PB Union	-	-	5	62.5	2	18.2	5	33.3	3	33.3	4	25.0	19	30.2
Artefacts	-	-	-	-	1	9.1	-	-	-	-	-	-	1	1.6
Total	4	100.0	8	100.0	11	100.0	15	100.0	9	100.0	16	100.0	63	100.0

With the Mann-Whitney test, we did not distinguish significant statistical significance in the size of the PB angle. Based in gender (P = 0.633) in men the PB was from 5° to 61° with the average 36.9°, while the females from 4° to 90°, with the average 34.3° (Table 3).

Table 3: The angle between common bile duct and the major pancreas duct by sex

	Sex		
Size of PB angle	M	F	Total
N	32	31	63
Mean value	36.9º	34.3º	35.6º
SD	19.1º	23.8º	21.1º
Min	5º	4º	4º
Max	61º	90º	90º
Mann-Whitney test	P = 0.633		

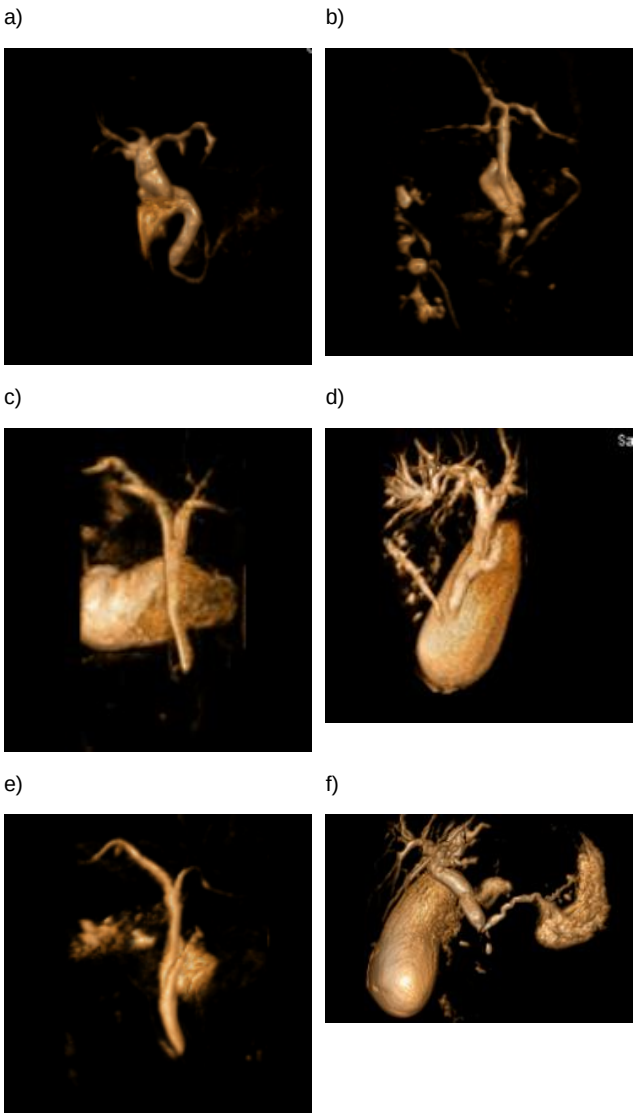

Figure 1: PB images of MRCP. a) Common bile duct joined main pancreatic duct (B-P Type); b) Common bile duct and major pancreatic duct are separated (V Type), narrow-angle between ducts is presented; c) Major pancreatic duct joined common bile duct (P-B Type); d) Common bile duct and major pancreatic duct are separated (V Type); e) Image shows narrow-angle between ducts; B-P Type is presented; f) Image presented right angle between ducts duct (V type)

We did not find any correlation between the age and size of the angle between common bile duct and the major pancreas duct (P = 0.782), (Table 4).

Table 4: The angle between common bile duct and the major pancreas duct by age

	Age by years						
Size of PB angle	20-29	30-39	40-49	50-59	60-69	70+	Total
N	4	8	11	15	9	16	63
Mean value	32.0º	61.5º	31.0º	43.4º	4.5º	32.2º	35.6º
SD	3.5º	2.3º	15.6º	15.8º	0.7º	14.9º	21.1º
Min.	29.0º	48.0º	15.0º	21.0º	4.0º	11.0º	4.0º
Max.	35.0º	63.0º	52.0º	61.0º	5.0º	58.0º	63.0º
Kruskal Wallis Test	K =1.32, P = 0.782						

The common channel in one case was 15 mm. In other cases, it was 3-12 mm (Table 5).

Table 5: Size of common channel (between common bile duct and major pancreatic duct)

Common channel (PB)	M	F	Total
N	12	13	25
Min (mm)	3	12	3
Max (mm)	10	15	15
Mean value (mm)	4	5	4.5

Discussion

The union of the common bile duct and the major pancreas duct in 31.7% of the cases was B-P Type (biliary pancreatic). The most common women were P-B Type with 12.9% of cases compared with 3.1% males.

At age 20-29 in 100.0% of cases, the union common bile duct and the major pancreas duct was "duodenal" or separated type, while the age over 70 years in 50.0% of cases was type B-P. The common channel in one case was 15 mm. In other cases, it was 3-12 mm. On MRCP, the length of the common channel was calculated to be 15 mm or longer in nine (82%) of 11 patients with anomalous PBJ. In patients with normal PBJ, MRCP identified PBJ with the channel measuring 0 mm in length [10]. The types of the anomalous pancreaticobiliary ductal union as determined on MR cholangiopancreatography (type A, n = 2; type B, n = 7; type C, n = 3) were concordant with those of intraoperative cholangiography in 11 of 12 patients. In five of eight patients with choledochal cyst (type Ia, n = 1; type IVa, n = 5; type IVb, n = 2) in whom MR cholangiopancreatography could not depict anomalous pancreaticobiliary ductal union, anomalous pancreaticobiliary ductal union was documented on intraoperative cholangiography that was performed after choledochal cyst resection [11].

The frequency of AUPBD varies from 1.5-3.2%. Its diagnosis needs a high index of suspicion and carefully performed investigations. Komi et al., in their new classification described 51 cases of AUPBD. Of these, 35.5% were Type I, 21.6% were Type II, and 43.1% were type III. Type III was sub-divided into three types (A, B and C). Type C is further divided into three sub-types (1, 2 and 3) [7].

The angle between common bile duct and the major pancreas duct had different sizes from 4° to 90° average 35.6° (SD ± 21.1°). With the Mann-Whitney test, we did not distinguish significant statistical significance in the size of the PB angle. Based in gender (P = 0.633) in men the PB was from 5° to 61° with the average 36.9°, while the females from 4° to 90°, with the average 34.3°.

The APJ (anomalous pancreaticobiliary junction) in group A was smaller than in group B (51.45° ± 13.51° *vs* 65.76° ± 15.61°, P < 0.05). According to the type of biliary duct and main pancreatic duct joining the duodenal wall, the

prevalence of acute pancreatitis in the V type and in the B-P type was higher than in the P-B type (12/17 *vs*. 10/29, or 18/26 *vs*. 10/29, respectively, all P < 0.05), whereas there was no significant difference in the prevalence of acute pancreatitis between the V type and B-P type (P > 0.05). The APJ were 59.32° ± 20.04°, 60.22° ± 11.06°, 57.13° ± 17.27°, respectively in V type, B-P type and P-B type joining of the main pancreatic duct (P > 0.05) [12]. We did not find any correlation between the age and size of the angle between common bile duct and the major pancreas duct (P = 0.782).

Misra and his colleagues reported that a common channel with a mean length of 4.7 ± 2.5 mm (range, 1.6 to 18.4mm) was present in 64 (63%) of 102 normal endoscopic retrograde cholangiopancreatography films [2]. The reported frequency of abnormal pancreaticobiliary junction ranged from 1.5% to 3.2% in different ethnic populations [13].

Wang et al. found an abnormal pancreaticobiliary junction in 59 (3.4%) of 1752 subjects undergoing endoscopic retrograde cholangiopancreatography [14]. In 1973, Babbitt et al. proposed abnormal pancreaticobiliary junction as the aetiology of choledochal cyst [15].

Kimura et al., studied 65 patients with the abnormal pancreaticobiliary junction and found that 49 (75.4%) had choledochal cyst alone, 11 (16.9%) had gallbladder cancer alone, and 5 (7.7%) had both choledochal cyst and gallbladder cancer [6].

These findings were confirmed by Wang et al., who found that 62.5% (5 of 8) of patients with gallbladder cancer and 33.3% (9 of 27) of patients with common bile duct cancer had an abnormal pancreaticobiliary junction [14].

AUPBD is a well described though uncommon, and often unrecognised, the cause of acute pancreatitis, especially in young patients. It has also been associated with gallbladder carcinoma. In one study, anomalous ductal union occurred in 16.7% of the patients with gallbladder carcinoma in comparison with an incidence of 2.8% among 641 consecutive patients with various hepatobiliary and pancreatic diseases. Gallbladder carcinoma occurred in 24.6% of the 65 cases of the anomalous ductal union in comparison with a 1.9% incidence of this cancer among 635 consecutive patients similarly studied and found to have a normal ductal union [2].

In conclusion, the union of the common bile duct and the major pancreas duct was B-P Type. The common channel was normal in most cases. The angle between common bile duct and the major pancreas duct had different size. We did not distinguish significant statistical significance in the size of the PB angle. PB angle on average in men was 36.9°, while in females 34.3°. No correlation between the age and size of the angle between common bile duct and the major pancreas duct.

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Basic Science

In Vitro Action of Meconium on Bronochomotor Tonus of Newborns with Meconium Aspiration Syndrome

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Abbreviations: MAS - Meconium Aspiration Syndrome; IL - interleukins (IL-1β, IL-6 and IL8); TNF-alpha - tumor necrosis factor; PLA2 - phospholipase A2; PAF - platelets activating factor; NO – Nitric Oxide; H & E - hematoxylin-eosin; X - the median value; SD - standard deviation; SEM - standard error

Abstract

AIM: Here we studied the role of meconium in the respiratory system on live and exited newborns (weight 250-3000 g). Throughout this study is followed the response of tracheal rings in acetylcholine and histamine in different molar concentrations (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} mol/dm^3).

METHODS: To study the smooth tracheal musculature we used 23 tracheal preparations obtained from the newborns exited from meconium aspiration.

RESULTS: Based on the functional analysis of the tracheal specimen we have concluded that the meconium aspiration did not change the smooth musculature response on acetylcholine and histamine when compared to control group, exited from lung inflammatory processes (e.g., pneumonia, bronchopneumonia, atelectasis, cerebral hemorrhage), where tracheal smooth musculature response is significant (P for other causes is not significant (P > 0.01).

CONCLUSION: The conclusions suggest that meconium did not potentiate the constrictor action of acetylcholine and histamine in the tracheobronchial system and did not cause modulation of bronchomotor tonus in case of his aspiration. Meconium causes mild relaxation of smooth tracheal musculature with a mechanism which is not mediated by cyclooxygenase products, from tracheal epithelium or proteins. Also, direct activity in the smooth musculature of several tested acids seems to have no significant impact in increasing the tonus of respiratory airway of smooth tracheal musculature.

Introduction

The mechanisms which contribute to increasing the reactivity of respiratory airways in Meconium Aspiration Syndrome (MAS) are very unclear. Meconium is a biologically active substance with very powerful contractile effect in vascular and airway smooth musculature, composed of leukotriene, PAF, ET-1 etc. Meconium contains high concentrations of fatty acids [1] and biliary acids [2] that may induce contraction of smooth musculature of respiratory airways. This contraction depends on the concentration of aspirated meconium [3]. Meconium aspiration syndrome (MAS) is an important cause of mortality and respiratory morbidity in newborns babies. Mechanic obstruction of respiratory airways, dysfunction of pulmonary surfactant, pulmonary inflammation and vasoconstriction are pathologic mechanism associated with MAS syndrome. Damage reactivity of respiratory airways also could be associated with MAS syndrome [4] [5].

The airway obstruction could affect reflexive alteration of bronchomotor tonus associated with bronchoactive substances. Interactions between individual pathogenetic factors are not yet known. Meconium is found to be present from 12 weeks of gestation. It is a product of amniotic fluid of fetus containing plaque cells of vernix caseosa secretion and gastrointestinal cells [6]. Meconium contains 4 different fatty acids (e.g. Choline, henodeoxicholic acid and lithocholic) and minerals from witch copper, zinc, manganese, calcium, iron and phosphorus are more frequent component [7] [8]. Also contains

plasma proteins (alpha 1 antitrypsin) [9] [10] and other active substances such as interleukins IL-1β, IL-6 and IL8, tumor necrosis factor (TNF-alpha) [11] and phospholipase A_2 (PLA_2) [12] which may induce directly or indirectly pulmonary inflammation, by increasing production of cytokines and by activating white blood cells or epithelial/endothelial cells of lungs. In vitro exposure of meconium increase release of IL-8, TNF-alpha [13], endothelin-1, platelets activating factor (PAF), leukotrienes, thromboxane A_2, induced of NO synthetase [14], NO [15], PLA_2 and other substances which affect the reactivity of respiratory airways and inflammation.

Recent studies of meconium aspiration in rats have shown the increased response of smooth musculature of respiratory airways to methacholine after seven days, and also to histamine in rabbits after 5.5 hours from meconium application. Mechanisms of increased reactivity of respiratory airways in MAS syndrome are unknown. Direct inflammatory effects of meconium on the release of broncho active substances (PAF, leukotrienes, etc.) and hypoxia accompanied by oxidative damage from oxygen therapy may be operative in this process [12].

Purpose of the study is to demonstrate the action of meconium in newborn babes with MAS syndrome in the inflexion of acetylcholine and histamine action in the smooth musculature of the tracheobronchial system in alive and the dead newborn babes.

Material and Methods

The research was conducted in cooperation with the Gynecology Obstetrics Clinic, Pathologic Anatomy Institute and Experimental Unit of Medical Faculty in Prishtina, with permission of the Ethics Commission by respecting principles of Helsinki Declaration.

Classification of tracheal preparation of newborn babies in different weeks of gestations is made by histopathological examination of tracheal preparation (in blocks of paraffin). The preparations have been stained with the standard: hematoxylin-eosin (H & E) methods.

Research has been conducted in 33 subjects of in vitro isolated tracheas of babies in different weeks of gestations (weight 250 to 3000 g). Tracheas were taken immediately after autopsies. Above bifurcation of the trachea were taken 6 tracheal rings and placed in Krebs solutions DIP (pH = 7.4). The water bath temperature was kept at 37^0C, and solution in the bath is aerosolised continuously with a gas mixture (95% CO_2 and 5% O_2**),** which has flowed in continual mode through the bath solution. Tracheal rings are serially connected with each other. The series consisting from 6 rings are placed in the bath for isolated organs (volume 50 ml), so that lower part of the ring is connected to the holder, while the upper part is connected to the transducer (" Force transducer" Statham UC_2). The smooth musculature response is registered in the multi-channel recorder (Watanabe HSE 6600). After 30 minutes is registered tonus of tracheal rings, then preparation is exposed to different molar concentration (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, mol/dm^3) of acetylcholine and histamine. Doses are changed every 15 minutes, while effects of broncho constrictor agents are followed 3 minutes, after application. Then preparation is rinsed several times with Krebs solution, before application of another substance.

Results are processed with statistic computer program GraphPad InStat III with comparing t-test for two working groups.

Results

In Table 1 and Figure 1 is showed the acetylcholine dose response on smooth tracheal musculature in newborn babies of different age groups of meconium aspiration syndrome vs control group ($P < 0.01$).

Table 1: Acetylcholine dose response of smooth tracheal musculature in newborn babes of different age groups of meconium aspiration syndrome MAS (Mean ± SEM)

Groups	Ach log^{-4}	Ach log^{-3}	Ach log^{-2}	Ach log^{-1}
MAS	2.91 ± 0.98	4.79 ± 1.56	10.75 ± 3.33	19.70 ± 4.73
Control	4.5 ± 1.53	7.18 ± 2.21	13.37 ± 3.46	23.96 ± 4.89

In Table 2 and Figure 2 is showed the cumulative response of histamine in smooth tracheal musculature in newborn babies of different age groups of meconium aspiration syndrome vs control group ($P < 0.01$).

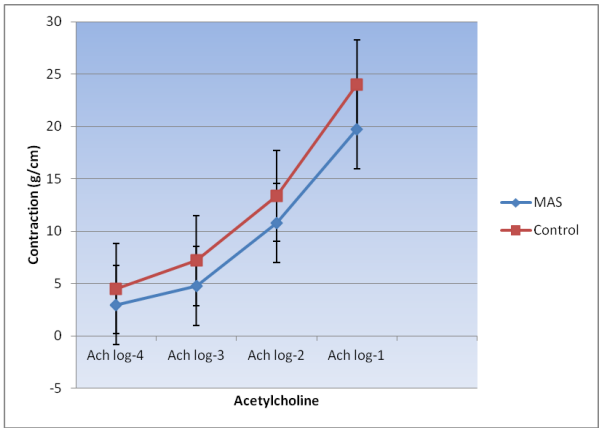

Figure 1: the Cumulative action of acetylcholine in smooth musculature in newborn babes with different age groups of Meconium Aspiration Syndrome (Mean ± SEM)

Table 2: Histamine dose response of smooth tracheal musculature in newborn babes of different age groups of meconium aspiration syndrome MAS (Mean ± SEM).

Groups	Histamine log^{-4}	Histamine log^{-3}	Histamine log^{-2}	Histamine log^{-1}
MAS	4.161 ± 1.76	8.0 ± 2.81	14.75 ± 4.04	22.0 ± 5.60
Control	5.25 ± 1.68	10.18 ± 2.56	19.06 ± 3.72	27.08 ± 5.06

Figure 3 is presented comparative effect of acetylcholine and histamine action in newborn babies of different age groups of meconium aspiration syndrome vs control group (P < 0.1).

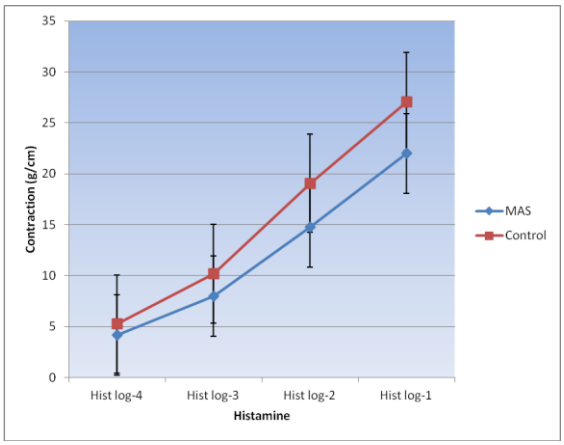

Figure 2: the Cumulative action of histamine in smooth tracheal musculature in newborn babes of different age groups of Meconium Aspiration Syndrome (Mean ± SEM)

Discussion

The studies of newborn babies affected with MAS during neonatal period have shown the abnormality of functionally pulmonary tests, diminished obstruction of respiratory airways, an episode of bronchospasm, and need for bronchodilator therapy.

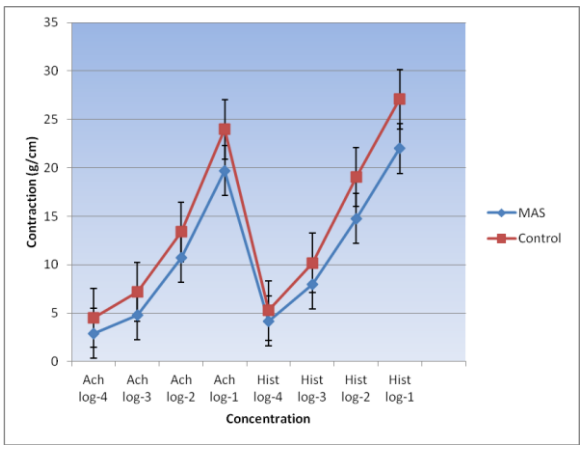

Figure 3: the Cumulative action of histamine and acetylcholine in smooth tracheal musculature in newborn babes of different age groups of Meconium Aspiration Syndrome (Mean ± SEM)

Progressive lung inflammation may increase the reactivity of respiratory airways. Thus the use of bronchodilators together with anti-inflammatory drugs may be useful in MAS syndrome. Meconium stasis of the amniotic fluid happens at least in 8% of all births. Incidence varies from 5-8 % before 39 weeks of gestation. Incidence could increase by 12 % after 39 weeks of gestation [15]. Prematurity is not a risk factor. MAS is very rare in newborn babes before 34 weeks of gestation. Meconium stasis presents a risk for newborn babes; MAS proceed in 10-30% and 19-34% cause mortality. Risk factors in MAS syndrome include newborn babes 1-3 weeks after birth date period, maternal diabetes, hypertension in gestation, difficult births, respiratory distress syndrome (RDS) in newborn babes, intrauterine hypoxia [16].

Symptoms and signs of MAS syndrome include tachypnea, nasal secretion, retraction, cyanosis or increased desaturation, also heavy stasis in umbilical cordon with skin spots. Meconium stasis can be noticed in oropharynx, larynx and trachea. The prophylactic suction of nose and mouth before the birth of body of the babes decreases the risk of MAS [17].

Recently, new therapeutic strategy for the treatment of MAS is suggested [18] including anti-inflammatory drugs, such as antagonists of prostaglandins, high-frequency ventilation, exogenous surfactant, nitric oxide and water inhalation [18].

The rings of tracheal tissue and lungs of guinea pigs are incubated for one hour in a water bath for isolated organs, in three different concentrations with human meconium to investigate whether there is a connection between the cumulative dosages of acetylcholine, histamine and meconium concentrations and contraction response [19]. Current studies show that the contractile response of the rings of tracheal and lungs tissue gradually increases with cumulative doses of acetylcholine and histamine in different concentrations of meconium and control condition [19]. However, there is no complete response in decreased concentration of meconium, which has lower tracheal reactivity to histamine and acetylcholine [19]. High concentration of meconium tends to increase tracheal reactivity. Incubation in a lower concentration of meconium 1 mg/ml in rabbit trachea act by lowering reactivity in vitro of acetylcholine and histamine [19]. In vitro relaxation of smooth tracheal musculature has been demonstrated in rats [20], but relaxation response increases with increasing of meconium concentration. Presence of reduced concentration of meconium in amniotic fluid may present a sign of physiologic maturity in newborn babes and do not present inflammatory response of tissues. On the other side, a high concentration of meconium may cause harmful changes resulting in inflammations and related to constriction of vascular and respiratory airways of the smooth musculature. While a lower concentration of meconium increases secretion of surfactant in isolated alveolar tip II cell [20] and inhibit oxidative blast of neutrophils and

phagocytosis [21]. This antioxidative capability of meconium could be partially responsible for the lower incidence of MAS syndrome in case of amniotic fluid aspiration of newborn babes.

In vitro condition, the aspiration of 20% of meconium in rats significantly increases the response of the respiratory airways to methacholine after 7 days and is associated with the lymphocytic and eosinophilic inflammation, metaplasia of goblet cells and increased concentrations of IL-5 and IL-3 in bronchoalveolar wash [1]. Is noticed progression of the polymorphonuclear inflammation in ventilated rats for 5.5 hours after inhalation of meconium, who also was associated with increased tracheal reactivity to histamine [2]. The above results suggest that the contraction of smooth musculature of the respiratory airways is associated with concomitant mechanisms at MAS syndrome, such hypoxia, cytokine production and the reactive products during inflammations.

There is no full correlation between contractile response and meconium concentration. Tracheal reactivity of the respiratory airways to the cumulative dosage of histamine and acetylcholine increases with increased concentration of meconium, but the reactivity of lung tissue tends to decreases. Mechanisms of reactivity in affected respiratory airways in MAS syndrome are unclear, and further experiments show for constrictor response of smooth musculature of the respiratory airways to meconium.

In the present studies, during increased concentrations of meconium, reactivity response of smooth tracheal musculature to the acetylcholine and histamine is shown with a tendency to partial decreases depending from increases dosage of these mediators. The different responses may be related to the duration of exposure to meconium. Exposure for a short time in vitro may present vasodilator and bronchodilator effects, while exposure for a long time may have mainly constrictor effects in smooth musculature that depends on the time of inhibiting medium. For a better understanding of above mechanisms, incubation for a short period in vitro of pulmonary rings and blood vessels in different meconium concentrations would be necessary.

Based on the results obtained experimentally can be concluded that:

Tracheal smooth musculature response in MAS syndrome to acetylcholine in cases that have exited without aspiration of amniotic fluid is significant ($P < 0.01$).

Meconium in MAS syndrome does not potentiate the constrictor action of acetylcholine in smooth tracheal musculature insignificant way ($P > 0.1$).

Tracheal smooth musculature response in MAS syndrome to histamine in cases that have not exited from meconium aspiration (but for other reasons) is significant ($P < 0.01$).

Meconium in MAS syndrome does not potentiate the constrictor action of histamine compared to group control ($P > 0.1$).

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Morphology of the Ovaries in Condition of Inhalation Intoxication with Dust-Saline Aerosols of the Aral Sea in Female White Rats

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Keywords: Dust-saline aerosols; Aral Sea; Electron microscopy; Ovaries; Follicular apparatus

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Abstract

BACKGROUND: After some clinical studies of the reproductive health of the female population of the Aral Sea region, we concluded that it is necessary to confirm by experiment the theory of the direct influence of dust-saline aerosols of the Aral Sea on the reproductive system of female individuals.

AIM: The purpose of this work is to study the effect of dust-saline aerosols of the Aral Sea on the folliculogenesis process in rats at inhalation intoxication.

METHODS: Inhalation in rats was carried out for 30 days for 4 hours a day for 5 days a week in special inoculating cylindrical chambers with the extra-chamber placement of animals in individual boxes and dynamic aerosol delivery. Morphological changes were assessed using electron microscopy.

RESULTS: Ultrastructural changes in the ovaries of the experimental study group were characterised by the pathology of all structures of the cortical substance of the organ. The accumulation of lutein pigment, the utilisation of lipid inclusions and the destruction of the complete cell reflected the pathology of estrogen production-an important factor in the postovulatory phase of the ovarian cycle.

CONCLUSIONS: Taking into account the proven effect of dust-saline aerosols on the production of estrogen, the violation of the postovulatory phase of the ovarian cycle, we trace the mechanism of folliculogenesis disturbance. This confirms the data of our previous studies on primary and secondary infertility in women living in the Aral Sea region and the necessity of creation and development of preventive measures for the inhabitants of the region.

Introduction

The climate of the Aral Sea region and the bordering regions is sharply continental and is characterised by sharp temperature changes. Winter is cold and severe; summer is hot. There is a deficit of atmospheric precipitation and low air humidity.

A consequence of a sufficiently developed industry of South Kazakhstan, Kyzylorda, and Aktobe regions is a high anthropogenic impact on the environment, which is expressed in the pollution of atmospheric air, soil and large rivers, the accumulation of industrial waste and solid domestic waste. The oil-producing areas are characterised by soil contamination with petroleum. Chromium, boron, phosphorus is the main pollutants for areas with production and processing of minerals, in agricultural areas. The main pollutants are fluorine, phosphorus, etc. [1] [2] [3].

Negative anthropogenic factors become destructive not only for ecosystems but also a real threat to human life and health, an obstacle to sustainable social and economic development. Ecologically dependent shifts and pathological changes occur in various organs and systems under the influence of negative environmental factors-life expectancy decrease, an increase in the degree of psychophysiological and genetic stress, an increase in specific pathology, and the emergence of new forms of environmental diseases [3].

Against the background of global ecological changes affecting all components of the ecosystem, human health deserves more attention, as human resources are the main component of development [4]. The necessity to improve the quality of diseases prevention and treatment that are associated with the impact of adverse environmental factors is dictated by the increased damage that causes environmentally dependent diseases to all of humanity.

According to the monitoring of recent years and scientific research, the complex environmental pollution in the studied regions has been identified and established; it was revealed it negatively effects on the population of the Aral Sea area of Kazakhstan. A high salt load and the effect of some ecotoxicants on the organism of the population in the zones of ecological tension in the Aral Sea region have been established, and it requires an additional systematic study of public health [3] [4] [5].

The results of our study, which was conducted over a period of 3 years and examined 2527 women of reproductive age, who live at least 5 years in the Aral region and engaged in occupations with hazards of no higher than 2nd class, have the following conclusions: in the Aral region, a later attack is observed menarche, over 16 years of age in 36.6% of cases. Perinatal losses according to the history of the population 23.5%, that is, almost every third women have a history of spontaneous abortion and undeveloped pregnancy. According to our study in the Aral Sea region, 3% of women with primary infertility and 5% infertility were identified. Late menarche was observed in the absolute majority of girls, a regular cycle of menstruation is not established for a long time; in a third of them, rare and/or scarce menstruation can persist until the 18th birthday, and in such cases, the primary oligomenorrhea is diagnosed. Later, menarche is most common for girls with body weight deficiency, who have chronic diseases, increased mental and physical stress. Also, the cause of late menarche can be the effect of toxic substances on the immature organism from the environment. Such girls need a careful examination and carrying out activities aimed at preventing disorders of the sexual system in the future, including infertility, complicated course of pregnancy and childbirth, early occurrence of other abnormalities in the reproductive system. After a number of clinical studies of reproductive health of the female population of the Aral Sea region, we came to the conclusion that it is necessary to confirm by experiment the theory of the direct influence of dust-saline aerosols of the Aral Sea on the reproductive system of female individuals [6] [7] [8] [9].

The purpose of this work is to study the effect of dust-saline aerosols of the Aral Sea on the folliculogenesis process in rats at inhalation intoxication.

Materials and Methods

The sampling of the study subjects was realised taken into account the weight, age, content and diet of 30 white female rats in the dioestrus phase. Immediately before the experiment, vaginal smears of animals were studied to determine the phase of the oestrous cycle. The females in the di-estrus phase were taken into the experiment. Smears (vaginal smears) were taken with a thin eye dropper, were placed on a slide and examined under a microscope at a magnification of 300 times in a darkened field.

The inhalation effect on rats was carried out for 30 days in special inoculated chambers according to the method of L. B. Borisova by standard modification of N. T. Yelevskaya for 4 hours a day for 5 days a week in cylindrical chambers with the extra-chamber placement of animals in individual boxes and dynamic aerosol delivery. The holes of the boxes are directed by head part inside the chamber. The air compressors are fixed-mounted compressors. Shallow was placed in the distributor for ¾ of its volume. The air was drawn in and out of the chamber at a rate of 50 l/min. A sampling of air to control the concentration of aerosol in the chamber was carried out every 30 minutes on the filters using a portable dust analyser «Prima 01». The euthanasia of the experimental and control animals was carried out by decapitation. The excretion of animals from the experiment was carried out in the diestrus phase for a more accurate result. The work with animals was approved by the Committee on Bioethics at the Karaganda State Medical University and corresponded to the international rules for the use of animals in the experiment.

The material for the study was the ovaries of mature females of white rats. For transmission electron microscopy, pieces of the ovaries were fixed in glutaraldehyde on phosphate buffer and fixed in 1% solution of osmium tetroxide. Semi-thin sections stained with blue toluidine were studied by the light-optical method. Ultrathin sections (30-60nm) were obtained by ultramicrotome Leica U70, after Reynolds contrast, they were examined and photographed on a transmission electron microscope Libra 120 Carl Zeiss.

Results

Electron microscopic examination of the ovaries of experimental animals in the control group showed that the flattened follicular epithelium of the primordial (primary) follicle contained an elongated form of the nucleus with initially located

heterochromatin and an enlarged perinuclear space. There was large mitochondrion (M) with an electronically transparent matrix and cristae in the cytoplasm. Well-developed elements of the Golgi complex-a stack of flattened cisterns and small vesicles were near the nucleus. The tubules of the granular endoplasmic reticulum (GER) were enlarged with the flaky material in the lumen (Figure 1). Also in the cytoplasm, there were lysosomes, free ribosomes and large lipid granules. The single-row follicular epithelium had a thin basal membrane and a smooth apical surface.

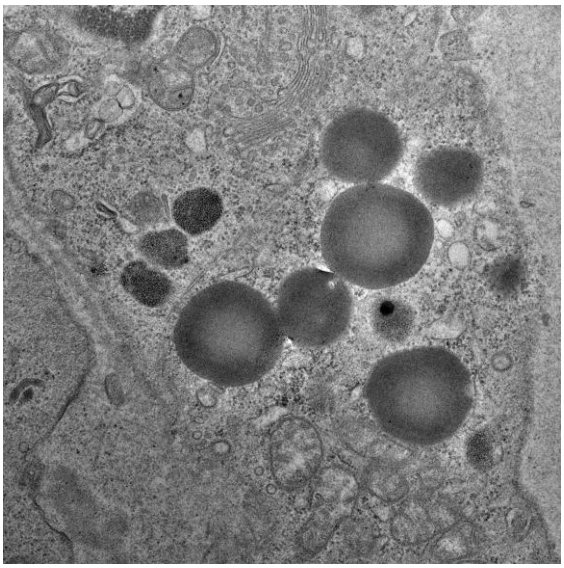

Figure 1: Control; Mitochondria (M), the Golgi complex (GC) and lipid granules (LG) are seen in the cytoplasm of the flattened primary follicle; BM-basal membrane; Electron diffraction pattern; Magnification x4000

Theca interna cells under the epithelium had an elongated shape with thin processes (Figure 2). There were polymorphs in the form of mitochondria and tubules of the GER, small lysosomes in the centre of the cytoplasm. Microtubules and pinocytosis vesicles were visible on the periphery of the cytoplasm.

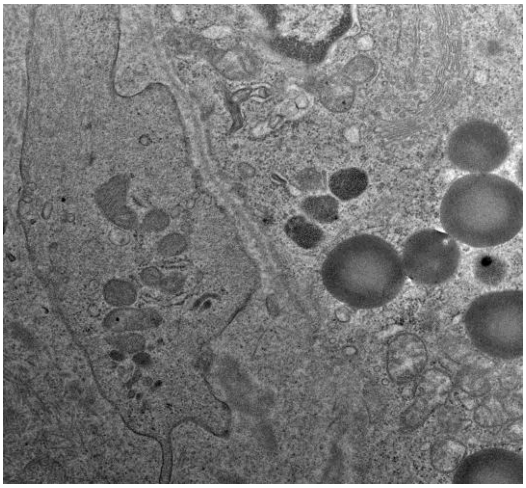

Figure 2: Control; Fibroblast-like theca interna cell; Electron diffraction pattern; Magnification x4000

The follicular epithelium of secondary follicles, represented by several layers of proliferating epithelial cells, was characterised by a large irregularly shaped nucleus with a high proportion of euchromatin and an enlarged perinuclear area. The cytoplasm contained well-developed tubules of the GER and large mitochondria (Figure 3).

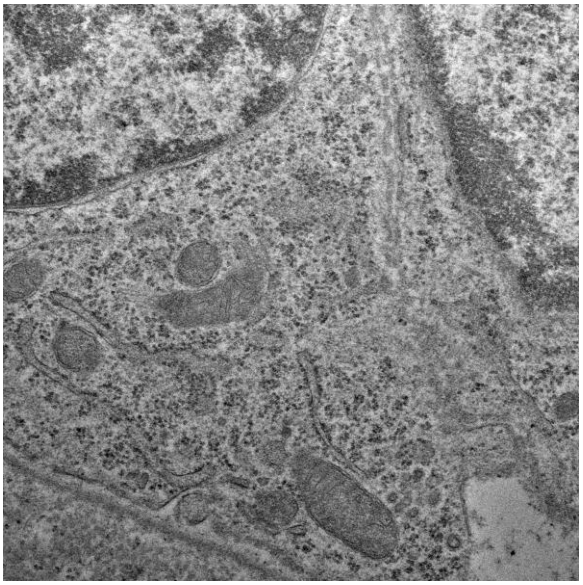

Figure 3: Control; Epithelial cell of the developing follicle; N-nucleus; M-mitochondria, granular endoplasmic reticulum (GER); Electron diffraction pattern; Magnification x4000

Slightly spaced apart follicular fluid cells had a smooth cytoplasmic surface. The apical surface of superficially located cells was characterised by small processes (Figure 4). Near the apical pole of the cell were located the dichotomies of the Golgi complex.

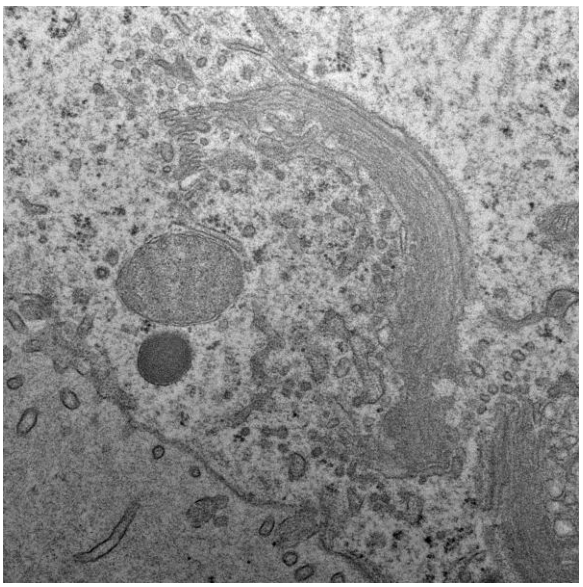

Figure 4: Control; the Apical surface of the follicular cell with the Golgi complex (GC) and microvilli; Electron diffraction pattern; Magnification x8000

Maturing large follicles had a large number of

cell layers, marked by the structure of the epithelium. Large nuclei contained euchromatin, the content of mitochondria, GER, and polyribosomes increased sharply in the cytoplasm (Figure 5).

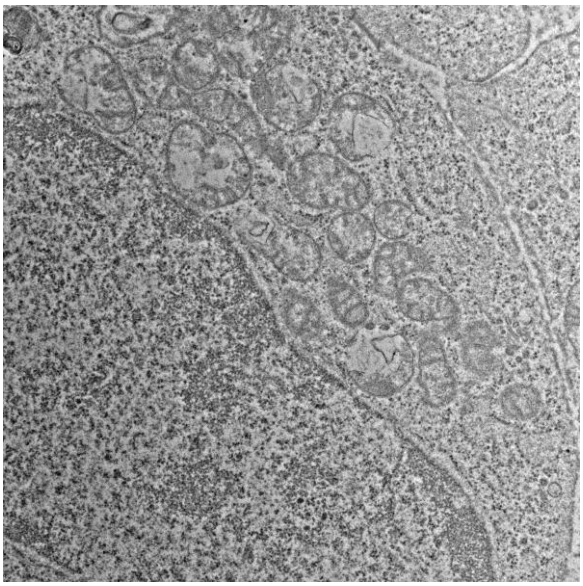

Figure 5: Control; Epithelial cell of a mature follicle; N-nucleus; M-mitochondria; Electron diffraction pattern; Magnification x8000

The ovule located in the centre of the follicles had a large pale nucleus and a cytoplasm with numerous yolk grains. Often the ovum did not fall into the plane of the ultrathin section of the follicle, more often it was found on semi-thin sections. Stroma of the ovary contained numerous spindle-shaped and luteal cells. The latter contained large mitochondria with vesicular crystals and lipid granules of a homogeneous structure of increased electron density (Figure 6).

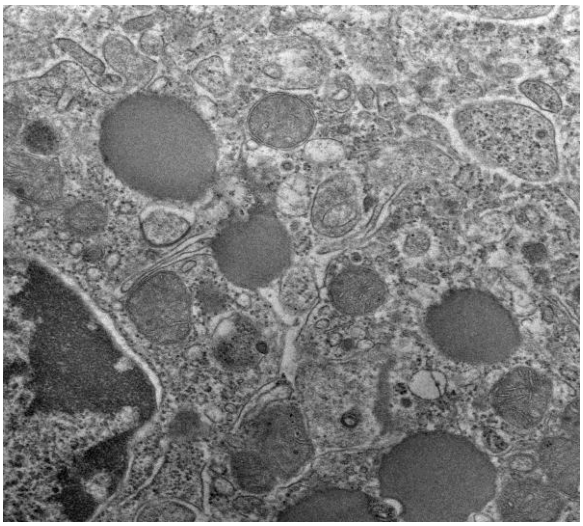

Figure 6: Control; Luteal cell of stroma with large lipid inclusions (L); N-nucleus; Electron diffraction pattern; Magnification x8000

Blood capillaries had preserved endothelial cells with a large nucleus and well-developed organelles (Figure 7). The apical surface was provided with thin cytoplasmic outgrowths.

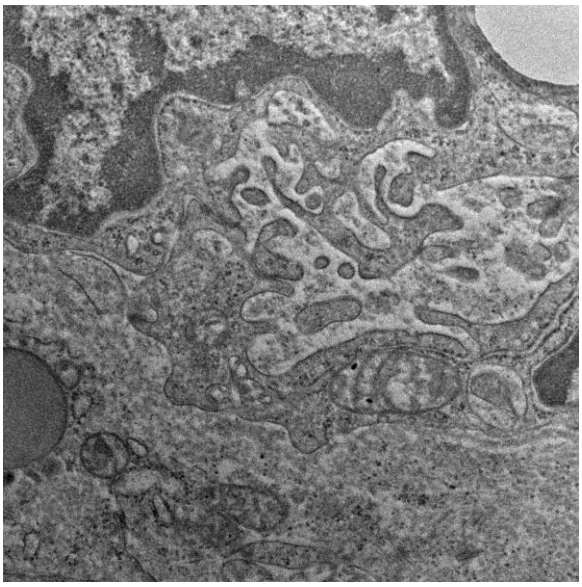

*Figure 7: Control**;** Endothelium of the blood capillary of stroma; N-nucleus, M-mitochondria; CP-cytoplasmic processes; Electron diffraction pattern; Magnification x8000*

In the experimental group, the epithelium of the primary follicles was sharply flattened and stretched. In the cytoplasm of the epithelium, secondary lysosomes accumulated, formed on the site of degenerated organelles (Figure 8).

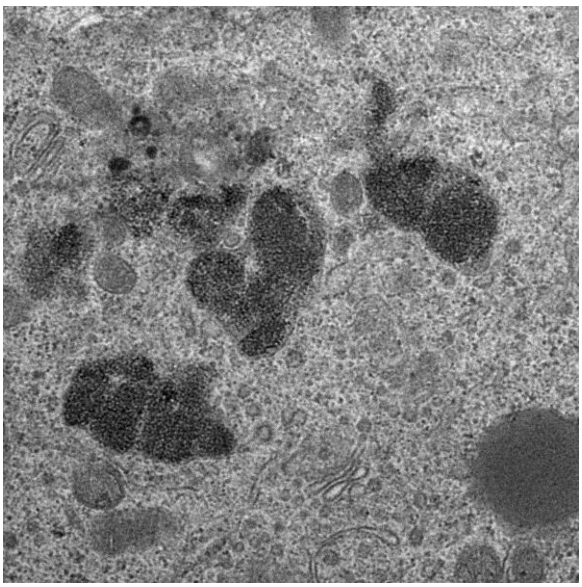

Figure 8: Experiment; Cytoplasm of the primary follicle with lysosomes (LS) and degenerately altered organelles; Electron diffraction pattern; Magnification x8000

Complete destruction of the nucleus and all parts of the cytoplasm of the follicles in the form of ruptures was noted (Figure 9). The fragments of the destroyed cells were seen in the lumen of the follicles. Cellular elements of theca interna «overgrew» with collagen fibrils (Figure 10).

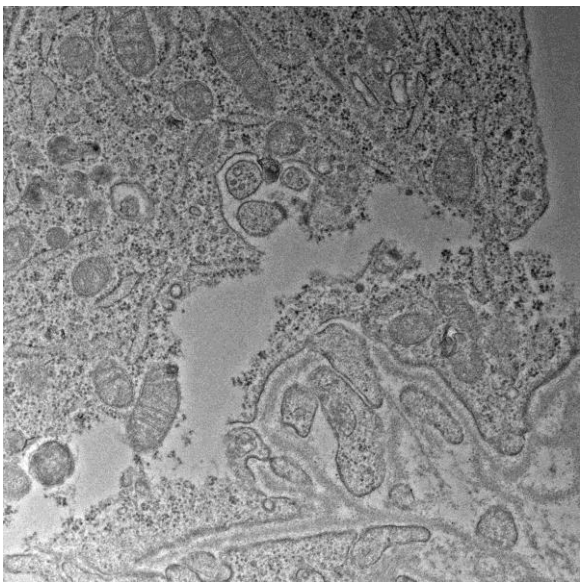

Figure 9: Experiment; Destruction of the cytoplasm of the basal part of the epithelial cell of the follicle in the rupture form; BM-basal membrane; Electron diffraction pattern; Magnification x8000

The wall of fibroblasts was destroyed; the organelles were in a state of necrosis. Numerous bundles of collagen fibrils and fibroblasts were observed in the stroma of the ovaries.

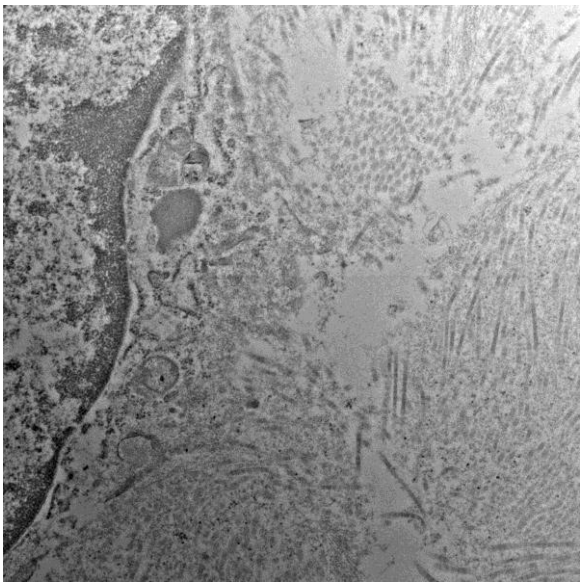

Figure 10: Experiment; Theca interna cell in a state of necrosis; CF-collagen fibrils; Electron diffraction pattern; Magnification x12000

Luteal cells of the stroma were characterised by expressed utilisation of lipids (Figure 11). The luteal pigment accumulated in the cytoplasm. The lipid granules glowed and merged, turning into unformed structures filling almost the entire volume of the cell (Figure 12). Many luteal cells were destroyed (Figure 13). Small blood vessels were necrotic (Figure 14). The capillary wall was a mass of necrotic material of the destroyed organelles. Endothelium of arterioles was perpendicularly elongated concerning the vessel's axis and often necrotic.

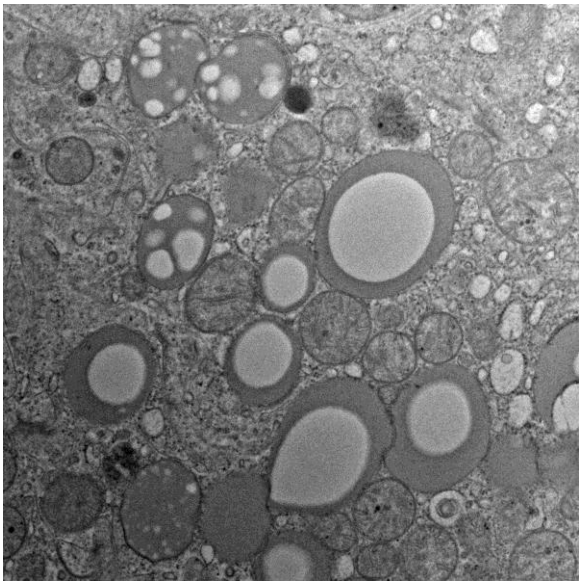

Figure 11: Experiment; Cytoplasm impacted by lipid granules; Electron diffraction pattern; Magnification x8000

Discussion

Ultrastructural changes in the ovaries of the experimental study group were characterised by the pathological changes of all structures in the cortex of the ovary.

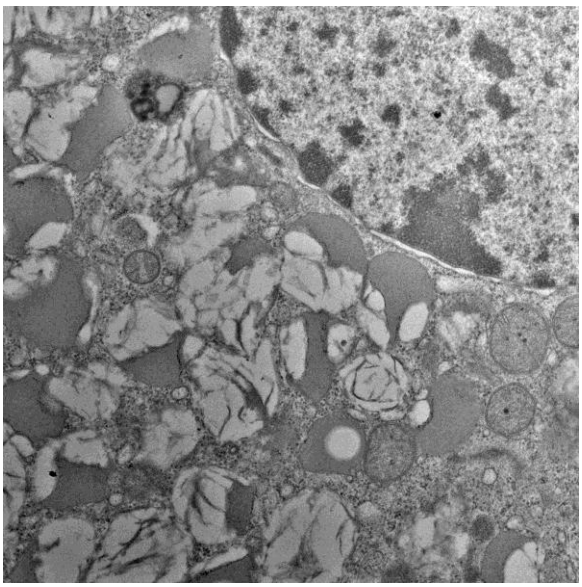

Figure 12: Experiment; Large confluent structures in place of the recycled lipid granules; Electron diffraction pattern; Magnification x8000

Against the background of destruction, necrosis of blood vessels and stromal fibrosis, follicular epithelium of primary follicles underwent dystrophic changes in the form of destabilisation of lysosomal membranes.

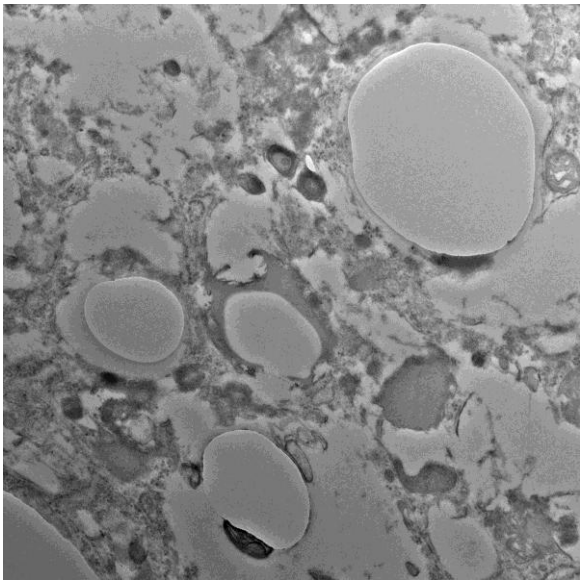

Figure 13: Experiment; Complete destruction of luteal cells; Electron diffraction pattern; Magnification x6000

The destruction of the nucleus and all parts of the cytoplasm in the form of ruptures indicated atresia of the follicles. Theca interna cells, associated with the production of estrogen, were also severely degraded. Ultrastructural changes were noted in the luteal cells of the stroma.

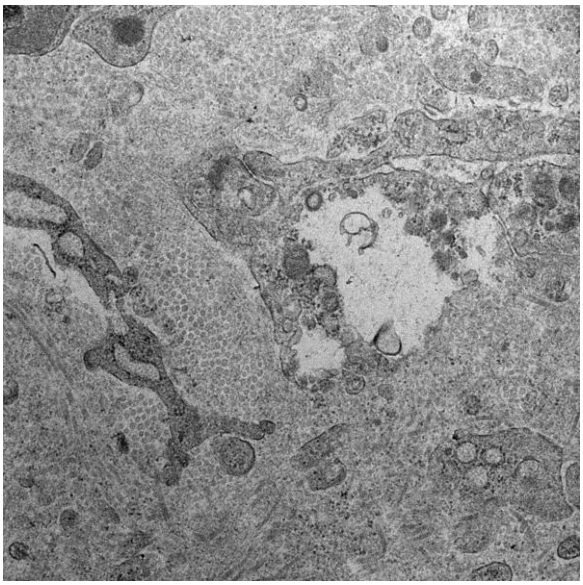

Figure 14: Experiment; Edema, endothelial necrosis of the blood capillary; Electron diffraction pattern; Magnification x6000

The accumulation of lutein pigment, the use of lipid inclusions and the complete destruction of cells reflect a disruption in the production of an estrogens-an important factor in the postviral phase of the ovarian cycle. In the course of the experiment, we concluded that during intoxication of female, white rats with dust-saline aerosols of the Aral Sea, there is a disturbance of folliculogenesis, which manifests itself as a destruction of the follicular composition of the ovaries and blood supply to the ovarian tissue.

The results presented in the course of the research allow us to expand scientific views on the disturbance of folliculogenesis processes when the dust-saline aerosols of the Aral Sea are exposed to the body. The results of the experiment show the expediency of studying the influence of this ecological factor on the reproductive health of the female body to identify possible preventive measures.

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Basic Science

Tumour Lymphocytic Infiltration, Its Structure and Influence in Colorectal Cancer Progression

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Abstract

BACKGROUND: The role of the immune system in the control of tumour progression has been stressed, recently. Many studies indicate the fact that the immune system can prevent tumour progression in several types of human malignant neoplasms including colorectal cancer. According to some authors, a higher density of "tumour-associated lymphocytes" (TAL), in malignant neoplasms, correlate with prolonged survival of patients.

AIM: This study aims to determine the structure and the influence of the immune cells, TAL, in the progression of colorectal cancer (CRC).

PATIENTS AND METHODS: The study included 103 patients with CRC operated at the University Clinic of Digestive Surgery in Skopje, whose operative material was analysed at the Institute of Pathology, Medical Faculty in Skopje. The structure of tumor-associated cells and their density were determined and were correlated with neoplasm's grade, local growth (T), positive lymph nodes, lymphatic invasion and stage of the disease.

RESULTS: CD4+, CD8+ and CD20+ lymphocytes (Ly) were found in TAL. The density of TAL was significantly different in neoplasms with different T status, lymphatic invasion, patients with and without nodal metastasis and patients with a different stage of the disease. The density of CD4+, CD8+, and CD20+ cells were significantly different in neoplasms with different T. The density of CD8+ and CD20+ lymphocytes was lower in patients with nodal metastasis and higher stage.

CONCLUSION: The density of tumor-associated lymphocytes can anticipate the disease progression in patients with colorectal cancer, and the density of TAL influences the control of tumour progression.

Introduction

Colorectal cancer (CRC) is the third most common malignant disease in the humans, with 570,000 new cases in the world each year. It is the second most common cause for the lethal outcome of malignancies in the West European countries and eighth in the developing countries. Despite the new surgical techniques and treatment with neoadjuvant and adjuvant chemotherapy, colorectal cancer is the most common tumour of the gastrointestinal tract which incidence and mortality continually increase [1] [2]. Prognosis of colorectal cancer mostly depends on the stage of disease (local growth, positive lymph nodes and distant metastasis-TNM classification) and depends on many other factors such as lymphovascular invasion, histological grade, positive surgical margins, preoperative serum values of carcinoembryonic antigen (CEA) bowel obstruction and many others [3] [4] [5] [6]. It is considered that new biomarkers such as APC, K-ras, DDS, p53, BRAF, TGF- β, CIMP and others can give more pieces of information for disease progression. The finding that some patients with certain mutations (KRAS), who have metastatic disease, do not respond

to the treatment with monoclonal antibodies represents the basis in the efforts for discovering personalised therapies [7] [8] [9] [10] [11]. This is a motivation for continued investigation of new prognostic factors to determine the disease progression and patient outcome as well as to improve the knowledge for developing new therapies. The role of the immune system in the control of disease progression is the basis for the development of immunotherapy in patients with malignant diseases and is a current topic for research in the field of oncology [9] [10] [11].

The idea that the immune system can recognise a neoplasm was introduced by Paul Ehrlich and was supported by Lewis Thomas and Macfarlane Burnet. Over the course of time, this idea was turned into a concept in which it is considered that the immune cells are capable of eliminating malignant cells. This is how the theory for “immune surveillance” was born. This theory supports hypotheses that the immune system is capable of surveillance of the newly appeared malignant cells and their elimination [12] [13] [14] [15] [16].

It is well known that colorectal cancer progression is influenced by inter-reaction between cancer cells and tumour microenvironment belonging to the patient [12] [13] [14].

It is considered that the immune cells present in tumour-associated stroma are in correlation with tumour progression and patient outcome. According to some authors, the high density of memory T-cells and cytotoxic T-cells in malignant neoplasms (tumour-associated lymphocytes-TAL) is in correlation with prolonged survival of patients [17] [18] [19].

Analysis of stromal inflammatory infiltrate, and tumour-infiltrating lymphocytes in CRC has not attracted the attention of researchers in the Republic of Macedonia recently, but new therapeutic approaches in the treatment of patients with CRC need new basic investigations to provide better management of these patients. According to that, we aimed to determine the density of TAL, the type of some immune cells, “tumour-associated lymphocytes”, and their influence on CRC progression.

Material and Methods

The study included 103 patients with CRC, 68 (66.2%) male, and 35 (33.98%) female with the age of the patients ranged from 33 to 97 years, the mean age 64.57 ± 11.5 years. All of them underwent surgery in our institution, and their operative material was routinely analysed for histopathological diagnosis and pTNM staging at the Institute of Pathology and was additionally analysed for TAL.

In this study the following parameters were examined: localisation of a tumour, local growth (T status), and grade of tumour differentiation (G), the presence of positive lymph nodes (LN) as well as the stage and the density of TAL.

The type of TAL was defined by immunohistochemical staining with antibodies against CD4 (Dako Monoclonal Mouse Anti-Human CD4, Clone 4B12); CD8 (Dako Monoclonal Mouse Anti-Human CD8, Clone C8/144B); CD20 (Dako Monoclonal Mouse Anti-Human CD20, Clone L26); with a standard procedure using Immunoperoxidase LSAB + system.

The density of TAL was determined semiquantitatively. The density of all cells (TAL) was determined in the first step in 4 grades and the density of positively stained cells from the total number of TAL for each marker individually was determined in the second step in the same manner as absent (-), scanty (+), moderate (++) and abundant (+++). To confirm consistency of grading, the cases were scored independently by two investigators. Examples of grading TAL and immune cell staining and their grading are shown in Figure 1.

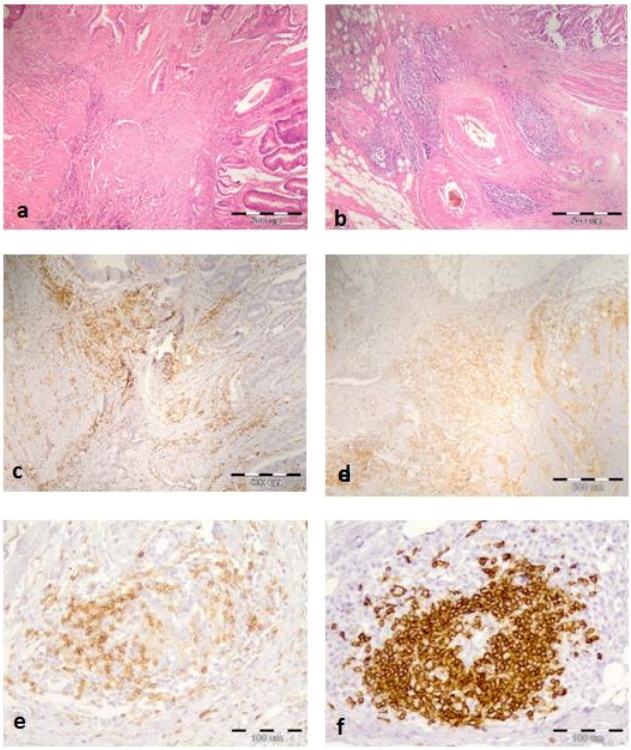

Figure 1: a) Scanty (+) Ly infiltration in the tumor invasive front (HEx40) b) Abundant (+++) Ly infiltration in the tumor invasive front (Hex40) c) Moderate (++) CD4 Ly infiltration (x40) d) Abundant CD4+ Ly infiltration (x40) e) Higher magnification of CD4+ cells (x200) f) Higher magnification of CD20+ cells in the tumor invasive front of CRC (x200)

Statistical analysis

Descriptive statistical methods were used for

statistical analysis of the data. Categorical variables are presented with absolute and relative numbers (%). Fisher's exact test was used for comparison of categorical variables. Spearman's correlation coefficient was used to determine the degree of correlation between analysed parameters. The statistical program SPSS for Windows, version 19.0 was used.

Results

Density of TAL

Distribution of patients and density of TAL according to the localisation of a tumour are shown in Table 1.

Table 1: Distribution of patients and density of TAL according to the cancer localisation

Localization		Quantity of TAL		
	No of patients	Scanty (No = 59)	Moderate (No = 32)	Abundant (No = 12)
Coecum	12 (11.65%)	8 (66.67)	3 (25)	1 (8.33)
Colon asc	9 (8.74%)	6 (66.67)	3 (33.33)	0
Flex hepat	3 (2.91%)	1 (33.330	2 (66.67)	0
Colon trans	5 (4.85%)	2 (40)	2 (40)	1 (20)
Flex lienlis	3 (2.91%)	2 (66.67)	1 (33.33)	0
Colon des	3 (2.91%)	3 (100)	0	0
Sigma	29 (28.16%)	17 (58.62)	7 (24.14)	5 (17.24)
Rectosig	8 (7.77%)	5 (62.5)	2 (25)	1 (12.5)
Rectum	31 (30.09%)	15 (48.39)	12 (38.71)	4 (12.9)

Stage of the disease and local growth of CRC, i.e. T category according to the TNM classification for colorectal cancer (AJCC, 2010), number of patients with negative and positive lymph nodes, number of cases with and without lymphatic invasion, and the grade of differentiation in the analysed series are illustrated in Table 2. The largest number of examined patients was with a tumour in T3 and T4 local growth without metastatic deposits in the local lymph nodes, without lymphatic invasion and with moderately differentiated neoplasms. The density of tumor-associated lymphocytes about the parameters mentioned above is presented in the same Table.

Table 2: Density of TAL according to the stage of disease, T category and nodal status and lymphatic invasion

Stage		Quantity (Density) of TAL			P-value
	No of patients	Scanty	Moderate	Abundant	
I	11	1 (9.09)	5 (45.45)	5 (45.45)	<0.0001**
II	45	15 (35.71)	23 (22.33)	7 (16.67)	
III	41	37 (35.92)	4 (9.09)	0	
IV	6	3 (50)	3 (50)	0	
T category					
1	5	0	4 (80)	1 (20)	0.0018**
2	7	1 (14.29)	2 (28.57)	4 (57.14)	
3	60	35 (58.33)	20 (33.33)	5 (8.33)	
4	31	23 (74.19)	6 (19.35)	2 (6.45)	
N category					
N0	56	17 (30.36)	27 (48.21)	12 (21.43)	0.0001**
N1/N2	47	42 (89.36)	5 (10.64)	0	
L category					
L0	54	20 (37.04)	26 (48.15)	8 (14.81)	0.00005**
L1	49	39 (79.59)	6 (12.24)	4 (8.16)	

P (Kruskal-Wallis test) *P < 0.05; **P < 0.01.

The density of TAL was higher in patients with lower stage I and II of the disease, in tumours with less local invasion, in patients without nodal metastasis, and in tumours with no lymphatic invasion. On the contrary, in stage III and IV of the disease, the density of TAL was low in the most of the analysed cases and the tumour infiltrating lymphocytes was scanty in patients with advanced local growth, with nodal metastasis and with lymphatic invasion.

Statistical analysis did not show statistical differentiation in the quantity of TAL according to tumour differentiation (P > 0.05).

Different cell types of TAL and their density in CRC

CD20, CD4 and CD8 positive cells in different quantity were found in all analysed cases.

CD20+ Lymphocytes

The density of CD20+ tumour-associated lymphocytes about the stage of disease, T and N category, and lymphatic invasion is presented in Table 3.

Table 3: Density of CD20+ cells according to the tumour stage, T and N category, and lymphatic invasion

Stage			The quantity of CD20+ Ly			P-value
	N° of patients	CD20+ Ly not present	Scanty	Moderate	Abundant	
I	11	0	0	6 (54.55)	5 (45.45)	<0.0001**
II	45	1 (2.38)	24 (23.30)	16 (38.1)	4 (9.52)	
III	41	0	36 (34.95)	4 (9.09)	1 (2.27)	
IV	6	0	6 (100)	0	0	
T category						
1	5	0	0	3 (60)	2 (40)	0.0002**
2	7	0	0	4 (57.14)	3 (42.86)	
3	60	1 (1.67)	41 (68.33)	13 (21.67)	5 (8.33)	
4	31	0	25 (80.65)	6 (19.35)	0	
N category						
N0	56	1 (1.79)	25 (44.64)	21 (37.5)	9 (16.07)	<0.0001**
N1/N2	47	0	41 (87.23)	5 (10.64)	1 (2.13)	
L category						
L0	54	1 (1.85)	26 (48.15)	18 (33.33)	9 (16.67)	0.001**
L1	49	0	40 (81.63)	8 (16.33)	1 (2.04)	

P (Kruskal-Wallis test) *P < 0.05; **P < 0.01.

We found a significant difference in the density of CD20+ lymphocyte (Ly) in all analysed parameters.

Scanty CD20+ Ly infiltrates found in patients with advanced local growth, with nodal metastases, lymphatic invasion and with a higher stage of the disease (100% Stage IV, 88.64% Stage III, 50% Stage II and 0% of patients in Stage I). The density of CD20+ Ly infiltrate lower (scanty) in patients with poorly differentiated tumours but without a statistically significant difference (P = 0.089).

CD4+ Lymphocytes

The density of CD4+ tumour-associated lymphocytes about the stage of disease, T and N

category, and lymphatic invasion is presented in Table 4.

Table 4: Density of CD4+ tumour-associated lymphocytes about the stage of disease, T and N category, and lymphatic invasion

Stage	The quantity of CD4+ Lymphocytes				P-value
	No of patients	Scanty	Moderate	Abundant	
I	11	0	10 (90.91)	1 (9.09)	P = 0.11 ns
II	45	10 (23.81)	26 (25.24)	9 (21.43)	
III	41	4 (9.09)	34 (33.00)	3 (6.82)	
IV	6	0	6 (100)	0	
T category					
1	5	0	4 (80)	1 (20)	P = 0.034*
2	7	0	7 (100)	0	
3	60	6 (10)	46 (76.67)	8 (13.33)	
4	31	8 (25.81)	19 (61.29)	4 (12.9)	
N category					
N0	56	10 (17.86)	36 (64.29)	10 (17.86)	P = 0.055 ns
N1/N2	47	4 (8.51)	40 (85.11)	3 (6.38)	
L category					
L0	54	11 (20.37)	37 (68.52)	6 (11.11)	P = 0.11 ns
L1	49	3 (6.12)	39 (79.59)	7 (14.29)	

P (Kruskal-Wallis test) *P < 0.05; **P < 0.01.

The low density of CD4+ Ly was detected in 100% of tumours with advanced local tumour growth (T4 category) and on contrary CD4+ low Ly density was not detected in tumours with T1. The statistical analysis confirmed that the density of CD4+ lymphocytes in the infiltrative front of a tumour significantly depended on the local growth of the disease.

Patients with regional lymph nodes metastasis had a lower density of CD4+ Ly infiltrate than patients without metastasis, but there was no a statistically significant difference in the density of CD4+ Ly according to the presence of lymph nodes metastasis. No statistical significance was found in the density of CD4+ Ly infiltrate according to the Stage, lymphatic invasion and grade also.

CD8 Lymphocytes

The density of CD8+ tumour-associated lymphocytes about the stage of disease, T and N category, and lymphatic invasion is presented in Table 5.

Table 5: Density of CD8+ tumour-associated lymphocytes about the stage of disease, T and N category, and lymphatic invasion

Stage	The quantity of CD8+ Lymphocytes					p-value
	No of patients	CD8+ Ly not present	scanty	moderate	abundant	
I	11	0	0	11 (100)	0	0.0055**
II	45	1 (2.38)	12 (28.57)	28 (66.67)	1 (2.38)	
III	41	0	24 (54.55)	20 (45.45)	0	
IV	6	0	3 (50)	3 (50)	0	
T category						
1	5	0	0	5 (100)	0	0.006**
2	7	0	0	7 (100)	0	
3	60	1 (1.67)	22 (36.67)	37 (61.67)	0	
4	31	0	17 (54.84)	13 (41.94)	1 (3.23)	
N category						
N0	56	1 (1.79)	15 (26.79)	39 (69.64)	1 (1.79)	0.024*
N1/N2	47	0	24 (51.06)	23 (48.94)	0	
L category						
L0	54	0	18 (33.33)	35 (64.81)	1 (1.85)	0.36 ns
L1	49	1 (2.04)	21 (42.86)	27 (55.1)	0	

P (Kruskal-Wallis test) *P < 0.05; **P < 0.01.

We found a significant difference in the quantity of CD8+ Ly in all analysed parameters except lymphatic invasion.

The density of CD8+ Ly significantly differs according to the local tumour growth. The low density of CD8+ Ly infiltrate significantly more often was present in patients with T4 and T3 status (54.84%, 36.67% respectively); there were no patients with T1 and T2 with a low density of CD8+ Ly infiltrate.

The density of CD8+ Ly was significantly lower in patients with nodal metastasis, lymphatic invasion and with an advanced stage of the disease.

We did not find a statistical difference in the density of CD8+ Ly according to the histological differentiation of a tumour.

The moderate density of CD8+ lymphocytes decreased when the grade of the differentiation decreased, but the difference was not significant.

Correlations

The correlations of the density of tumour-associated lymphocytes with T tumour status, the involvement of the regional lymph nodes, lymphatic invasion, stage and the grade of differentiation were done using the Spearman's correlation test. Correlations were determined between the different T cell types also.

The results showed a significant negative correlation between the density of CD20+ Ly and T status, stage and G, i.e. the density of CD20+ Ly was lower in less differentiated tumours with advanced local growth and higher stage. The density of CD20+ Ly was in positive correlation with the density of CD8+ Ly and the density of TAL (Table 6).

The density of CD8+ Ly was lower in tumours with advanced local growth and higher stage of the disease. Correlations were not statistically significant except for the density of CD8+ Ly and the stage of the disease (Table 6).

Table 6: Correlations of the quantity of TAL and all other analysed parameters

CD20	Spearman R	t-test, P-level
CD20 / CD8	0.367	3.97, P = 0.00013**
CD20 / TAL	0.436	4.87, P = 0.000004**
CD20 / T	-0.407	4.48, P = 0.00002**
CD20 / Stage	-0.564	6.87, P < 0.0001**
CD20 / G	-0.220	2.27, P = 0.025*
CD4		
CD4 / CD 8	0.387	4.21, P = 0.000055**
CD4 / CD 20	0.016	0.16, P = 0.87
CD4 / TAL	0.342	3.66, P = 0.00041**
CD4 / T	-0.155	1.57, P = 0.12
CD4 / Stage	-0.034	0.34, P = 0.74
CD4 / G	-0.091	0.92, P = 0.36
CD8		
CD8 / TAL	0.381	4.14, P = 0.000073**
CD8 / T	-0.271	2.83, P = 0.0056**
CD8 / Stage	-0.324	3.44, P = 0.00084**
CD8 / G	-0.107	1.08, P = 0.28

*P < 0.05; **P < 0.01.

Statistically, a significant correlation was found between CD4+ Ly and CD8+ Ly and the total

amount of TAL (Table 6). The density of CD8+ Ly increased when the density of Total amount of TAL increased and when the density of CD4+ Ly increased (Table 6). There was also a significant positive correlation between CD20+ cells and CD8+cells.

Discussion

Rudolf Virchow, 150 years ago, was the first who described inflammatory cells in tumours and established the theory that tumours arise at chronic inflammation sites. It is known that the risk of developing colorectal cancer is greater in patients with ulcerative colitis and those with Crohn's disease. Despite the knowledge that chronic inflammation is a risk for development of a malignant neoplasm, many clinical and experimental studies stress the protective role of immune cells in cancer progression, hence, the statements and cognitions that immune response plays a key role in fighting tumour growth and its dissemination [7].

Some authors consider that the type, density and location of the immune cells in colorectal cancer can be superior and independent prognostic factor for tumour progression [7].

Cancer progression is a complex process that is influenced by genetic, epigenetic and environmental factors, but at the same time, it is a process of interaction between cancer cells and their microenvironment, that is, stroma. Tumour stroma is built of different cells among which are inflammatory cells that belong to congenital and inherited immune system [20] [21] [22]. Former investigations have shown that lymphocytes that infiltrate the tumour-tumour infiltrating lymphocytes (TIL/TAL) are predominantly T lymphocytes among which two subtypes are identified CD4+ and CD8+ T lymphocytes. CD4+ T lymphocytes are T helper cells, whereas CD8+ T lymphocytes are cytotoxic cells capable of killing the "target cell", i.e. tumour cell which exposed antigens CD8+ T lymphocytes are capable of recognising. They are able to destroy tumor cells in direct cell interaction by releasing lytic components [22] [23] [24] [25] [26] [27]. CD4+ T lymphocytes are helper cells that regulate their proliferation and coordinate the immune response by stimulating the other immune cells. They are stratified in subgroups T helper 1 (Th1) and T helper 2 (Th2) cells. Th1 CD4+ T lymphocytes are responsible for the proliferation of cytotoxic T lymphocytes that participate in the killing of tumour cells.

In addition to T lymphocytes, B CD20+ lymphocytes arranged in aggregates have been found among TAL together with other immune cells. In lymph glands and the remaining lymphoid tissue, B lymphocytes receive signals for growth, differentiation and affinity maturation from follicular T helper immune cells. They differentiate into plasma cells and memory cells with long lifespan [28]. The process of lymphoid neogenesis is described in infectious diseases, chronic inflammation processes, autoimmune diseases and chronic graft rejection [29] [30] [31]. In these conditions, T and B cells cooperate to create a strong immune response when B cells enhance T cell response by antigen presentation, costimulation and modulation of migration and function of dendritic cells [28], 29].

Lymphoid neogenesis is also described in malignant neoplasms, while the role of CD20+ lymphocytes in tertiary lymphoid structures that are found in these neoplasms has not been completely clarified and it is still in the phase of research [29]. Nowadays many patients with malignant neoplasms develop tumour-specific antibodies, and B cells have a significant role in the immune response to neoplasm both by exacerbating cancer-associated inflammation and by the effect they have on T lymphocytes, macrophages and myeloid stem cells.

We found a statistically significant correlation between the examined subtypes of lymphocytes with a negative trend between CD4+ and CD20+ cells and CD8+, CD20+ cells and also a positive trend between CD4+ and CD8+. The correlation implies mutual connection in the function of these cells in one common immune response to tumour antigens.

TAL-tumour infiltrating lymphocytes are concentrated in the stroma of an invasive tumour front. Many authors examined the role of TAL in tumor progression in relation to survival of patients with colorectal cancer [23] [24] [25] [26] [32] [33].

Even though for a long time it was considered that colorectal cancer is a bad immunogenic tumor, the new cognitions suggest that there is a substantial immune response to this disease and that the presence of the immune response is connected with a better prognosis of patients with colorectal cancer, that is, the increased presence of T cytotoxic lymphocytes is associated with absence of early metastases and absence of early recurrence, as well as that the type of density and location of the immune cells have prognostic significance in colorectal cancer [7] [33] [34].

Therefore, tumour-associated lymphocytes can provide significant prognostic information about patients with colorectal cancer, which could be used for treatment with adjuvant therapy in a different stage of cancer [29].

The results from the analysis of TAL in the invasive front of cancer in our group of patients with colorectal cancer showed the presence of T (CD4+ and CD8+) and B (CD20+) lymphocytes and their density significantly correlated with clinically-pathologic parameters for disease progression. The density of TAL was higher in patients with lower stage

(I and II) of the disease, in tumours with less local invasion, in patients without nodal metastasis, and in tumours with no lymphatic invasion. The density of CD8+ and CD20+ lymphocytes in a neoplasm was higher in patients without metastases in lymph nodes and with a lower stage of the disease. Lower density of CD20+ lymphocytes was found in neoplasms of patients with advanced local growth and lymphatic tumor invasion. Thus, it can be concluded that the density of tumor-associated lymphocytes can be a parameter for neoplasm progression in patients with colorectal cancer and the density of tumor-associated lymphocytes influences the control of tumor progression.

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Serum Markers of Iron Metabolism in Chronic Liver Diseases

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Keywords: Steatosis; Serum ferritin; Serum iron; Transferrin saturation; Chronic liver disease

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Abstract

BACKGROUND: Disorders in the metabolism of iron in the direction of iron overload are observed not only in primary hemochromatosis but also in some chronic liver diseases other aetiology. Elevation of serum iron, ferritin and transferrin saturation is reported in nonalcoholic fatty liver disease and alcohol, chronic hepatitis C and liver cirrhosis.

AIM: Aim of the study was to evaluate and compare the frequency of the iron serum markers in patients with various chronic liver diseases.

MATERIAL AND METHODS: The study included a total of 246 persons -186 patients with chronic liver disease without cirrhosis (-115 men, women -71; average age of 50.41 ± 12.85, from 23 to 77 years) and 60 healthy controls (-30 men, women -30, middle-aged 50.50 ± 11.31, from 29 to 83 years). Medical history, physical examination and demographic data including height, weight, laboratory and instrumental studies were performed.

RESULTS: The highest incidence of elevated serum iron, transferrin saturation and ferritin and decreased serum hepcidin found in cases of alcoholic liver disease (ALD), nonalcoholic fatty liver disease (NAFLD) and chronic hepatitis C (CHC).

CONCLUSION: Finally, analysis of the changes in serum markers of iron metabolism shows that the difference between healthy and sick with liver disease is primarily due to changes in alcoholic and nonalcoholic fatty liver disease, particularly steatohepatitis, and chronic hepatitis C.

Introduction

The liver is an important organ for the homeostasis of the iron. Reticuloendothelial macrophages and hepatocytes are the main depots more than iron. In the liver is performed the synthesis of the hormone hepcidin and thus it became the central authority for the regulation of the body's reserves of iron [1] [2] [3].

Disorders in the metabolism of iron in the direction of iron overload are observed not only in primary hemochromatosis but also in some chronic liver disease with another aetiology [4] [5] [6] [7]. Increases in serum levels of iron, ferritin, and transferrin saturation are reported in nonalcoholic fatty liver disease and alcoholic fatty liver disease, chronic hepatitis C and liver cirrhosis [8] [9] [10] [11] [12] [13].

In recent years, there accumulated a lot of new data, some of them contradictory, about the clinical significance of serum parameters of iron metabolism as surrogate markers of siderosis and severity of liver disease [8] [10] [14] [15] [16] [17]. There remain open questions regarding the clinical significance of serum parameters of iron metabolism and hepcidin in various chronic liver diseases and the role of some genetic factors and environmental factors for organic liver damage during overload syndrome iron.

The study aimed to evaluate and compare the frequency of the iron serum markers in patients with various chronic liver diseases.

To implement the above objective we set ourselves the following tasks:

1. To evaluate the change of serum parameters of iron metabolism in patients with the chronic liver disease compared to healthy subjects
2. To carry out comparative serum markers of iron metabolism in various chronic liver diseases

Material and Methods

Investigated persons

The study included a total of 246 persons - 186 patients with chronic liver diseases without cirrhosis (- 115 men, women - 71; average age of 50.41 ± 12.85, from 23 to 77 years) and 60 healthy controls (men -30 women -30 middle-aged 50.50 ± 11.31, from 29 to 83 years) in seven groups:

Group I - 38 patients with primary fatty liver disease, non-alcoholic (male 20, female 18, mean age of 12.45 ± 53.13, from 30 to 76 years) -nonalcoholic steatosis (n = 22) and non-alcoholic steatohepatitis (n = 16). The diagnosis was based on standard criteria, ultrasound evidence of steatosis and histologically verified in 22 cases and ruled out other etiologies.

Group II - 45 patients with alcoholic liver disease (31 men, 14 women; mean age of 10.80 ± 53.42 from 30 to '73) - alcoholic fatty liver (n = 13) and alcoholic steatohepatitis (n = 32) with the intake of absolute alcohol over 40 g/day, histologically confirmed in 39 of them. The diagnosis was based on standard criteria and excluded other causes of liver damage.

Group III - 38 patients with chronic hepatitis C (21 men, 17 women; mean age of 13.61 ± 52.66 from 27 to 77 years). All patients with active viral replication and the diagnosis were histologically verified in 29 of them.

Group IV - 35 patients with chronic hepatitis B (29 men, 6 women, middle-aged 14.40 ± 44.15 from 23 to 74 years) with evidence of viral replication and histologically confirmed the diagnosis in 32 of them.

Group V -13 patients with chronic hepatitis D (7 men, women 6; middle-aged 45.08 ± 9.57, from 30 to 60 d), serologically demonstrated chronic infection in any histologically verified only in 3 of them.

Group VI -17 patients with autoimmune liver disease (male 10, female 7; middle-aged 47.59 ± 11.52, from 26 to 62 years) - primary biliary cirrhosis (n=7) and chronic autoimmune hepatitis (n=10). In all patients, the diagnosis was confirmed histologically and immunologically.

All included patients with chronic liver diseases no significant comorbidities, interfering with the syndrome of iron overload.

Group VII - 60 healthy volunteers participating in clinical trials. All respondents have not a history, physical, laboratory, serological (test for HIV, HBV, HCV and acute HAV), ECG and ultrasound evidence of past or present diseases of the liver and biliary system.

Written informed consent for research is taken by all persons involved in the study.

Methods

History, physical examination and demographic data incl. Height, weight, BMI, abdominal circumference. Estimates for daily consumption of absolute alcohol are based on recalculation in one standard alcoholic unit, which equates to about 10-12 g of absolute alcohol.

They were investigated following standard and specific to the disease laboratory parameters, incl. Immunological and virological parameters.

Conventional ultrasound of abdomen and pelvis was performed in all respondents, and other pictorial or research tools-necessarily. Percutaneous or surgical liver biopsy with histologic evaluation of grades of steatosis and activity and stage of fibrosis was conducted at a total of 142 studied patients with chronic liver diseases judged by the criteria of Brunt, METAVIR and other standard histological criteria (Table 1).

Table 1: Frequency of histological parameters in patients with different groups chronic liver diseases

Histological parameters	NAFLD			Ald			Chc (n=29)	Chb (n=32)	Chb Hdv (n=3)	Chronic autoimmune hepatitis (n=17)	Total (n=142)
	Nas (n=6)	Nash (n=16)	Total (n=22)	As (n=7)	Ash (n=32)	Total (n=39)					
					Steatosis						
Steatosis (-) (n)	0	0	0	0	2	2	16	25	2	14	59
Steatosis (+),(n)	6	16	22	7	30	37	13	7	1	3	83
1 degree, (n)	*1*	*-*	*1*	*-*	*3*	*3*	*10*	*5*	*1*	*1*	*21*
2 degree (n)	*5*	*12*	*17*	*7*	*19*	*26*	*3*	*2*	*0*	*2*	*50*
3 degree (n)	*-*	*4*	*4*	*-*	*8*	*8*	*-*	*-*	*-*	*-*	*12*
					Activity						
Activity (-),(n)	6	-	6	7	13	20	0	0	0	1	27
Activity (+),(n)	0	16	16	0	19	19	29	32	3	16	115
1 degree , (n)	*-*	*-*	*0*	*-*	*2*	*2*	*5*	*13*	*0*	*5*	*25*
2 degree , (n)	*-*	*10*	*10*	*-*	*11*	*11*	*16*	*13*	*3*	*11*	*64*
3 degree , (n)	*-*	*6*	*6*	*-*	*6*	*6*	*8*	*6*	*0*	*0*	*26*
Fibrosis											
Fibrosis (-),(n)	6	1	7	7	1	8	0	6	0	8	29
Fibrosis (+),(n)	0	15	15	0	31	31	29	26	3	9	113
1 stage *(n)*	*-*	*8*	*8*	*-*	*-*	*13*	*14*	*19*	*3*	*9*	*67*
2 stage *(n)*	*-*	*7*	*7*	*-*	*-*	*12*	*11*	*7*	*0*	*0*	*37*
3 stage *(n)*	*-*	*-*	*0*	*-*	*-*	*6*	*4*	*0*	*0*	*0*	*10*
3 stage *(n)*	*-*	*-*	*0*	*-*	*-*	*0*	*0*	*0*	*0*	*0*	*0*

All patients received the standard treatment for the disease.

The credibility of the results evaluated by the following statistical analysis: parametric -t-test for paired differences to assess the changes in dynamics; -Mann-Whitney nonparametric comparison of averages for non-Gaussian distribution of data; ANOVA, correlation analysis: Pearson and Spearman, χ-square test and other (SPSS v.13). The results evaluated statistically significant at a threshold level of significance $p < 0.05$.

Results

The highest incidence of elevated serum iron, transferrin saturation and ferritin and decreased serum hepcidin was found in cases with AFLD,

NAFLD and CHC. Increased serum iron was found in 53% of NAFLD in 64% - at AFLD, 42% - at CHC but only 6% in CHB, 8% for CHB+HDV infection and 6% in autoimmune liver diseases. Increased serum ferritin was reported in 42% for NAFLD in 49% -in AFLD and 29%-CHC, but only 3% on CHB and 0% in CHB+HDV infection and autoimmune liver diseases. Increased transferrin saturation was established in NAFLD in 18%, 36% - at AFLD, 29% - at CHC, 14% - on CHB and 8% - at CHB+HDV infection. Decreased serum hepcidin was reported in 47% at AFLD, 60% - at NAFLD, 53% - at CHC, 26% - on CHB, 8% - at CHB+HDV infection and 6% - at autoimmune liver diseases. Share distribution in the different groups chronic liver diseases with simultaneous increase of 2 or 3 of serum parameters of iron metabolism is shown in Table 2.

Table 2: **Values of iron metabolism in different groups chronic liver diseases**

Values of iron metabolism	NAFLD (n=38)	AFLD (n=45)	CHC (n=38)	CHB (n=35)	CHB, HDV (n=13)	Autoimmune liver diseases (n=17)	Total (n=186)
↑ iron + ↑ ferritin (n, %)	16 (42%)	22 (49%)	10 (26%)	1 (3%)	0 (0%)	0 (0%)	49 (26%)
↑ iron + ↑ transferrin saturation (n, %)	6 (16%)	16 (36%)	9 (24%)	2 (6%)	1 (8%)	0 (0%)	34 (18%)
↑ ferritin + ↑ transferrin saturation (n, %)	4 (11%)	14 (31%)	5 (13%)	1 (3%)	0 (0%)	0 (0%)	24 (13%)
↑ iron + ↑ ferritin + ↑ transferrin saturation (n, %)	4 (11%)	14 (31%)	4 (11%)	1 (3%)	0 (0%)	0 (0%)	23 (12%)

The most common abnormal iron metabolism found in the group with AFLD, followed by that of CHC and NAFLD. In all these cases there was reduced and serum hepcidin.

Serum iron was significantly higher in patients with NAFLD compared to controls (Z = -2,2494, P = 0.013) and CHB (Z = -2,357, P = 0.018), and that at AFLD-compared to controls (Z = -4,151, P = 0.0001), CHB (Z = -2,125, P = 0.034), CHB+HDV (Z = -4.002, P = 0.0001) and autoimmune liver diseases (Z = -2,864, P = 0.004) (Figure 1).

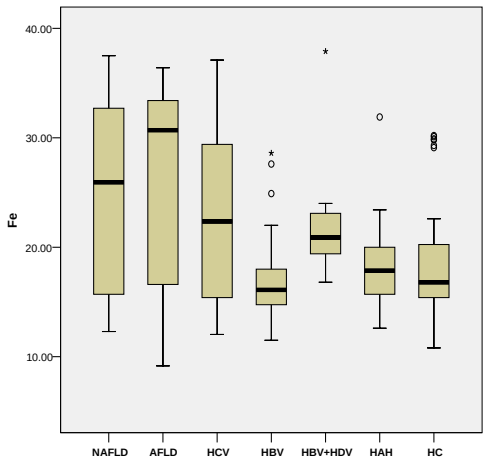

Figure 1: Serum iron (mmol/l) in different groups

Significantly higher values were in CHC compared to controls (Z = -2,173, P = 0.030) and CHB (Z = -2,205, P = 0.027). Values of iron binding capacity were significantly higher in patients with NAFLD compared to controls (Z = -5,565, P = 0.0001) and CHB (Z = -4,736, P = 0.0001) at AFLD - compared to controls (Z = -7,844, P = 0.0001), CHC (Z = -3,442, P = 0.001), CHB (Z = -6,578, P = 0.0001) and autoimmune liver diseases (Z = -2,036, P = 0.042) (Figure 2).

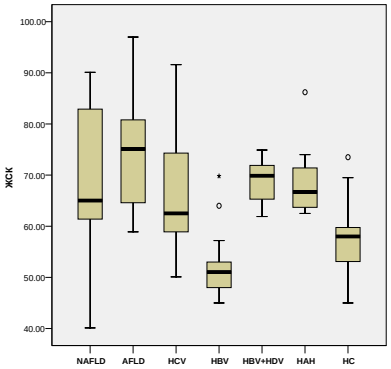

Figure 2: Values of iron binding capacity in different groups

Significantly higher values were in CHC compared to controls (Z = -4,825, P = 0.0001) and CHB (Z = -4,319, P = 0.0001), and in CHB, compared to autoimmune liver diseases (Z = -3,921, P = 0.0001) as well as in autoimmune liver diseases compared to controls (Z = -5,475, P = 0.0001). Ferritin values were significantly higher in: NAFLD compared with controls (Z = -1,965, P = 0.05), CHB (Z = -3,832, P = 0.0001), autoimmune liver diseases (Z = -3,561, P = 0.0001); AFLD when compared to controls (Z = -2,953, P = 0.003), CHB (Z = -3,895, P = 0.0001), and the autoimmune liver diseases (Z = -3,740, P = 0.0001), p = 0.0001); CHC when compared to the CHB (Z = -2,892, P = 0.004), and the autoimmune liver diseases (Z = -2,942, P = 0.003); CHB relative to controls (Z = -3,683, P = 0.0001) and autoimmune liver diseases (Z = -2,088, P = 0.037) (Figure 3).

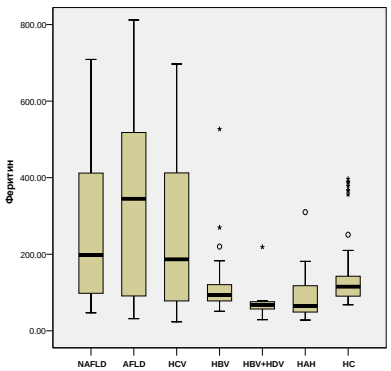

Figure 3: Values of serum ferritin in different groups

The values of transferrin saturation were significantly higher in: NAFLD compared with

autoimmune liver diseases (Z = -2,343, P = 0.019); AFLD compared with autoimmune liver diseases (Z = -3,032, P = 0.002); CHC compared with autoimmune liver diseases (Z = -3,035, P = 0.002); CHB compared with autoimmune liver diseases (Z = -3,409, P = 0.001); autoimmune liver diseases and compared to controls (Z = -3,450, P = 0.001) (Figure 4).

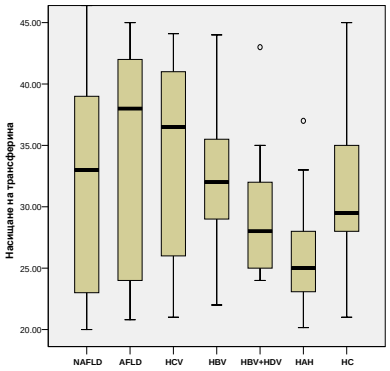

Figure 4: Values of transferrin saturation in different groups

Serum levels of hepcidin were significantly lower in patients with NAFLD, AFLD and CHC in comparison to controls (respectively Z = -2,326, P = 0.02, Z = -3,312, Z = -2,559, P = 0.001 and P = 0.01), CHB (respectively Z = -2,122, P = 0.034, Z = -2,991, P = 0.003 and Z = -2,295, P = 0.023) and autoimmune liver diseases (respectively Z = -2,277, P = 0.023, Z = -2.840, P = 0.005 and Z = -2,295, P = 0.022), but no statistically significant difference in the comparison between them (Figure 5).

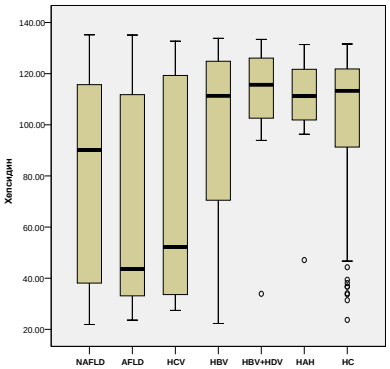

Figure 5: Values of serum hepcidin in different groups

In all groups chronic liver diseases, serum iron, ferritin, transferrin saturation and hepcidin showed correlation (r = 0.470-0.875, P = 0.003-0.0001). Increased serum iron, ferritin, transferrin saturation and serum decreased hepcidin, and combinations thereof, found more often in metabolic steatohepatitis or alcoholic aetiology when comparing the cases with steatosis (Table 3).

Mean serum iron, transferrin and transferrin saturation in patients with nonalcoholic and alcoholic steatohepatitis showed significantly higher values compared with steatosis (respectively Z = -4.155, P = 0.0001, Z = -3.474, P = 0.001, Z = -4.110, P = 0.0001 and Z = -3.628, P = 0.0001 (Figures 6, 7, 8).

Table 3: **Values of iron metabolism in NAFLD and AFLD**

Values of iron metabolism	NAFLD			AFLD		
	NAS (n=22)	NASH (n=16)	Total (n=38)	AS (n=13)	ASH (n=32)	Total (n=45)
↑ Serum Iron (n, %)	5 (23 %)	15 (94%)	20 (53 %)	3 (23%)	26 (81%)	29 (64%)
↑ Serum ferritin (n, %)	2 (9 %)	14 (87%)	16 (42 %)	1 (8%)	21 (65%)	22 (48.9%)
↑ transferrin saturation (n, %)	2 (9 %)	5 (31%)	7 (18%)	1 (8%)	15 (47%)	16 (36%)
↑ Serum Iron + ↑ Serum ferritin (n, %)	2 (9 %)	14 (87%)	16 (42%)	1 (8%)	21 (66%)	22 (49%)
↑ ↑ Serum Iron + ↑ transferrin saturation (n, %)	1 (4%)	5 (31%)	6 (16%)	1 (8%)	15 (47%)	16 (36%
↑ Serum ferritin + ↑ transferrin saturation (n, %)	0 (0%)	4 (25%)	4 (11%)	0 (0%)	14 (44%)	14 (31%)
↑ ↑ Serum Iron + ↑ Serum ferritin + ↑ transferrin saturation (n, %)	0 (0%)	4 (25%)	4 (10%)	0 (0%)	14 (44%)	14 (31%)
↓ hepcidin (n, %)	5 (23 %)	13 (81%)	18 (47%)	3 (23%)	24 (75%)	27 (60%)

Serum hepcidin was significantly lower in patients with NASH and ASH compared in groups with NAFLD and AFLD, respectively (respectively Z=-3.252, p=0.001 and Z=2.304, p=0.021) (Figure 9).

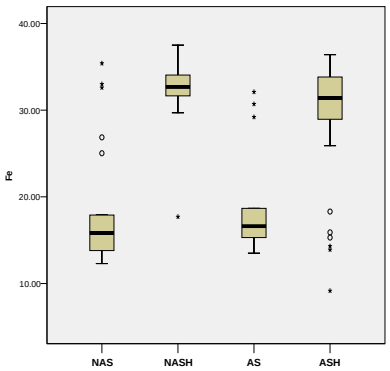

Figure 6: Values of serum iron in NAFLD and AFLD

Discussion

The results of our study show that chronic liver diseases detect abnormalities in all serum parameters of iron metabolism-elevated serum iron, ferritin and transferrin saturation, as well as reduced hepcidin compared with healthy individuals. Considered changes in parameters of iron metabolism separately, the most frequent and approximately equally are variations in serum iron and hepcidin, followed by those of ferritin and transferrin saturation.

Deviations from the normal range in healthy persons are identified only in serum hepcidin, which was reduced in 20% of cases. If we use standard laboratory parameters, the frequency of the syndrome

of iron overload is estimated at 22% based on transferrin saturation above 45%.

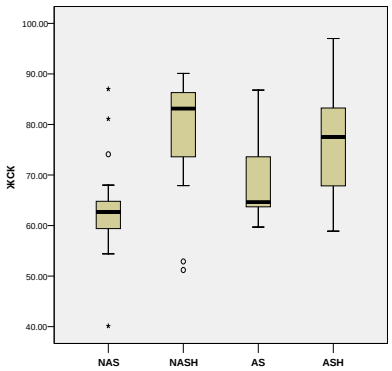

Figure 7: Values of iron binding capacity in NAFLD and AFLD

Approximately at this frequency is the combined increase in serum levels of the ferritin and the iron (26%), as well as the simultaneous increase in the iron and transferrin saturation (18%).

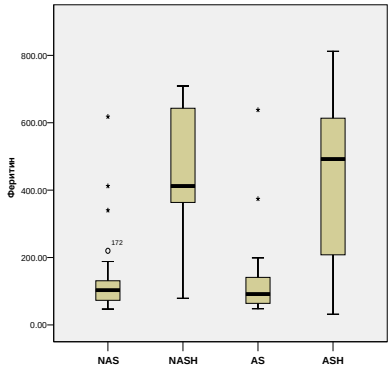

Figure 8: Values of serum ferritin in NAFLD and AFLD

Two times lower is the frequency of the simultaneously elevated transferrin saturation and the ferritin -13%, as well as the simultaneous increases of the three serum parameters -iron, transferrin saturation and ferritin to -12%. This frequency does not change significantly if these combinations are added and reduced hepcidin.

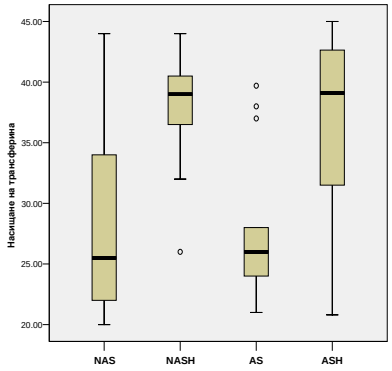

Figure 9: Values of transferrin saturation in NAFLD and AFLD

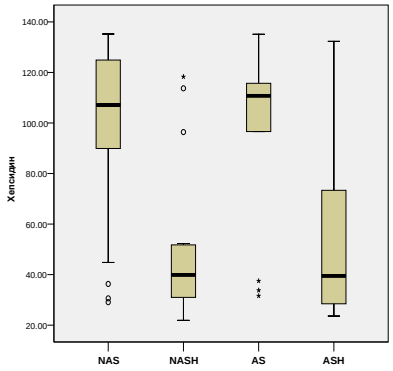

Figure 10: Values of serum hepcidin in NAFLD and AFLD

This applies both to frequency deviations and for their serum levels. At most this is true for serum hepcidin. Changes in these groups are more apparent, in comparison with those of patients with chronic hepatitis B and D, chronic autoimmune hepatitis and primary biliary cirrhosis. In all cases of combined changes in standard serum markers of iron metabolism and all groups HLD is presented and reduced hepcidin. We did not find a difference in the serum levels of the examined parameters of iron metabolism in patients with nonalcoholic and alcoholic fatty liver disease. In patients with chronic hepatitis B, alone or in combination with chronic hepatitis D, as well as autoimmune diseases a change in serum markers of iron metabolism is reported only in individual cases.

Our data shows that unlike healthy, in which serum iron, ferritin and IBC are higher for men than for women, in case of a liver disease gender difference in the level of serum iron and IBC disappears. Deviations of the serum indicators of iron metabolism were more pronounced with age (predominantly in HH C) and increased BMI. Relationship with BMI was mainly at the expense of patients with NAFLD and HH C. Only in patients with NAFLD all indicators were more obviously deviated in obesity. This can be explained by the relationship of these diseases with insulin resistance [17] [18].

For the first time, we found different correlations between serum parameters of iron metabolism in healthy patients and those with the disease. While in healthy individuals only have a positive relationship between the level of iron and IBC and feedback between serum hepcidin and transferrin saturation, then in HLD as a whole and individual subgroups of liver disease serum levels of iron, IBC, ferritin, transferrin saturation and hepcidin showed a strong correlation.

In HLD is proved a link between indicators reflecting liver damage and liver function and serum parameters of iron metabolism. We found a correlation between serum markers of iron metabolism and liver enzymes AST, ALT and GGT, as the abnormalities were higher in groups with diverted

indicators of iron metabolism in comparison to the group with normal levels. Further-more serum markers of iron metabolism correlated with total bilirubin and direct bilirubin, prothrombin time, and platelet count. These results confirm established by other authors interconnections between the parameters of the syndrome of iron overload and liver enzymes and liver function [19] [20] Also, we have established a link with IgA. The advantage of our study is that we proved the greatest relationship of these indicators with the global assessment of severity of liver disease-index weight of liver damage. Serum iron, IBC, ferritin and transferrin saturation increases with TLD and values of hepcidin-reduces. We proved and their relationship with HDL-cholesterol, triglycerides and glucose. All correlations are determined primarily by changes in patients with NAFLD and ALD. Also, in patients with NAFLD and ALD, we found another correlation between AF and albumin. The relationship between KKLI-indicator to assess the severity of alcoholic liver disease and the serum parameters of iron metabolism is determined by patients with alcoholic steatohepatitis. In patients with proven HH C, we proved a connection mainly with indicators for severity of liver damage-total and direct bilirubin, albumin and prothrombin index.

In our study, we examined various liver diseases, and we examined the overall changes in iron metabolism in the control group of healthy persons and in between them. In NAFLD is often increased serum iron (53%), followed by a decrease in serum hepcidin (47%) and elevated ferritin (42%), and about 2 times less frequently elevated transferrin saturation (18%). The combined standard deviation of serum markers of iron metabolism is most often the simultaneous increase in serum iron and ferritin-42%, followed by increases in serum iron and HT-16%. In 11% had elevated serum iron, ferritin and HT. Our results show that the incidence of the syndrome of over-saturation with iron, as measured according to the standard serum markers is 18% based on the increased NT and 11%-based on changes in any serum markers. The higher incidence of abnormal serum iron and ferritin corresponds to the hepcidin, but can be explained by non-specific changes related to the underlying disease.

In ALD increased serum iron (64%) together with reduced serum hepcidin (60%) are the most common abnormalities. Ferritin was promoted to ½ of the cases (49%). And in this condition as in NAFLD transferrin saturation above 45% is the rare deviation-36%. The simultaneous increase in serum iron and ferritin was 48%, but the combined tolerances of increased serum iron and LT, as well as increased iron, transferrin and HT, are the most common in alcoholic aetiology, respectively in 36% and 31%. The incidence of the syndrome of over-saturation with iron was 36% by elevated HT and 31%-based on changes in any serum markers. This frequency is the highest in comparison with all other chronic liver diseases, and it is about 2 to 3 times greater in comparison with patients with NAFLD. Here, as in NAFLD incidence of abnormal serum iron corresponds to the hepcidin, but about half of these cases can certainly be considered an overload of iron based on changes in the standard serum markers of iron metabolism. It is more pronounced changes in hepcidin to meet in earlier changes in the liver before a heavy load of iron.

Interest is the comparison of the results in patients with steatosis and steatohepatitis inside NAFLD groups and ALD and between them. We found more pronounced deviations in all serum markers of iron metabolism in both non-alcoholic and alcoholic steatohepatitis when compared to cases with steatosis, and no difference between patients with NAD and ALD, and between those with NASH and ASH. The values of serum iron parameters of metabolism in patients with nonalcoholic steatosis and alcohol were close to the controls and other groups HLD, but transferrin saturation in them is higher. The values of the parameters of iron metabolism in alcoholic and nonalcoholic steatohepatitis are highest.

Increased serum iron, ferritin, transferrin saturation and decreased serum hepcidin, as well as a combination between them, we found more often in steatohepatitis with alcohol or metabolic aetiology compared to cases with steatosis. Thus the frequency of the overload of iron in NASH increases to 31% (on NT) and 25% (all indices), and in ASH-respectively 47% and 44%. The increase in serum iron and ferritin and reduction of hepcidin were found in over two-thirds of those surveyed with NASH and ASH.

In HH C deflected (low), the most are serum hepcidin (53%), followed by increased serum iron (42%). Elevated transferrin saturation and ferritin are detected equally-29%. Differences in the rate of change of the different parameters are less in comparison to the alcoholic and nonalcoholic fatty liver disease. Almost 2 times less frequent is the combination of increased serum iron and increased ferritin. On the other hand at the same time, an increase in the iron and HT (24%), ferritin and HT (13%) and the deviation in all the indicators (11%) are close to that of NAFLD. The frequency of iron overload by elevated HT is 29% and 11%-13%-based on the above latter two combinations.

Unlike HH C in HH B increased serum iron is found only in 2 of 35 patients (6%) and in the same proportion (1 out of 13 patients-8%)-for chronic HDV infection, and the ferritin-in 1 patient (3%), and none of the patients with HHD. At higher levels, we found elevated transferrin saturation (14%) and decreased serum hepcidin (26%). However, this frequency corresponds to that in healthy individuals. Variation in these indicators we found in only 1 case (8%) with accompanying HDV infection. A combination of increased iron with increased HT we reported in 2 patients with HH B (respectively 1 patient with HHD), and when one of them combined with the rest of the

parameters. Accordingly to the above changes in 2 patients with HH B and 1-with HHD maintained overload with iron according to the increased transferrin saturation, and in one of the patients with HH B-and according to the combination of the remaining markers.

In autoimmune diseases, increased serum iron we found in only 1 out of 17 tested cases (6%) diagnosed with PBC. In another case, we found reduced serum hepcidin (6%). Data for immune-mediated liver diseases are extremely small. Similar to our observation, it is reported a very low incidence of biliary cirrhosis [16] [21].

In conclusion, the analysis of data from our study shows changes in every serum marker of iron metabolism, including reducing of serum hepcidin in nonalcoholic and alcoholic fatty liver disease, and chronic hepatitis C in comparison with healthy persons, and other chronic liver diseases associated with HBV, HDV or autoimmune pathogenesis. It is proved a strong correlation between them. The syndrome of iron overload correlates mainly with liver enzymes, liver function tests, and metabolic parameters. None of the surrogate serum markers of iron overload in chronic liver disease does reflect fully and accurately haemosiderosis. However, we recommend the evaluation to be carried out by transferrin saturation above 45%, alone, especially NAFLD or together with the increased transferrin-in alcoholic liver disease and chronic hepatitis C. Thus, with the highest probability is confirmed iron overload.

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Clinical Science

Upregulation of Twist2 in Non-Muscle Invasive Urothelial Carcinoma of the Bladder Correlate with Response to Treatment and Progression

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Keywords: Bladder cancer; Epithelial-mesenchymal transition; Twist2; NMIBC; BCG

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Abstract

BACKGROUND: Twist2 is a transcription factor and an epithelial-to-mesenchymal transition that plays an important role in cell polarity, cell adhesion, and has a role in tumour invasion and metastases.

AIM: In this study, we examined the expression of Twist2 in non-muscle invasive bladder carcinoma (NMIBC) and correlated the expression with response to treatment and tumour progression.

METHODS: Data of 305 patients with NMIBC of Ta, T1 were retrieved from hospitals archives. Twist2 expression was examined in tissue samples by immunohistochemistry at initial diagnosis and final follow-up, normal control was 10 normal urothelium, 10 patients with muscle-invasive bladder cancer (MIBC) were a positive control. Treatment of NMIBC was implemented according to the European Association of Urology guidelines on NMIBC. The descriptive statistical analysis included means, standard deviation, p-value; Univariate and multivariate Cox regression analyses.

RESULTS: Twist2 expression score was identified as negative, low (1-15%); medium (15-40%); and high (40-100%). Patients who had low or low medium scores at the initial diagnosis had a good response and a favourable prognosis. Expression of a high score of Twist2 in patients having high-grade T1 tumours showed non-responsiveness to repeated courses of intravesical bacillus Calmette Guerin (BCG) therapy and was upstaged to MIBC.

CONCLUSION: Twist2 expression in tissue samples of NMIBC would indicate the tumour response to therapy, upgrading and upstaging in the follow up after intravesical BCG therapy.

Introduction

Bladder cancer (BCa) is seventh cancer in the male population, 75% of patients with BCa present at initial diagnosis with non-muscle invasive bladder cancer (NMIBC) of stage Ta and T1 respectively [1]. Muscle invasive bladder cancer (MIBC) of stages T2-T4 has the potential of lymph nodes invasion and distant metastases. High-grade Ta and T1 tumours have a high potential for recurrence and upstaging to MIBC. The twist is a basic helix-loop-helix transcription factor, two Twist-like proteins, Twist1 and Twist2, are sharing structural details, the N-termini of both Twists is different where Twist1 lacks glycine-rich region that consequently leads to a different function of Twist2 [2]. The epithelial-mesenchymal transition (EMT) leads to the loss of cell polarity and cellular adhesion that facilitates cancer cells migration, invasion, and metastases in different types of cancer including BCa. Twist2 is a regulator of EMT that has an important role in tumorigenesis where malignant cells acquire the ability of invasion, metastasis in addition to resisting cancer therapy [3] [4] [5]. In breast cancer, Twist2 expression was significantly increased, the cytoplasmic Twist2 in cancer cells at the tumour centre of primary carcinomas contributes to the maintenance of epithelial cancer characteristics, invasion and metastasis. Heterogeneous expression

of Twist2 in tumours may have a functional link to tumour progression, Twist2 promotes breast cancer invasion through loss of E-cadherin, and there is a strong link between nuclear Twist2 represented as nuclear positivity with an overexpression score and cancer progression and metastases [6] [7]. EMT process depends on Twist2 cellular location [5]. EMT is a dynamic process in the pathogenesis of BCa, despite significant advancements in diagnosis and treatment, the outcomes remained more or less the same. Expression of EMT markers E-cadherin, N-cadherin, Vimentin, Snail, Twist, Zeb, and Slug in BCa showed that increased expression of EMT transcription factors correlated significantly with tumour grade and stage; therefore EMT marker profile would provide a sensitive and effective prognostic tool for investigation of BCa [8]. Expression levels of EMT markers Twist in tumour specimens taken by transurethral resection of bladder tumour of patients with NMICB and measured by immunohistochemistry(IHC) staining showed that expression level was significant predictors of intravesical recurrence-free survival, the nuclear positivity of Twist2 related to recurrence and progression [8]. In a Long-term follow-up of the patient to determine whether EMT related markers can predict patient survival and progression in NMIBC, it was shown that EMT markers E-cadherin, Twist, and Vimentin detected by IHC had statistically significant correlations with grade, recurrence, tumour progression, and progression-free survival [9]. The twist is considered as a potential oncogene promoting the proliferation and inhibiting the apoptosis in BCa; it promotes the synthesis of a pro-angiogenic factor, a vascular endothelial growth factor which is involved in tumour progression and metastasis [10]. Upregulation of Twist2 was related to the aberrant expression of E-cadherin and the increased expression of Vimentin, which were reported as important indicators of EMT. Twist2 regulates EMT by depriving the epithelial cell phenotype of E-cadherin and endowing the mesenchymal cell phenotype with Vimentin, which may be involved in the progression and prognosis [11]. Tumour progression in colorectal cancer was significantly correlated to overexpression of Twist2 and Twist1 [12]. Twist2 expression was correlated to the progression of cervical cancer [13]. The purpose of this study was to determine whether Twist 2 upregulation was the promoter of NMIBC not to respond to intravesical bacillus Calmette Guerin therapy (BCG) and whether the overexpression was correlated positively with recurrence and upstaging. The goal of this study was to evaluate Twist2 expression by IHC in order to track disease response and progression during therapy, as well as the evaluation of association between Twist2 value and the clinical outcome, and/or determination of whether Twist2 expression contributes to providing additional information about the likely clinical outcome beyond the information provided by clinicopathological data.

Material and Methods

A cohort of the study was 305 patients with NMIBC whom tumour samples were stored in tumour archives; the study ran between the years 2008 and 2016. Patient's clinical data were analysed, and the selection was made for those who had at least 3 years of potential follow-up or had frequent recurrences, upgrading or upstaging to MIBC that indicated immediate cystectomy, inclusion criteria were no metastatic disease at diagnosis, any previous chemotherapy or radiotherapy. Ten patients with MIBC tumours were considered as positive control, 10 patients who had benign prostatic hyperplasia and undergone trans-urethral resection of the prostate (TURP) and had no associated cancer were taken as negative control.

The institutional review board approved the study and patients gave informed consent to use the medical data; the study was to evaluate tumour markers in diagnosis and follow-up for NMIBC.

Patients were adults aged from 32 to 83 years, diagnosis of NMIBC was based on urine cytology, abdominal ultrasound, non-contrast computed tomography of the urinary tract, cystoscopy and transurethral resection of bladder tumours (TURB), pathological diagnosis of NMIBC was made by two independent pathologists. Patients received treatment in accordance with the European Association of Urology guidelines for the treatment of NMIBC [14], patients who had low papillary grade Ta tumors were treated with TURB, recurrent low-grade Ta and low-grade T1 tumors were treated with complete TURB and 1 year of full-dose BCG treatment, patients with recurrent tumours with high grade and high-risk tumours, a full-dose of intravesical BCG for 1–3 years were indicated. Tumour progression to a higher grade or upstaging an immediate cystectomy was done. Follow up was done with urine cytology and cystoscopy and biopsy every 3 months for 36 months, patients who had a recurrence after the first course underwent a re-TURB and second course of intravesical BCG. Patients showed repeated recurrence, upgrading, or upstaging to muscle-invasive bladder cancer categorised as non-responder to BCG intravesical therapy.

Patients characteristics

A group of 305 patients with NMIBC who had been treated with either TURB alone for low papillary grade Ta a tumour, or TURB and Intravesical intravesical BCG for patients with high-grade Ta, and for T1 tumours, follow-up took place for 3 years. Patients were categorized into 4 groups; low grade Ta (n = 32) were group 1, high grade Ta (n = 67) were group 2, low grade T1 (n = 47) were group 3, high grade T1 (n = 114) were group 4 (Table 1).

Table 1: Demographic and Clinical Characteristics of patients having Ta-T1 non-muscle invasive bladder carcinoma at baseline and at follow-up following treatment with TURB* and BCG‡

Features	Ta, Low grade	Ta,High grade	T1,low grade	T1, high grade
Number	32	67	92	114
Gender				
Male	27	50	69	78
Female	5	17	23	36
Age-years				
Range-mean	32-76 (51)	48-76 (62)	47-82 (63)	45-83 (65)
Treatment	TURB*	TURB + BCG‡	TURB + BCG	TURB + BCG
Response to treatment	Cured	Responder (68.6%)	Responder (54.3%)	Recurrence (42.4%)
		No response (31.3%)	No response (45.6%)	Upstaging (67.5)
Follow-up Diagnosis	Cured	Ta, low grade, n = 46	T1, low grade, n = 50	Recurrent T1, n = 37
		T1, high grade, n = 21	T1, high grade, n = 30	T1-T3, n = 77
			T2-T4, n = 12	

*TURB, transurethral resection of a bladder tumour; ‡ BCG, intravesical instillation of bacillus Calmette Guerin.

Response to treatment was either good response with no recurrence or residual diseases in the 3 years follow-up, or non-responder with repeated recurrences, upgrading, or upstaging. According to response criteria, the 305 patients were categorised into 6 groups in consideration of initial diagnosis and endpoint of being responder or non-responder. All patient's tissue samples at initial diagnosis and at last follow-up were studied for Twist 2 expression by immunohistochemistry, the follow-up groups were: group1, low grade Ta responder (n = 32); group2, High grade Ta non-responder (n = 46); group3, high grade Ta non-responder (n = 21); group 4,low grade T1 responder (n = 50),group 5, Low grade Ta non-responder (n = 42), group 6, high grade T1 non-responder (n = 114) (Table 2).

Table 2: Twist2 expression in non-muscle invasive bladder cancer at baseline in the intention-to-treat and at follow up with correlation to response to BCG therapy*

Group characteristics	no	Twist 2 expression at initial diagnosis Range	Mean ± SD¶	Score†	Twist2 expression at final follow-up Range	Mean ± SD	Score	p[9] value
1.Ta low grade responder ‡	32	0	0	Negative	0	0	Negative	0.001
2.Ta high grade responder	46	3-13	1 ± 1	Low	3-6	3 ± 1	low	0.001
3.Ta high grade nonresponder‡	21	21-30	26 ± 2	Medium	27-38	35 ± 1	Medium	0.001
4. T1 low grade responder	50	25-36	27 ± 3	Medium	20-25	23 ± 1	Medium	0.001
5.T1 low grade nonresponder	42	45-55	50 ± 1	High	60-75	65 ± 1	High	0.001
6. T1 high grade nonresponder	114	57-83	65 ± 3	High	74-92	88 ± 3	High	0.001

*Expression of Twist2 by immunohistochemistry was evaluated at initial diagnosis and final follow-up of Ta-T1 non-muscle invasive bladder cancer, patients were treated with Trans-urethral resection and intravesical BCG for 3 courses, at the end of follow-up patients were stratifies into 6 groups according to response to treatment. ‡ Response was assessed at the final follow-up, responder was identified as complete response after treatment, Responders are patients who had complete response with no or minimal residual disease, nonresponders are patients who received maximum of three courses of transurethral resection of a tumour and three courses of intravesical instillation of BCG, this patient had upgraded, upstaging, and or repeated recurrences. †Twist2 expression score was measured according to the percentage of cytoplasmic and nuclear staining: negative staining, low score 1-15%, medium 15-40%, high 40-100 % positive. The P value was calculated with the use of chi-square test, P= 0.001 was statistically significant. ¶ SD standard deviation. BCG; Bacillus Chalmette Guerin intravesical installation.

Immunohistochemistry

Total number of cases was of 305 patients; tissue samples that were taken by TUR-BT in the initial diagnosis and in the follow up and were examined for expression of Twist2 by immunohistochemistry (IHC), Normal control were tissue samples of patients having normal urothelial tissue and had not bladder cancer or other malignancies (*n* =10), positive control were muscle invasive urothelial bladder cancer who underwent cystectomy (*n* = 10) (Table 3).

Table 3: Clinical features and Twist2 expression in the negative and positive control groups

Group	Number	Age Range (mean)	Gender Male	Female	Twist2 expression Range	Mean ± SD	Score
Normal urothelium	10	58-76 (67)	10	0	0	0	Negative
Muscle invasive T2	10	57-76 (62)	8	2	86-98	95±4	High

SD: standard deviation.

Tissue samples at the initial diagnosis and in the following periods were fixed for 24 hours in 10% neutral buffered formalin solution and then processed for preparing paraffin blocks. Tissue samples were examined for histopathological evaluation with Hematoxylin and eosin stain and evaluated according to the international histological classification of urinary bladder tumours of WHO-1999, tumour grade according to WHO grading system, 2004***,*** and tumour stage according to TNM staging system of UICC-2004 2015.

Paraffin-embedded sections of 4mm thickness were processed using Anti-Twist2 concentrated antibody (Abcam, ab 57997, USA), antigen retrieval was performed in all cases by steam-heating the slides in 1 mmol/l solution (pH 9.0) for 45 min. After blocking of endogenous biotin, staining was performed using an automated immunostainer (Ultra Benchmark, Ventana, USA), followed by detection using Ultraview detection Kid (Ventana, USA). Positive and negative control sections were used for each assay.

Immunohistochemistry scoring

The entire section was examined to find the area with maximum positivity, and stained nuclei for Twist2, positively stained cytoplasm and nuclei were scored using the 40X objective in 20 fields. Immunohistochemistry evaluation was done by two independent observers. For evaluation of a Twist2 expression, each slide was scored according to the percentage of positively stained cytoplasm and nuclear staining. The following ranges were used: negative score was that to cells not stained, low score was that 1 to 15% positive cytoplasmic stain, medium score was to 15-40% stain, high expression score was to over 40% cytoplasmic expression associated with nuclear expression, normal urothelium was negative

for Twist2 expression, muscle-invasive urothelial carcinoma showed high expression of Twist2 (Figure 1). Negative external control was done by omitting primary antibodies. The acquisition of images was done with Nikon Eclipse E600 software program Lucia 5.

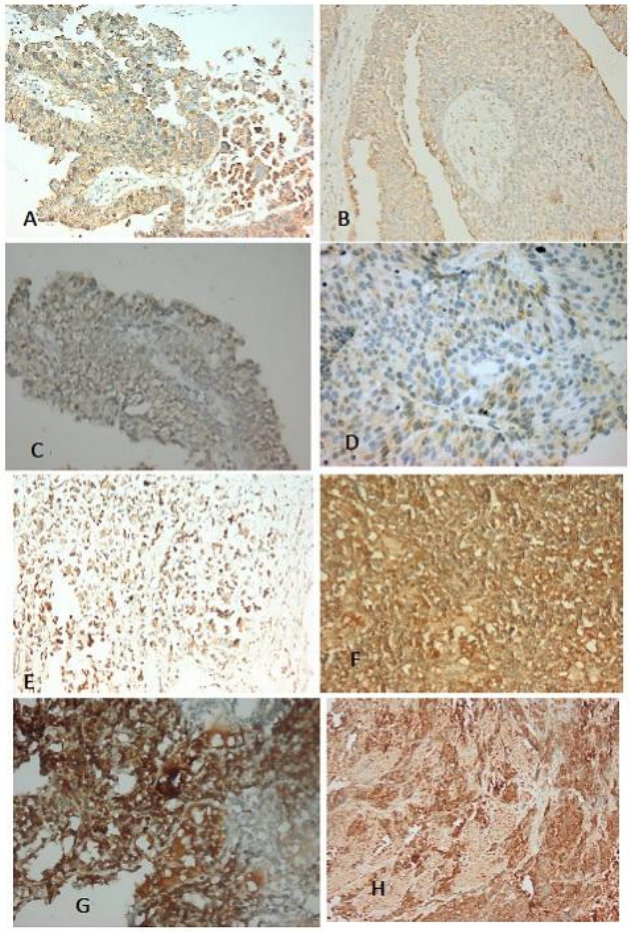

Figure 1: Immunohistochemical (IHC) staining of Twist2 expression on paraffin-embedded tissues of non-muscle invasive bladder carcinoma. No expression of Twist2 could be seen in normal urothelial tissues, while positive expressions of Twist2 have mainly localised in the cytoplasm in high-grade Ta, and in both cytoplasm an nucleus of high-grade T1 and T2. Magnitude ×200, ×400. (a) High-grade Ta, positive for Twist2, with medium expression, IHC x200; (b) Low-grade Ta, positive for Twist2, with low expression, IHC, x200; (c) High-grade Ta, positive for Twist2, with high expression, IHC x200; (d) Low grade papillary Ta, positive for Twist2, with low expression, IHC x 400; (e) High grade T1, positive nuclear and cytoplasmic staining with overexpression, IHC x200; (f) High grade T1, Positive for Twist2,with overexpression, IHC x400; (g) High grade T1, Positive for Twist2,with overexpression, IHC x200; (h) muscle invasive carcinoma T2, highly positive for twist2, with overexpression, IHC x200

Statistical analysis

A descriptive study of variables in the study cohort was done; Chi-square test was performed to compare categorical variables with the expression of Twist2, dependent variables were recurrence and progression-free survival. Differences between categories were evaluated using a log-rank test. To determine the way by which a tumour was not responding to BCG intravesical immunotherapy, recurrence, and how tumour progression was affected by upregulation of Twist2 expression, a Cox's proportional hazards analysis was performed. A $p < 0.05$ was accepted as statistically significant. Unavailable Cox regression analysis was performed with theTwist2 marker treated as a continuous variable, as proposed by Almman et al., [15] and Keegan et al., [16], trying to avoid extreme bias. The expression of Twist2 variables was analysed in function of recurrence-free and progression-free survival in response to treatment.

Results

The 305 patients with NMIBC were 224 men and 81 women, with age ranging from 32 to 83 years; patients with Ta tumors were 99 cases, 32 with low-grade papillary tumor, and 67 with high grades Ta; patients with T1 amounted to 206 cases, 92 of low grade, and 104 were high grade (Table1). The expression of the Twist2 in the tissue samples was measured in the initial pathological diagnosis as well as in the follow-up, Patients were categorised according to initial diagnosis and final diagnosis in the follow up after therapy with TURB alone or TURB and intravesical BCG for 2-3 courses. Clinical data reported cure, recurrence, upgrading, and upstaging, during the 3 follow-up years, tumour responded to therapy was considered responder, non-responders were the tumours that showed upgrading and upstaging (Table 2). IHC for expression of Twist2 was done in the initial diagnosis, and in the final follow-up, patients were categorised into six groups (Table 3). Interpretation of the Twist2 expression showed that the higher is the stage and grade of a tumour, the higher the Twist2 expression scores. Patients who did not respond to treatment and had upgrading and upstaging showed higher expression of Twist2 in the initial diagnosis the more likely the poor response therapy, these findings were observed in groups 3,5, and 6 who were high-grade Ta, part of low-grade T1, and high-grade T1. High score at initial diagnosis in patients with high-grade T1 was correlated with non-responsiveness to therapy and upstaging to MIBC. Low-grade Ta papillary tumours had a low score in the initial and follow-up diagnosis and had a good prognosis.

High grade Ta tumor were two subdivisions, those with low medium score in the initial diagnosis (n = 47) who had responded to therapy and had the same low medium score in the follow up, the second subdivision were those who had high medium score in the initial diagnosis (n = 33), they were upstaged to high-grade T1 in the follow-up. Comparison between mean values of Twist2 expression in initial diagnosis of NMIBC and MIBC was highly significant (0.001),

comparison between mean values of Twist2 expression in initial diagnosis of NMIBC and non-responders that had upstaged and upgraded in the follow-up was highly significant (0.001) (Table 2) Univariable analysis of Twist2 expression in the initial diagnosis in relation to tumor grade and stage was highly significant (P 0.001); univariable analysis of Twist2 expression in relation to sex and gender was insignificant. Univariate logistic regression model showing Twist2 Expression measured at initial diagnosis is a predictor of disease prognosis (P-value 0.002), Adjusted R Square (0.571), odds ratio (1.416) (Table 4)

Table 4: Univariate logistic regression model showing Twist2 measured at initial diagnosis as a predictor of disease prognosis

	Adjusted R Square	B	P value	Odds ratio	95% CI
Twist2 expression at initial diagnosis	0.571	0.348	0.002	1.416	1.138-1.761

Discussion

Epithelial-to-mesenchymal transition (EMT) is characterised by loss of cellular adhesion and polarity and is responsible for cancer metastasis and upstaging, EMT regulates urothelial tumour progression and sensitivity to drug therapy [16]. The present study showed that the Twist2 which is one of EMT transcriptional repressors of E-cadherin when measured at initial diagnosis of NMIBC would indicate a response to drug therapy, progression, upgrading and upstaging. Twist2 was reported to be good prognostic markers for tumour progression in urothelial bladder cancer [6] [7] [8] [9] [16]. The present study explored the significance of measurement of Twist2 expression by IHC at initial diagnosis of NMIBC and at follow up following treatment with TURT and intravesical BCG therapy, our finding showed that Twist2 expression score is a predictive marker for tumor response to therapy and would indicate future favorable response or progression, present work is the first study to show the significance of Twist2 expression in NMIBC to predict response to treatment. Overexpression and high medium Twist2 expression score was a determinant factor for non-response to therapy, upgrading and upstaging; overexpression of Twist2 with over 40% score was significant with a p-value ($p < 0.001$). Previous reports show that heterogeneous nuclear expression of Twist2 in cancer tissue will indicate upstaging, progression, metastases, and poor survival [2] [3] [4] [5] [6] [10] [11] [12] [13]. Present results on the cytoplasmic and nuclear expression of Twist2 confirmed the previous reports. There was a significant difference in the expression of Twist2 in NMIUBC and positive control of MIUBC. Using the ROC curve for the diagnostic index of Twist2 with a cut-off > 40, area under the ROC curve was 0.994 with a sensitivity of 100 and specificity of 97.5. The positive predictive value was 87, and the negative predictive value was 100. The percentage of cases that had upstaged or upgraded in the follow-up assessment amounted to 62.95%. The univariate logistic regression model showing Twist2 expression measured at first as a predictor of disease progression showed that the adjusted R square was 0.571, the p-value was 0.002, the Odds ratio was 1.416 with 95% confidence interval of 1.138-1.761. Present results are by previous studies that showed the high positive expression of Twist2 indicating progression, metastases and poor survival in carcinoma of the bladder and other cancers as breast cancer, colorectal cancer, and ovarian cancer [5] [9] [10] [11] [12].

In conclusion, the high expression of Twist2 with intense cytoplasmic and nuclear staining with duplication or triplication of its value in the follow-up for patients with NMIUBC indicated recurrence, upstaging and progression of Ta and T1 tumours and unfavourable response to intravesical instillation of BCG. The study concluded that Twist2 expression in the initial diagnosis and the follow-up of treatment of NMIBC might be useful for stratifying patients with Ta and T1 into risk categories. The results may have an important clinical implication. Twist2 as a marker for EMT would be considered as a tumor marker to predict bladder cancer pathway.

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Clinical Science

Nitric Oxide and Pre-Eclampsia: A Comparative Study in Ghana

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Abstract

BACKGROUND: Preeclampsia is one of the commonest aetiologies of foetal and maternal mortality and morbidity. Though common, the aetiology of preeclampsia has remained unknown with several inconclusive theories surrounding the disease. Recent studies have implicated vascular endothelial dysfunction and possibly nitric oxide in preeclampsia.

AIM: To compare plasma nitric oxide levels in pre-eclampsia and healthy pregnant women in a large tertiary hospital in Ghana.

METHODS: This was a case-control study conducted among pre-eclampsia and healthy pregnant women in Korle-Bu Teaching Hospital over a four-month period. Thirty (30) pre-eclamptic and 30 healthy pregnant women aged 18-35 years with over 30 weeks' gestation were consecutively recruited into the study after obtaining informed consent. Plasma nitric oxide levels were determined using the Griess Reagent system. Data were analysed using Statistical Package for the Social Sciences (SPSS) software version 20.0 and results were compared using the independent t-test. A P-value of ≤ 0.05 was considered statistically significant.

RESULTS: The parity and body mass index (BMI) of the participants were similar. There was a significant difference in the blood pressure of the pre-eclamptic compared to healthy pregnant women. There was no statistically significant difference (P-value = 0.160) in the plasma levels of nitric oxide in pre-eclamptic (Mean = 1178.78; SD = 89.70 nM) compared to healthy pregnant women (Mean = 1365.43; SD = 95.46 nM).

CONCLUSION: Plasma nitric oxide levels may not play a significant role in the aetiology of pre-eclampsia.

Introduction

Two to eight per cent (2-8%) of all pregnancies worldwide are complicated by pre-eclampsia causing over 63,000 maternal deaths annually [1]. The maternal mortality rate of pre-eclampsia is highest in low and middle income countries. However, pre-eclampsia is still a life-threatening disorder even in developed countries [2]. There is a five-fold increase in perinatal deaths from intrauterine growth restriction and prematurity as a result of pre-eclampsia [3]. Preterm birth in itself is responsible for the majority of neonatal deaths and nearly one half of all cases of congenital neurologic disability [4]. Fifteen percent (15%) of all premature deliveries in the United States (US) are as a result of pre-eclampsia [3]. The aetiology of pre-eclampsia lies in the placenta though it remains unknown [5]. Pre-eclampsia is known to occur only in the presence of a placenta as in for example molar pregnancy and resolves after its delivery. Placental growth is a regulated process and it is vital for normal foetal development and for maintenance of successful pregnancy. Normal placental development requires that cytotrophoblast invades the maternal spiral arterioles [5]. There is an impairment of cytotrophoblastic invasion of the myometrial portion of the spiral arteries in pre-eclampsia leading to narrowing of the spiral arteries with limited blood supply to the foetus [6]. This eventually causes placental ischaemia and microinfarction with subsequent release of placental factors leading to an imbalance in angiogenic factors and therefore widespread endothelial dysfunction that is seen in pre-eclampsia [6]. The ability of the maternal system to handle the deficits in placentation and subsequent challenge to the maternal cardiovascular system partly depend on the immune system, as systemic

inflammatory stress plays a key role in endothelial cell activation [6].

Nitric oxide (NO), a vascular endothelial relaxant may be involved in the development of pre-eclampsia. An endothelial form of NO synthase has been localised to the syncytiotrophoblast and villous endometrium in term pregnancies [5] [6]. The placenta is, therefore, an important source of NO during pregnancy. Various animal models in which NO synthesis has been inhibited have been associated with symptoms such as hypertension, proteinuria, thrombocytopenia and restricted foetal growth [5] [6]. The main placental vasodilator is nitric oxide, and it regulates placental vascular resistance and reactivity, apoptosis and invasion by trophoblast, and aggregation and adhesion of platelets in the placental bed [7]. Numerous studies hold the view that pre-eclampsia is a multisystem disorder with vascular endothelial dysfunction, however, as to whether the change in the function of the endothelium noted in pre-eclampsia results in a decrease, an increase or an unchanged endothelial NO synthesis is still debatable [8].

Literature has reported inconsistent results as far as serum nitric oxide levels in pre-eclampsia compared to healthy pregnant women is concerned. Various studies have reported raised serum nitric oxide levels [9] while others have reported non-significant change [8] [10], and others too, a reduced serum level of nitric oxide [5] [6] [11] in pre-eclampsia compared to normal pregnant women. There is also conflicting literature as to whether the alteration in the function of the endothelium seen in pre-eclampsia results in a pathophysiologic decrease in NO synthesis [8] [10]. Notwithstanding the above controversies, systematic reviews and meta-analyses have shown that pre-eclamptic have a significant increased risk of incidence of cardiovascular diseases, obesity, diabetes and insulin resistance later in life [12]. Thus pre-eclampsia has a huge global and economic burden. There is, therefore, the need to carry out this study to find out the role NO play in the pathophysiology of pre-eclampsia in Ghanaian women.

Methods

This was a case-control study undertaken at the Korle-Bu Teaching Hospital (KBTH), Ghana between March and June 2016.

The study was conducted at the Korle-Bu Teaching Hospital, the premiere Teaching Hospital and the largest tertiary hospital affiliated with the University of Ghana School of Medicine and Dentistry. The 2000 bed capacity hospital has a 350 bed capacity with 3 operating theatre suites obstetrics and gynaecology department. The department has 65 doctors, 200 nurses and midwives, with a daily antenatal attendance of 100 patients, and a total annual delivery of between 10,000 and 12,000.

Ethical Approval for the study was obtained from the Ethical and Protocol Review Committee of University of Ghana School of Medicine and Dentistry (Protocol Identification Number: CHS-Et/M.4-P4.5/2015-2016). Clearance was also received from the Management of the Korle-Bu Teaching Hospital and Head of Obstetrics and Gynaecology department where the study was conducted.

The study population included third-trimester healthy pregnant women and pre-eclamptics aged 18-35 years attending the obstetrics and gynaecology clinic at the Korle-Bu Teaching Hospital. Patients not eligible for inclusion were:

1. Pregnant and pre-eclamptic on any medical treatment other than iron and folic acid

2. Pregnant and pre-eclamptic with chronic hypertension, history of kidney disease, diabetes mellitus, cardiac diseases and neuromuscular problems.

Pre-eclampsia was diagnosed using the onset of hypertension after 20 weeks of gestation with blood pressure > 140/90 mmHg measured on two separate occasions with the coexistence of proteinuria of at least 2+ on dipstick [13].

The plasma nitric oxide level for healthy pregnant women and pre-eclamptics has been found to be 63.8 and 73.3 µmol/l respectively [14], with a mean difference (d) of 9.5 µmol/l. Using the formula by Charan and Biswas [15], sixty (60) pregnant women in their third trimester (gestation > 30 weeks), consisting of 30 pre-eclamptic as cases and 30 healthy pregnant women as controls were recruited consecutively into the study after obtaining informed consent.

The participants were interviewed using a structured questionnaire to obtain their demographic characteristics after signing an informed consent form. The information collected included their age, parity and gestational age. Participants subsequently had their weight and height measured using mechanical patient weighing scale with height rod (Product: 6003, Italy).

Three ml of blood was drawn from the cubital vein using a sterile 19G hypodermic needle fixed on a 5 ml syringe after cleansing the site to be punctured with methylated spirit. Aseptic conditions were adhered to. The blood sample was transferred into a sodium ethylenediamine tetraacetate (Na EDTA) test tube and prevented from clotting by gently inverting the tube 4 times manually. Nitric oxide levels were assessed in the plasma samples using the Griess Reagent system (Promega, Madison, USA). The

assay relies on a diazotisation reaction that was originally described by Griess in 1879.

Patients' age, weight, height, parity, BMI and plasma nitric oxide levels were entered into Microsoft® Access database 2010 (Microsoft® USA), and analysis was done using statistical package for social science (SPSS®) software version 20.0.

The age, BMI and parity of participants, were presented as means (standard deviations) in a tabular form. The plasma nitric oxide levels between the two groups were presented in a bar chart. Independent t-test was employed to compare the difference between the mean plasma nitric oxide level of pre-eclamptic and healthy pregnant women. A *p*-value ≤ 0.05 was considered statistically significant.

Results

The mean systolic, diastolic and arterial pressures were high in the pre-eclamptic compared to healthy pregnant women (Table 1). Age, parity and BMI were similar among the pre-eclamptic and the healthy pregnant women.

Table 1: Demographic and clinical characteristics of the study sample

Characteristic	Pre-eclamptic Mean(SD)	Healthy pregnant women Mean(SD)	*P*-value
N	30	30	
Age (years)	30.97 (5.51)	29.93 (2.60)	0.358
Parity	1.70 (1.42)	1.13 (1.41)	0.567
BMI	32.03 (7.52)	30.50 (5.50)	0.374
SBP	170.13 (23.69)	116.47 (13.38)	<0.001*
DBP	106.30 (18.79)	67.57 (8.54)	<0.001*
MAP	126.20 (20.86)	83.87 (8.85)	<0.001*

*Significant at *P* < 0.05; n-sample size; SD-standard deviation; BMI-body mass index (kg/m^2); SBP-systolic blood pressure (mmHg); DBP-diastolic blood pressure (mmHg); MAP-mean arterial pressure (mmHg).

There was no statistically significant difference in plasma nitric oxide levels in pre-eclamptic compared to healthy pregnant women (*P* = 0.160). The plasma nitric oxide levels in pre-eclamptic and healthy pregnant women were 1178.78 (89.70) nM and 1365.43 (95.46) nM respectively (Figure 1).

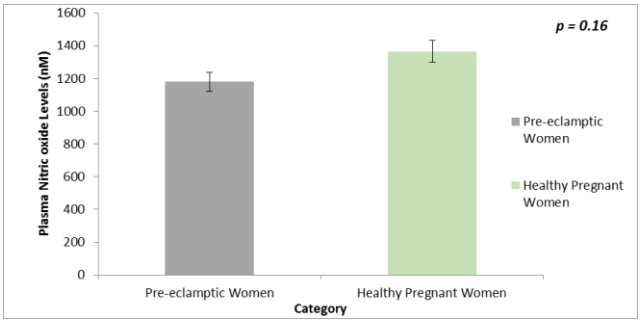

Figure 1: Mean plasma nitric oxide levels for pre-eclamptic and healthy pregnant women

A non-significant negative correlation between mean arterial pressure and plasma nitric oxide levels in pre-eclamptic was noted (Pearson Correlation Coefficient *r* = -0.072; *P* = 0.712) (Figure 2).

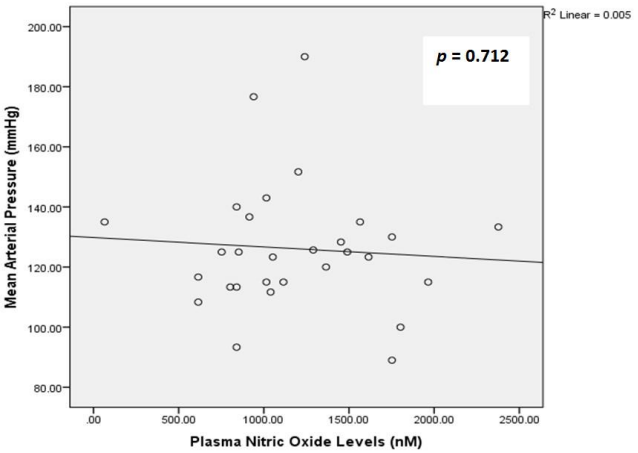

Figure 2: Correlation between mean arterial pressure and plasma nitric oxide levels in pre-eclamptic

Discussion

This study showed a statistically non-significant difference (*P* = 0.358) between the maternal age of pre-eclamptic compared to healthy pregnant women and therefore no association of maternal age with pre-eclampsia. This is similar to the findings of other studies [16] but disagrees with the findings of Macdonald-Wallis and colleagues [17]. The difference in findings may be attributable to sample characteristic differences between the study populations.

This study observed a statistically non-significant difference (*P* = 0.374) in BMI between pre-eclamptic and healthy pregnant women. Therefore BMI may have no association with pre-eclampsia. This is similar to the findings of Onyebule and colleagues [18] but contradicts the observations of other studies which have noted an association of elevated BMI with pre-eclampsia [19].

The systolic, diastolic and mean arterial pressures of the pre-eclamptic were significantly higher compared to that of the healthy pregnant women (*P* < 0.001). This was expected given the criteria used for diagnosis of pre-eclampsia. Mean arterial pressure is said to be predictive of pre-eclampsia even though other studies have noted otherwise [20].

Our study showed a statistically non-significant reduction in plasma nitric oxide levels in pre-eclamptic compared to healthy pregnant women (*P* = 0.160) agreeing with the observations of other studies [8] [10]. Previous studies designed to search for a relationship between nitric oxide production in

pre-eclampsia and healthy pregnancy has shown inconsistent conclusions [9] [11]. The supporters for increased nitric oxide levels in pre-eclamptic compared to healthy pregnant women argue that the increase is as a result of a compensatory mechanism for the occurring endothelial damage in pre-eclampsia hence an attempt to correct the vasospasm effect.

However, the supporters for decreased nitric oxide levels in pre-eclamptic as compared to healthy pregnant women suggest that the reduction is as a result of down-regulation of the nitric oxide synthase enzyme and/or occurrence of endothelial damage in the development of the disorder. The results of a statistically non-significant difference in plasma nitric oxide levels between pre-eclamptic and healthy pregnant women in this study is supported by other studies [8] [10]. This finding, however, may not mean that there is no association between plasma nitric oxide levels and pre-eclampsia but then as per this study, the change in nitric oxide level may not significantly impact the pathophysiology of pre-eclampsia. This may be because the determination of nitric oxide levels is confounded by several factors including a source of sample (plasma, serum, urine), a method of assaying, diet, alcohol consumption, atmospheric pollution, exercise and cigarette smoking [21].

In disorders where there may be a very small difference in nitric oxide production, it may be impossible to find a significant change over the uncontrolled external factors stated above. Considering the study site, it may be very difficult eliminating the above inter-subject variations, and therefore a longitudinal study on selected subjects where inter/intra personal variations can be seen as well as any differences in nitric oxide levels throughout pregnancy between pre-eclamptic and healthy pregnant women is advised. Other studies have also admitted the contradictory reports regarding the involvement of nitric oxide in maternal adaptation to pregnancy and suggested possible multi-mechanism physiology acting in concert to maintain the pregnant mother and the foetus with the input from each mechanism being genetically determined [22].

A non-significant (P = 0.712) negative correlation (Pearson correlation coefficient r = -0.072) was found between mean arterial pressure and plasma nitric oxide levels of pre-eclamptic with an R^2 value of 0.5% implying that changes in plasma nitric oxide levels are a poor predictor of mean arterial pressures in pre-eclamptic patients.

In conclusion, this study failed to demonstrate any significant difference in plasma nitric oxide levels in pre-eclamptic compared to healthy pregnant women. Therefore, plasma nitric oxide levels may not play a significant role in the pathophysiology of pre-eclampsia.

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Obstetric Outcome in Pregnant Patients with Low Level of Pregnancy-Associated Plasma Protein A in First Trimester

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Keywords: Factor screening; First trimester; Pregnancy-associated plasma protein A; the Perinatal outcome

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Abstract

BACKGROUND: Pregnancy-associated plasma protein A (PAPP-A), is a protease which releases Insulin-like growth factor. The role of this factor is stimulation of cell mitosis, differentiation and trophoblastic invasion of deciduas. Identification of patients with low PAPP-A (under 0.4 MoM in the first trimester has an influence on birth weight, attenuation of fetal growth, preeclampsia, birth and fetal demise.

AIM: The main issue in the study is evaluating an influence of PAPP-A, calculated in the first trimester on the unfavourable outcome of pregnancy.

MATERIAL AND METHODS: Seventy pregnant women with singleton pregnancy underwent first-trimester biochemical screening. The target group were women with PAPP-A below 0.4 MoM, and in control group, PAPP-A were over 0.4 MoM. There was an assessment of the influence on the mode of delivery, gestational week, the presence of intrauterine growth restriction, preeclampsia, temporary birth, intrauterine fetal demise and newborn condition.

RESULTS: In target group, consisted of 35 patients, 16 were delivered at term. From 28 to 37 g.w.- were 7 patient, 22-28 g.w.- 4 and 8 patients were under the 22 g.w (all with fetal demise) there were 19 pretemporary deliveries - 9 with Cesarean Section (SC). In the target group: 5 newborn were with IUGR, 6 women had preeclampsia, 1 had placental abruption. In control group were 35 patients: 28 delivered at term, 9 with SC, 26 vaginal deliveries; with IUGR were 4 newborns. Two newborns were hypertrophic.

CONCLUSION: There is a significant difference in unfavourable outcome in the cases with PAPP-A under 0.4 Mom, particular in the group, with a PAPP-A value under 0.2 MoM. The patients delivered with SC with the main indications in utero hypoxia, growth restriction and elevated blood pressure had PAPP-A between 0.3-0.4 MoM. The patients with intrauterine fetal death and placental abruption in the most of the cases have PAPP-A value under 0.2 MoM. There is a need to be aware in these pregnancies to achieve the preventions of adverse outcome, to decrease perinatal morbidity and mortality.

Introduction

The concentration measurement of Pregnancy-associated plasma protein A in the first trimester of pregnancy (PAPP-A), is one of the combined biochemical screening methods for aneuploidies, according to the recommendations of the Fetal Medicine Foundation (FMF), the combined screening method in the first trimester of pregnancy [1].

PAPP-A is a protease for insulin-like growth factor binding protein 4 (IGFBP 4), which facilitate the degradation of this protein, resulting in the release of insulin growth factor (IGF). IGFBP has a great ability for modification of its structure and function, as a result of proteolytic degradation. The intact IGFBP 4 is a powerful inhibitor of IGF in vitro, suggesting that proteolysis acts as a positive regulator of the IGF-availability. PAPP-A isolated from the serum of the pregnant woman has an IGF-dependent protease activity of IGFBP-4. This protease has an important role in the local proliferative answer, acting by accelerating the cell division. It increases the bioavailability of IGF, which in return mediates the

trophoblast invasion of the decidua and modulates the transport of glucose and amino acids in the placenta [2]. A disorder in the release of IGF might be a cause for inappropriate placental perfusion, which would affect the fetal growth and other adverse conditions in pregnancy [3] [4].

PAPP-A is a placental secretory product, and its value is low in the first trimester. The syncytiotrophoblastic deficient forming can have a role in the abnormal placental secretion in affected pregnancies. Normally, the media for PAPP-A rises from 0.4 MoM in the 10th gestational week to approximately 0.7 MoM in the 13th gestational week, so the PAPP-A increases as the pregnancy progress.

The low value of PAPP-A (< 0.4 MoM) could predict an adverse perinatal outcome that includes fetuses with intrauterine growth restriction (IUGR), preeclampsia, preterm birth, miscarriage and stillbirths [5] [6] [7].

Material and Methods

This study was submitted and approved by the Ethical Review Committee of the Medical University in Skopje and is in adherence to the laws and regulations of the country in which the research was conducted. Written consent with patient permission was obtained from each patient.

This prospective cohort study was conducted at the University Clinic for Obstetrics and Gynecology in Skopje included 75 successively admitted and delivered patients during the period of one year from January 2017 to December 2017. All delivered neonates were without a sign of congenital infection, malformation and chromosomopathies.

The study was taken at the University Clinic of Gynecology and Obstetrics, Skopje. The analyses were performed at the Biochemical laboratory in the Clinic, till some patients (70 patients) was fulfilled. The selection of the patients was made consecutive from patients in whom a combined biochemical screening in the first trimester was performed. The patients were divided into two groups: *the target group* - 35 patients who delivered with values of PAPP-A below or equal to 0.4 MoM and a *control group* - 35 patients who delivered with values of PAPP-A over 0.4 MoM. All the patients had fetuses without chromosomal abnormalities. The data for the patients was collected by questionnaire that included anamnestic, demographic information, information about present pregnancy, personal, familial and obstetric history.

Inclusion criteria for PE were the presence of proteinuria at least 0.5 g/L/24 hours, increase in systolic pressure for minimum 30 mmHg, and diastolic pressure 15mmHg, measured two times apart for six hours, compared with blood pressure before pregnancy.

Exclusion criteria were underlying presidential morbidity: chronic hypertension, diabetes, renal disease, autoimmune and metabolic disease (NICE guidelines). Inclusion criteria for IUGR were birthweight less than 5th percentile for gestational age and sex, and exclusion criteria were the presence of congenital infection, anomalies and chromosomopathies and mother who took medication, alcohol and with toxicomania. The placental abruption was clinically and histopathological proven, and exclusion criteria were rupture of membrane, uterine fibroid or other operation of the uterus.

Methods

The concentrations of free β-HCG and PAPP-A were measured from 5ml of the peripheral blood sample with vacutainer in a tube without anticoagulant. The samples were delivered to the laboratory at the Clinic. The present device-Siemens Healthier-Immulite 2000 XPi, with a method of chemiluminescence, and the risk was calculated by licensed software PRISCA version 5.2.1, which is integrated into the device and is used at the Clinic.

The results are presented in absolute values and percentages. *The target group* is the one with PAPP-A below 0.4 MoM, and *the control group* is the one with values of PAPP-A over 0.4 MoM. The difference in the incidence of the qualitative parameters was analysed by Chi-squared test. The values of $P < 0.05$ were taken for statistical significance.

SPSS V.20 was used for numeric and attributive parameters. Standard descriptive and analytical bivariant and multivariant methods were used. Statistical significance among attributive parameters was determined wit Chi-square test and numerical parameters with Student T-test.

Results

There is no significant difference in the prevalence of the patients in both groups, i.e. the age is not a risk factor that affects the value of PAPP-A in patients without chromosomopathies.

The percentage of term deliveries in the target group was 47%, compared to the control group in which it was 80%. There was a significant percentage of deliveries before 22nd gestational weeks in the target group - 22% compared to the same parameter in the control group - 0%.

In the control group, the number of patients who delivered by Caesarean section was 9, and the number who delivered spontaneously was 26.

Table 1: Prevalence of complication in the patients

PAPP-A value (MoM)	0.0 -0.10	0.11-0.20	0.21-0.30	0.31-0.40	Total	> 0.4 Control group
Fetal loss	2	3	2	1	8	0
Hypertension			1	5	6	2
IUGR			3	2	5	4
Placental abruption		1			1	0
Preterm delivery (total= iatrogenic+ contractions)					11	7
Fetal hypoxia			5	4	9	4

In the target group, there were 8 pregnancies terminated with artificial abortion because of an intrauterine death of the fetus, 1 patient delivered a death fetus spontaneously, in the 31st gestational week with preterm placental abruption. Although there is no significant difference in the delivery with Caesarean section, it should be noted that in the control group there were 7 preterm deliveries, 4 of them were delivered by Caesarean section, and 3 were spontaneously delivered.

Table 2: Complication and PAPP-A value

Complication number (%)	Fetal loss	Hypertension with or without proteinuria	IUGR	Placental abruption	Fetal hypoxia	Without complication	Total
Target group	8 (22.8)	6 (17.1)	5 (14.3)	1 (2.8)	9 (25.8)	6 (17.2)	35 (100)
Control group	0 (0)	2 (5.7)	4 (11.4)	0 (0)	4 (11.4)	25 (71.5)	35 (100)
p value	< 0.05	< 0.05	> 0.05	> 0.05	< 0.05	1.00	

Discussion

The values of PAPP-A between 0.05 and 0.20 MoM are associated with adverse outcomes, such as stillbirths and preterm placental abruption [8]. The received results correlate with the study from 2010 [9]. There is a significant difference in the adverse outcome of life born with PAPP-A values between 0.3 and 0.4 MoM, compared to the control group. This group involves the patients who delivered a live-born infant by Caesarean section because of IUGR, fetal hypoxia and elevated blood pressure [10] [11]. There are many studies that prove the correlation between low values of PAPP-A below 0.4 MoM and the birth weight and fetal growth. In one retrospective cohort study, it is found positive predictive values for SGA of 2.97 (95% CI from 1.1 to 6.4) [12].

There is a significant difference in the adverse outcome in patients with values of PAPP-A below 0.4 MoM, especially with values below 0.2 MoM. In the patients, who delivered by Caesarean section with a life born because of a fetal growth restriction, fetal hypoxia and elevated blood pressure in the pregnancy, the value of PAPP-A is 0.3 to 0.4 MoM. Patients with intrauterine fetal death and preterm placental abruption, have a value of PAPP-A below 0.2 MoM, but having only 2 patients with this result, we can not interpret its statistical significance. The PAPP-A values interval between 0.3 and 0.4 MoM, showed more fetuses with growth restriction and preeclampsia, and iatrogenic preterm delivery because of the complications mentioned above. There is no significant difference in the number of spontaneous preterm delivery among the patients with a PAPP-A value below 0.4 MoM. The most of premature deliveries in control group are due a premature rupture of membrane and premature contractions.

To make an algorithm for following of these pregnancies, a huge series of a pregnant woman should be included, and more precise definition of the values of this protein should be done, that has a significant impact of the pregnancy outcome. In term of the fact that there is a national recommendation for follow up on patients with low values of PAPP-A in some countries, an attempt for its implementation in our country could be done [13]. The listed Australian recommendations for follow up of patients with low PAPP-A values (below 0.37 MoM - 5th percentile), can be applied for the beginning as it is national approved and it covers a big group of patients. Caution should be taken about the obtained results, with a purpose of prevention of an adverse pregnancy outcome and decreasing the perinatal mortality and morbidity. It is necessary to assess the cost-benefit if these recommendations are going to be implemented in our circumstances.

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Evaluation of the Clinical Proficiency of RDTs, Microscopy and Nested PCR in the Diagnosis of Symptomatic Malaria in Ilorin, North-Central, Nigeria

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Abstract

BACKGROUND: Accurate laboratory diagnosis of suspected malaria is the hallmark to the control of the disease.

AIM: The clinical proficiency of commercial Rapid Diagnostic test kits (RDTs) using nested PCR as quality control was evaluated among patients attending two public healthcare providing institutions in Ilorin, Kwara state, North-Central, Nigeria.

METHOD: A cross-sectional evaluation of finger prick blood samples of volunteer patients were accessed for malaria parasites with pLDH, HRP2, Pf, Pf/PAN and nested PCR molecular assays. The data derived were analysed using standard formulae for diagnostic accuracy, and the obtained predictive values were subjected to a comparison with one-way analysis of variance (ANOVA).

RESULT: Three hundred and sixty-eight (368) patients comprising 203 (55%) females and 165 (45%) males participated in this study. Routine microscopy revealed that 54 (32.7%) males and 80 (39.4%) was infected with Plasmodium falciparum. SD Bioline (pLDH) 47.4%; Carestart Malaria (HRP2) 49.8% recorded low sensitivities. Micropoint (pfPAN) 82.8% and Micropoint (Mal. Pf) 64.4% recorded a high sensitivity. SD Bioline (pLDH) 67.4%; Carestart Malaria (HRP2) 85.9%; Micropoint (PfPAN) 62.2% and Micropoint (Mal. Pf) 86.7% had high specificities. The positive predictive value (PPV) ranged from 67.7% to 85.94%, while the negative predictive values (NPV) of 64.4% for SD Bioline (pLDH); 86.7% for Carestart Malaria (HRP2); 89.3% for Micropoint (pfPAN) and 58.5% for Micropoint (Mal. Pf). Agarose gel analysis of *P. falciparum ssrRNA* gene (206 bp) for 28 specimens containing 10% concordant and discordant samples showed that all 12 negative specimens for RDTs and routine microscopy were truly negative for nPCR. However, the remaining 16 specimens were positive for nPCR and showed discrepancies with routine microscopy and RDTs. Cohen's interrater diagnostic measure analysis revealed that the weighted kappa for the RDTs was moderate 0.417 ($p=0.027$), 95%CI (0.756, 0.078) and good for nPCR 0.720 ($p < 0.001$), 95%CI (0.963, 0.477). The area under the curve (AUC) specify that nPCR has been more effective than the RDTs (nPCRAUC = 0.875; $p < 0.001$ and RDTsAUC = 0.708; $p = 0.063$).

CONCLUSION: A thorough large-scale quality control is advocated on all commercial RDTs being used in most sub-Saharan African countries. This is to avoid double jeopardy consequent upon misdiagnosis on unidentified positive cases serving as pool reservoir for the insect vector and cyclical infection and re-infection of the populace.

Introduction

In spite of the continued efforts at eradicating malaria in sub-Sharan Africa, it has remained a major public health concern in the region. One of the remarkable milestones left to be achieved for the complete eradication of malaria is the development of adequate biological and clinical diagnostic tools [1] [2] [3] [4]. Microscopy has been the usual clinical practice for the diagnosis of malaria in endemic regions, but this method of diagnosis is faced with so many issues bothering on expertise, quality of field microscopy, epileptic electricity supply and poor reagents to mention a few [5] [6] [7] [8]. Although, detection thresholds of 4–20 parasites per microlitre are achievable using a Giemsa-stained thick blood film in controlled laboratory situations [9], thresholds of 100–200 parasites per microlitre are more common in field

settings [7] [8]. The use of rapid diagnostic tests as an alternative to microscopy in remote areas has however been advocated [1]. RDT was developed to improve the timeless sensitivity, and objectivity of malaria diagnosis through less reliance on expert microscopy [10]. These kits come in strips and cassettes embedded with immuno-chromatographic cork material that has antibodies targeted for *Plasmodium* parasite antigens. RDTs work on the detection of Histidine-Rich Protein2 (HRP-2) from *Plasmodium falciparum* and Parasite-Specific Lactate Dehydrogenase (pLDH) or *Plasmodium* aldolase from the parasite glycolytic pathway found in all species [11] [13]. RDTs are increasingly being used for malaria diagnosis because they are rapid and easier to use especially in limited resource settings and do not require trained personnel or special equipment [1] [8] [14] [15] [16]. One major pitfall in the use of RDTs in clinical situations is the issue of false-positive/false negative results which may lead to misdiagnosis and over administration of antimalarial drugs which often culminate into increased financial costs, side effects, and selection pressure for development of resistance in malaria parasite populations [17] [18] [19]. Inadvertently, false-negative results may lead to excess morbidity and mortality and further transmission. Thus, quality diagnostics are essential [20]. There are many commercial RDTs in the market today, but their efficacy remains a subject of concern. Therefore, it is not clear which RDT is more appropriate for different epidemiological settings [5] [21] [22]. It has been estimated that a diagnostic test with 95% sensitivity and 95% specificity requiring minimal infrastructure would avert more than 100,000 deaths and about 400 million unnecessary treatments (Long, 2009). The ominous disparity in RDTs and routine microscopy is gradually becoming contentious, hence the need for a more appropriate clinical diagnostic technique. A new diagnostic technique undergoing clinical trials becomes evident. Several molecular detection methods (MDM) are increasingly being researched in clinical practice [11] [23] [24] [25] [26]. It operates on the basis of small nuclear subunit ribosomal (SSU) rRNA genes which are targets, extensively used for the molecular detection of human malaria parasites [25]. These genes are known to have highly conserved regions and their copy numbers ranging from 4 to 8. These characteristics ensure that they are suitable genes for phylogenetic studies and molecular detection of *Plasmodium* parasites [27].

There are two basic approaches for species detection, single polymerase chain reaction and nested polymerase chain reaction (PCR). In general, nested-PCR is more sensitive than single PCR because they detect malaria parasites at very low-level malaria parasitemia [28]. The nested PCR is highly sensitive and has been widely used for diagnosis, confirmation of diagnosis, epidemiological studies, drug efficacy assessment and to measure the accuracy of microscopy [23] [29]. The nested PCR is used as a quality control laboratory technique to evaluate the clinical proficiency of established RDTs for this study. There is a dearth of published information on the assessment of the diagnostic efficiency of malaria in Ilorin, north-central Nigeria, thus the need for the present study. This study, therefore, seeks to comparatively evaluate the diagnostic efficiency of the various malaria diagnostic methods in Ilorin, north-central Nigeria.

Materials and Methods

A prospective study was conducted from January to March 2016 in Ilorin, Kwara State. A total of 368 subjects (203 males and 165 females) were enrolled at the following comprehensive health centres: University of Ilorin clinic and Children Specialist Hospital (Center Igboro) in Ilorin metropolis. The above areas were used to avoid bias in the sample collection, and so both children and adults were included in the study. The patients were screened for *Plasmodium falciparum* parasites using RDTs, Microscopy and nested PCR techniques.

Peripheral blood samples were collected from voluntary donors at the hospitals mentioned earlier. Examined samples were categorized into 2 groups; concordant and discordant samples after the methods of [24] with slight modifications. The concordant samples were those samples that tested simultaneously positive or negative with PLDH, HRP2 (RDTs) and Microscopy. The discordant group1 were samples that tested positive for PLDH, HRP2 but negative with Microscopy. The discordant groups 2 were samples that tested negative with PLDH, HRP2 but positive with microscopy. Dried blood spot (DBS) from all blood samples with discordant results and 5% of randomly selected samples with concordant results were analysed using nested PCR technique.

Patients of all age groups, presenting with signs and symptoms of malaria infections were included in the study. Demographic information, clinical details and basic information regarding prevention measures of the patients were recorded using questionnaires to establish inclusion and exclusion criteria. Venous blood samples were collected in EDTA and Plain bottles from the patients with suspected malaria cases. A drop was spotted on filter paper (Whatman no. 1). Each filter paper was dried at room temperature and stored carefully in a plastic container to avoid cross-contamination at -20ºC.

The diagnostic accuracies of the following RDTs were assessed, viz; *p*LDH (SD Bioline), HRP2 (Carestart Malaria), Pf/pan (Micropoint) and Mal. *Pf* (Micropoint). Fresh blood samples were transferred directly from the EDTA bottle to RDTs sample pads

with 2 drops of buffer solution (according to manufacturers' instruction). The buffer solution allows the blood to migrate towards the diagnostics and control line.

Thin and thick blood films were prepared following the methods of Gilles [30]. The slides were fixed in methanol in order to allow lysis of red blood cells. The slides were then stained with 10% Giemsa solution for 30 minutes, and thereafter washed, and screened under oil immersion (X 1000 Mg) Olympus microscope for *Plasmodium spp.* Parasite density was determined as the number of parasites per 200 leukocytes (WBC).

Dried blood spot (DBS) from all blood samples with discordant results and 5% of randomly selected samples with concordant results were analysed using molecular (nested PCR) technique. The various techniques were carried out in separate rooms.

Two methods were used for the DNA extraction according to Bereczky *et al.,* [31]. Briefly:

Tris-EDTA buffer-based extraction: Each filter paper punch was placed in Eppendorf tube, soaked in 65 μl of TE buffer and incubated at 50ºC for 15minutes. The punches were then pressed gently at the bottom of the tube several times, using pipette tip for each punch and heated at 97ºC for 15 minutes to elute the DNA templates. The liquid condensing on the lid and the wall of the tubes were removed by short centrifugation. DNA extract was kept at 4ºC for use within a few hours or stored at 20ºC.

Chelate extraction: Each filter paper punch was incubated overnight at 4ºC in 1ml of saponin in phosphate buffered saline (PBS). The punches were washed for 30 minutes in PBS at 4ºC, transferred into new tubes containing 25 μl of stock solution (20% chelex and 75 μl of distilled water) and vortexed for 30 seconds. The tubes were heated at 99ºC for 15 minutes to elute DNA templates.

A Master Mix containing all the reagents is prepared and aliquoted into the reaction tubes and overlaid with mineral oil. DNA templates generated from each sample by the two respective methods were added last into the master mix. Two amplification reactions were carried out. In the first amplification reaction (nest 1), a pair of oligonucleotide primers, which hybridised to a sequence in ssRNA gene of *Plasmodium falciparum*, were used. The product of the first reaction is then used as DNA template for a second amplification reaction (nest 2). The second amplification reaction involves the use of genus-specific and species-specific primers. The genus-specific and species-specific primers (oligonucleotide primers) was used to indicate the presence of malaria parasites and the *Plasmodium* species in the samples.

The PCR products from the amplification reaction were subjected to agarose gel electrophoresis. The electrophoresed products (result) were then interpreted by molecular detection specialist.

Data was entered and analysed using Statistical package for social sciences (SPSS 16.0) to determine the discrepancies between RDTs and nested PCR. The Sensitivity (sn), specificity (sp), positive predictive values (ppv) and negative predictive value (npv) were calculated using standard formulae, and their differences were analysed by comparing their mean values with One way ANOVA. The level of significance was estimated at $p < 0.05$. An interrater reliability analysis using the Kappa statistic was performed to determine consistency among raters. Cohen's interrater statistics was used to validate a measure of agreement between the diagnostic outcomes among the laboratory diagnostic tools.

Results

A Total of 368 patients; 203 (55%) females and 165 (45%) males were randomly enrolled and screened for malaria parasites using microscopy and RDT test kits. Four commercial RDTs were employed for this study, viz; Malaria Ag Pf/Pan by Standard Diagnostics Inc. Hagandong, Korea; Malaria HRP2 (Pf) by Carestart™ by Access Bio, Inc., New Jersey, USA; Malaria PF by Micropoint Inc. The USA.

From the PLDH (SD BIOLINE test kit), 145 (39%) were infected consisting of 72 (49.7%) males and 73 (50.3%) females. Carestart (HRP2) showed that 146 (39.7%) with male (72, 49.3%) and female (74, 50.7%). Micropoint (pfPAN) reported 167 (45.4%) with male (87, 52.1%) and female (80, 47.9%). Results from microscopy indicated that 234 (63.6%) were infected, consisting of 111 (47.4%) males and 123 (52.6%) females. However, only Micropoint (pfPAN) and (*Mal.* Pf) were statistically significant as regards sex at $P > 0.05$ (Table 1).

Table 1: Diagnostic detection of *P. falciparum* based on gender

	Prevalence of Malaria infections			
Diagnostics	Sex	Infected n (%)	Uninfected n (%)	p-value
SD Bioline (pLDH)	Male	72 (49.7)	93 (41.7)	0.082
	Female	73 (50.3)	130 (58.3)	
Carestart Malaria (HRP2)	Male	72 (49.3)	93 (41.9)	0.098
	Female	74 (50.7)	129 (50.1)	
Micropoint (pfPAN)	Male	87 (52.1)	78 (38.8)	0.007
	Female	80 (47.9)	123 (61.2)	
Micropoint (Mal. Pf)	Male	72 (46.8)	93 (43.5)	0.028
	Female	82 (53.2)	121 (56.5)	
Microscopy	Male	111 (47.4)	54 (32.7)	0.112
	Female	123 (52.6)	80 (39.4)	

The diagnostic detection of *P. falciparum* across age-groups in the study revealed that there were discrepancies in the diagnostic accuracy of the four RDTs when compared to routine microscopy. For instance at age ≤ 5yrs; routine microscopy identified varying parasitaemia with all the subjects at that age group being infected (40, 100%). However, SD Bioline identified only two (2, 5.0%) as positive for *P. falciparum*. At age-group 6-15yrs, the results were not comparable as slight differences occurred with all the diagnostic methods. There was a change in trend at age 16-25 yrs where it was observed microscopy detected more subjects as malaria positive (56, 62.9%) when compared with the other four RDTs (SD Bioline 15, 38.5%; Carestart 13, 33.3%; Micropoint pfPAN 24, 27.0%; Micropoint Pf 26, 29.2%). The diagnostic accuracy of the RDTs appear to positively pick up with increasing age of the subjects, for instance at age 36-45 yrs, there was no appreciable differences in the diagnostic outcome of the subjects (SD Bioline 31, 34.8%; Carestart 23, 25.8%; Micropoint pfPAN 23, 59.0%; Micropoint Pf 23, 59.0%; Microscopy 17, 43.6%). Malaria positive subjects declined with routine microscopy at age ≥ 46 yrs (9, 34.6%) while the RDTs showed more positive results within this age group. However, the diagnostic tests were all statistically significant at $p < 0.001$ (Table 2).

Table 2: Diagnostic detection of *P. falciparum* across age-group

Age-grp.	*Diagnostics*	Prevalence of Malaria infections		
		Infected n (%)	Uninfected n (%)	p-value
≤ 5 yrs				
	SD Bioline (pLDH)	2 (5.0)	38 (95.0)	
	Carestart Malaria (HRP2)	4 (10.0)	36 (90.0)	
	Micropoint (pfPAN)	9 (22.5)	31 (77.5)	
	Micropoint (Mal. Pf)	9 (22.5)	31 (77.5)	
	Microscopy	40 (100)	0	
6-15 yrs				
	SD Bioline (pLDH)	57 (65.5)	30 (34.5)	
	Carestart Malaria (HRP2)	65 (74.7)	22 (25.3)	
	Micropoint (pfPAN)	65 (74.7)	22 (25.3)	
	Micropoint (Mal. Pf)	62 (71.3)	25 (28.7)	
	Microscopy	78 (89.7)	9 (10.3)	
16-25 yrs				
	SD Bioline (pLDH)	31 (34.8)	58 (65.2)	
	Carestart Malaria (HRP2)	23 (25.8)	66 (74.2)	
	Micropoint (pfPAN)	24 (27.0)	65 (73.0)	
	Micropoint (Mal. Pf)	26 (29.2)	63 (70.8)	
	Microscopy	56 (62.9)	33 (37.1)	
26-35 yrs				
	SD Bioline (pLDH)	28 (32.2)	59 (67.8)	
	Carestart Malaria (HRP2)	29 (33.3)	58 (66.7)	
	Micropoint (pfPAN)	27 (31.0)	60 (69.0)	
	Micropoint (Mal. Pf)	27 (31.0)	60 (69.0)	
	Microscopy	34 (39.1)	53 (60.9)	
36-45 yrs				
	SD Bioline (pLDH)	15 (38.5)	24 (61.5)	
	Carestart Malaria (HRP2)	13 (33.3)	26 (66.7)	
	Micropoint (pfPAN)	23 (59.0)	16 (41.0)	
	Micropoint (Mal. Pf)	23 (59.0)	16 (41.0)	
	Microscopy	17 (43.6)	22 (56.4)	
≥46 yrs				
	SD Bioline (pLDH)	12 (46.2)	14 (53.8)	
	Carestart Malaria (HRP2)	12 (46.2)	14 (53.8)	
	Micropoint (pfPAN)	19 (73.1)	7 (26.9)	
	Micropoint (Mal. Pf)	19 (73.1)	7 (26.9)	
	Microscopy	9 (34.6)	17 (65.4)	
Total				
	SD Bioline (pLDH)	145 (39.4)	223 (60.6)	<0.001
	Carestart Malaria (HRP2)	146 (39.7)	222 (60.3)	<0.001
	Micropoint (pfPAN)	167 (45.4)	201 (54.6)	<0.001
	Micropoint (Mal. Pf)	166 (45.1)	202 (54.9)	<0.001
	Microscopy	234 (63.6)	134 (36.4)	<0.001

Routine microscopy was used as a gold standard against the four RDTs. There was low sensitivity for the first two diagnostic RDTs, viz; SD Bioline (*pLDH*) 47.4%; Carestart Malaria (HRP2) 49.8%. However, Micropoint (*pf*PAN) recorded a high sensitivity of 82.8%, and Micropoint (Mal. *Pf*) had 64.4%. The probability that a diagnostic will indicate the absence of malaria parasite among those without the disease is called specificity and in the present study, the specificity of SD Bioline (*pLDH*) 67.4%; Carestart Malaria (HRP2) 85.9%; Micropoint (*Pf*PAN) 62.2% and Micropoint (Mal. *Pf*) 86.7%. PPV has determined the probability that the positive results were positive. In the following RDTs, the ppv ranged from 67.7% to 85.94%. The probability that the subjects truly does not have malaria reflected with NPV values of 64.3% for SD Bioline (*pLDH*); 86.7% for Carestart Malaria (HRP2); 89.3% for Micropoint (*pf*PAN) and 58.5% for Micropoint (Mal. *Pf*) (Table 3).

Table 3: Performance of RDTs using routine microscopy as the gold standard

Diagnostics	Sensitivity	Specificity	PPV	NPV
SD Bioline (pLDH)	0.4739	0.6739	0.7078	0.6438
Carestart Malaria (HRP2)	0.4979	0.8593	0.8593	0.8667
Micropoint (pfPAN)	0.8278	0.6223	0.6773	0.8929
Micropoint (Mal. Pf)	0.6438	0.8667	0.7905	0.5850

Keys: PPV=positive predictive value; NPV=negative predictive value.

Malaria sensitive groups were divided into the concordant groups and the discordant groups. The concordant group was further divided into Group 1: all True positives (i.e. positive for pLDH, HRP2 and microscopy) and Group 2: True negatives (i.e. negative for pLDH, HRP2 and microscopy). The discordant groups were also divided into two: Discordant group false positives (i.e. positive for pLDH and HRP2 and negative for microscopy), and the discordant group 2: false negatives (i.e. negative for pLDH and HRP2 and positive for microscopy). The clinical accuracy of the diagnostics was evaluated with nPCR by taking 10% each of Groups 1 & 2 and randomly picking 4 samples each from Groups I & II (Figure 1).

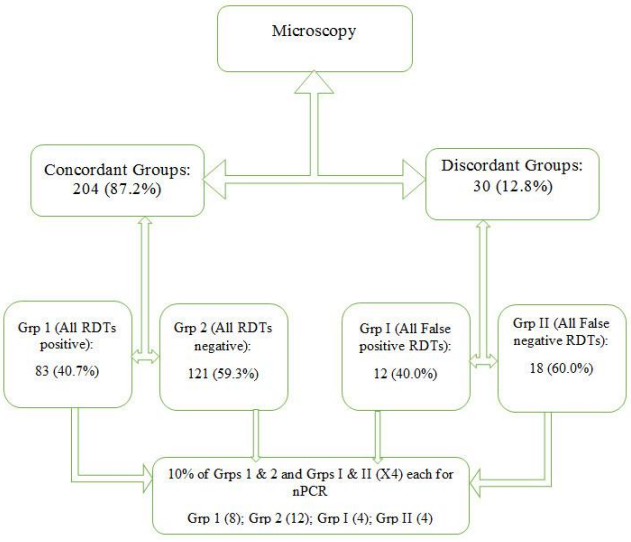

Figure 1: Flowchart describing the proportion of RDTs Sensitivity and samples subjected to nested PCR Analysis

The concordant group: Group 1: all True positives (i.e. positive for pLDH, HRP2 and microscopy) and Group 2: True negatives (i.e. negative for pLDH, HRP2 and microscopy). The discordant groups: Group 1: False positives (i.e. positive for pLDH and HRP2 and negative for microscopy), and Group 2: false negatives (i.e. negative for pLDH and HRP2 and positive for microscopy). The clinical accuracy of the diagnostics was evaluated with nPCR by taking 10% each of Groups 1 & 2 and 4 samples each from Groups I & II was randomly picked.

Agarose gel photograph of *P. falciparum* ssrRNA gene (206 bp) resolved on 1.2% Agarose gel from 28 clinical samples of patients with malaria suspected cases provided the following results: Lanes 3, 5, 6, 8, 11, 13, 15, 19, 22, 24, 27 and 31 were PCR negative for *P. falciparum*. All the 12 specimens diagnosed and grouped as negative for RDTs and routine microscopy was truly negative for nPCR. This further shows that the RDTs specificity can be relied upon. Lane 1 and 17 = DNA Ladder while Lane 2 and 18 = 3D7 Positive control (Figure 2). However, lanes 4, 7, 9, 10, 12, 14, 16, 20, 21, 23, 25, 26, 28, 29, 30, 32 showed *P. falciparum* positive cases meaning that the RDTs had low sensitivities as they initially reported non-malaria infections. To revalidate the present fallouts, the assays used as negative controls were subjected to nPCR and there was no amplification.

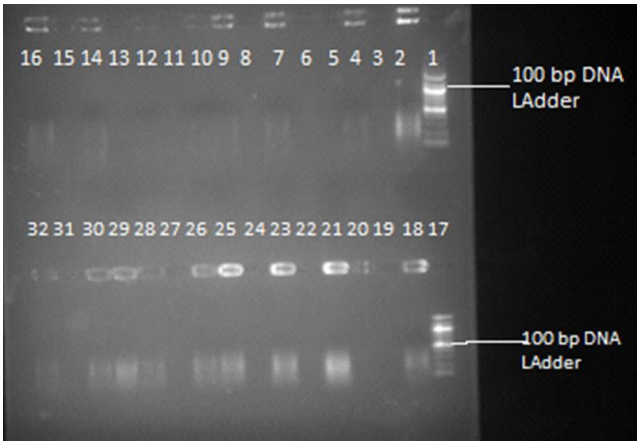

Figure 2: Schematic representation of Agarose gel photograph of P. falciparum ssrRNA gene (206 bp) from 28 clinical samples using species-specific oligonucleotide pairs for P. falciparum. Lanes 3, 5, 6, 8, 11, 13, 15, 19, 22, 24, 27 and 31 were PCR negative for P. falciparum. Lane 1 and 17 = DNA Ladder, while lane 2 and 18 = 3D7 Positive control. Lanes 4, 7, 9, 10, 12, 14, 16, 20, 21, 23, 25, 26, 28, 29, 30, 32 P. falciparum positive cases

Cohen's interrater diagnostic measure analysis revealed that the weighted kappa was estimated to assess the extent to which the sensitivity and specificity of RDTS and nPCR correspond with the routine microscopy in the diagnosis of malaria among a sampled population of the subjects. The kappa value for the RDTs was moderate 0.417 ($p = 0.027$), 95%CI (0.756, 0.078) and good for nPCR 0.720 ($p < 0.001$), 95%CI (0.963, 0.477). The area under the curve (AUC) is the percentage of randomly drawn pairs for which malaria is truly positive. The effectiveness of the RDTs and nPCR was also evaluated with AUC. The nPCR was found to be more effective than the RDTs ($nPCR^{AUC} = 0.875$; $p < 0.001$ and $RDTs^{AUC} = 0.708$; $p = 0.063$) (Table 4).

Table 4: Cohen's Interrater diagnostic measure for RDTs and nPCR with routine microscopy

Diagnostics	AUC	*p*-value	Kappa	95% CI	
				Upper Bound	Lower Bound
Population for Quality control					
RDTs	0.708	0.027	0.417	0.756	0.078
nPCR	0.875	<0.001	0.720	0.963	0.477

Key: AUC: area under curve -0.5: worthless, to 1, a perfect test; Kappa < 0.20: poor, 0.41-0.60: moderate, 0.61-0.80: good and 0.81-1: very good; *P* is significant < 0.05. 95%CI: Estimate ± 1.96 SE.

Discussion

The diagnostic accuracy of malaria laboratory test reagents and kits has been a major pitfall in the efforts towards complete eradication of the disease in sub-Saharan Africa where presumptive evaluation has remained the norm [15] [16] [20]. The present study identified some incongruities between the diagnostic reliability of the sampled RDTs cum routine microscopy and nPCR. The diagnostic detectability of SD Bioline (pLDH) and Carestart Malaria

HRP2 were not gender significant ($p > 0.05$) while that of Micropoint (pfPAN) and (*Mal.* Pf) were statistically significant ($p < 0.05$). Concerning the stratified age groups in the study; a retinue of diagnostic discrepancies were also observed especially at age ≤ 5yrs, the RDTs failed to detect *Plasmodium falciparum* antigens adequately. If the RDT results obtained for this age group were clinically relied upon; then diagnosis will be misleading the clinician. The low sensitivity recorded in the present situation among the ≤ 5 yrs group is in contrast with what was obtained in other studies [32] [33] [34] [35]. In previous studies, it was reported that RDTs failure to adequately indicate febrile illness usually occurs due to handling and storage [36] [37]. The RDTs on arrival in developing countries face challenges of immediate inspection and dispatch to health facilities and consequently are abandoned in the sun [38] [39].

The diagnostic accuracy picked up with increasing age in such a way as to believe that there exist a wider differences in addition to a substantial none overlap between the test methods. This assumption has also been reported by Endeshaw et al., [40]; Ojuroungbe et al., [15]; Osei-Yeboah et al., [16]. Although positive malaria subjects declined with routine microscopy (≥ 46yrs) which appears to be the normal occurrence in malaria endemic regions [41] [42] the results obtained with the sampled RDTs

indicated an array of false negatives. The sensitivity of the RDTs (Pf/HRP2 and Pf PLDH) were low and fell below the threshold approved by WHO [1]. The observed low sensitivity in this study is similar to reports in other field studies [4] [43] [44] [45] [46]. A probable reason for these might be because some of the RDT kits rely on *Pf*HRP2 antigen for the detection of *P. falciparum*, and *Pf*HRP2 gene deletions often lead to false-negative results [43] [47] [48]. On the other hand, Baker *et al.,* [49] conducted studies on *Pf*HRP2 DNA obtained from isolates from African and South American countries with extreme sequence variation but concluded that diversity of the protein was not a major cause of the varying sensitivities of RDTs. Other possible explanations for the poor RDTs sensitivity, despite significant parasitemia, may include substandard diagnostics [50] [51], defects in device membrane [29], anti RDTs antibodies in human [7] and genetic diversity of parasite antigen *Pf*HRP2 and *Pf* PLDH [52].

Routine microscopy results were equivalent to those obtained by nPCR, except for few microscopically negative, but positive for nPCR as also reported by these authors [15] [22] [43] [53]. From a clinical perspective, failure to establish high parasite density from blood samples of febrile malaria patients can pose grave fatality [12] [20]. Diagnostic output with low specificity is less serious when compared with low sensitivity because presentations with low specificity only translate to overdiagnosis and an over the treatment of non- malaria cases [7] [44]. Faint colour bands formation on RDTs may also contribute to underreporting as subjective interpretation may contribute to poor performance [54]. This study used the nPCR techniques as a quality control technique between routine microscopy and available commercial RDTs. While appreciating that nPCR techniques provide astounding results even detecting parasitaemia at low microlitre, the high cost, complexity, materials and laborious exercise required may not make it a candidate diagnostic tool in many resource-poor communities [22] [55]. We determine the ability of a nested PCR assay to detect *Plasmodium* DNA in stored dried blood samples. The authors only choose the nPCR to ascertain the clinical diagnostic accuracy of the use of routine microscopy and RDTs in our settings. Often, malaria presentations at primary health providing centres in our communities are usually misleading, and the diagnostic failure of the RDTs may contribute over or/and under therapy.

Moreover, this has far-reaching implications such as treating patients on clinical suspicion, the threat of drug resistance, as well as possibility of missing a malaria case and consequent complications and mortalities [5] [36]. The WHO minimum selection criteria for malaria RDTs, i.e. panel detection score (PDS) against *P. falciparum* samples should be at least 75% at 200 parasites/μL [56]. WHO recommends that RDTs must demonstrate high sensitivity and specificity [1].

In conclusion, the results obtained from this study has indeed proven that DNA amplification technique provides a better diagnostic outcome as compared with microscopy and antigen-antibody complex detection. However, some of the shortcomings of the DNA amplification include; time, cost, sample quality and probably the volume of the sample available for PCR analysis. Furthermore, the ambiguity and complexity associated with the suitable choice of adequate RDTs to meet up with the prompt and immediate diagnosis are major challenges, especially now that the clinical approach to management, control, elimination and patient's well-being are paramount. Several factors may be militating against the reliability of any of these RDTs, some of these include genetic diversity of parasite antigen, parasite detection threshold, parasite target and level of malaria endemicity. A regular and thorough large-scale quality control is advised on all commercial RDTs imported into most sub-Saharan African countries, this is imperative to avoid the double jeopardy consequent upon misdiagnosis on unidentified positive cases serving as pool reservoir for *Anopheles* mosquitoes leading to cyclical infection and re-infection among the inhabitants.

Ethics approval and consent to participate

This study was part of a study approved by the University of Ilorin Ethical Consideration Committee on 10th December 2012 with protocol approval number: UERC/ASN/2012/221. The consent form was administered, approved by the volunteers and documented before commencing the study.

Authors' Contributions

OSH designed the protocols, performed the statistical analysis and wrote the final draft, OAO collected literature and wrote the first manuscript, OBO and OF provided volunteers for the research and obtained consent, OA carried out the nPCR analysis, OSH and OAO performed the routine microscopy, RDTs and administered the questionnaire.

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Clinical Science

Proton Pump Inhibitors Diminish Barrett's Esophagus Length: Our Experience

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Abstract

AIM: Our main objectives were to evaluate the influence of two-year proton pump inhibitors (PPI) therapy in patients with Barrett's oesophagus on its length, in both types, short and long segment.

METHODS: In this single-centre, prospective interventional controlled study were analysed data collected prospectively over two years from patients with Barrett's oesophagus diagnosed by endoscopy. Patients who received continuous proton pump inhibitors (PPI) for 2 years were included. At each patient visit symptoms were recorded, and at each endoscopy, the length of Barrett's oesophagus (BE) was measured. Biopsies were taken along the length of the oesophagus at intervals of 1 cm. In total, 50 patients with Barrett's oesophagus were included in the study: 10 of whom had long-segment Barrett's oesophagus, and 40 patients had short-segment Barrett's oesophagus. The mean number of endoscopies performed was 3 per patient.

RESULTS: The length of Barrett's esophagus (BE) was influenced by PPI therapy: Circumferential extension in BE patients short-segment Barrett's esophagus (SSBE) (before treatment was 1.5 cm and after treatment was 0.8 cm Maximum proximal extension in SSBE group before treatment was 2.3 cm (SD ± 1.1 cm), and 1.1 cm (SD ± 0.9 cm), respectively. Squamous islands were detected in 25% of patients examined after 2 years on PPIs.

CONCLUSIONS: PPIs achieve a reduction to the length of Barrett's oesophagus, in both types, and the development of squamous islands is commonly associated with their use.

Introduction

In the USA, Barrett's oesophagus (BE) is defined as the displacement of the squamocolumnar junction proximal to the gastro-oesophagal junction with histological evidence of specialised intestinal metaplasia on biopsy specimens [1]. The British Society of Gastroenterology (BSG) has a different definition of Barrett's oesophagus. The BSG defines Barrett's oesophagus as "an endoscopically apparent area above the esophagogastric junction that is suggestive of Barrett's oesophagus (salmon-coloured mucosa) which is supported by the finding of the columnar lined oesophagus on histology". According to this definition, areas of intestinal metaplasia, although often present, are not a requirement for the diagnosis of Barrett's oesophagus [2]. This different definition has arisen for several reasons: results from multiple studies have demonstrated that specialized intestinal metaplasia may be difficult to detect unless thorough biopsies are taken; there seem to be cases of cancers arising in patients who have noninternalized epithelium; and molecular abnormalities are present even in nondysplastic Barrett's mucosa [3] [4] [5].

The need to standardise the classification of Barrett's oesophagus leads to the development of a system known as the Prague classification of Barrett's oesophagus. The Prague C (circumferential) and M (maximal extent) criteria were developed and validated by Sharma *et al*., [6] In this classification, both the maximal length (M) (including tongues) of Barrett's esophagus, as well as the length of the circumferential Barrett's segment (C) are measured during endoscopy. These numbers can then be used to track the length of the Barrett segment over time. This system has a high degree of overall validity for the endoscopic assessment of the visualised Barrett oesophagus segment when it is > 1 cm in length. If

the segment is < 1 cm in length, this classification system is less valid in its ability to define the length of the Barrett segment [6]. Gastroenterologists, at least in the USA, agree that endoscopic evidence (salmon-coloured mucosa proximal to the gastro-oesophageal junction) and histologic evidence of specialised intestinal metaplasia (that is, the presence of goblet cells) are required to make the diagnosis of Barrett's oesophagus [1].

The goals of gastroesophageal reflux disease treatment in patients with Barrett's oesophagus include control of symptoms, healing and maintenance of healed esophagitis and prevention of progression of Barrett's oesophagus toward cancer. The first two are fairly easy achievable using either medical therapy with proton pump inhibitors (PPI) or with antireflux surgery. We have much less data on prevention of progression of this disorder, but it would certainly appear that if treatment resulted in regression of the length of Barrett's oesophagus, it might also decrease the risk for progression [7].

This study aimed to show whether proton pump inhibitors have an impact in the reduction of length of Barrett's oesophagus, in both endoscopic types, short segment and long segment Barrett's oesophagus.

Material and Methods

This study was performed in University Clinical Center of Kosovo, Clinic of Gastroenterohepatology and Institute of Pathology in Prishtina and Skopje. The time of the investigation was June 2009-December 2011.

In this prospective study, 50 patients with BE were included. All patients were interviewed for their age, sex, reflux symptoms chronicity, medications used, weight, smoking and endoscopic determination of hiatal hernia presence. All endoscopic procedures were performed by 1 of 2 experienced endoscopists (having performed > 10.000 gastroscopies each). Upper endoscopy was performed by using the Videogastroscope GIF type Q 145 series.

Endoscopic respondents were investigated by the following dynamics:

In the first endoscopy, endoscopic BE diagnosis was obtained in the terrain of Gastro-esophageal Reflux Disease (GERD) changes, the presence of a hiatus hernia was diagnosed, as well as macroscopic classification of Barrett's esophagus as short (SSBE < 3 cm) and long (LSBE > 3 cm) segment of Barrett's esophagus. Patients for two years were treated with PPI with maximal doses (lansoprazole 2 x 30 mg or pantoprazole 2 x 40 mg, 30 min before meals).

Two months following the first endoscopy, repeat endoscopy with multiple biopsies from the BE zone, 10-15 biopsies, according to "settle-protocol". Patients were continued to be treated with PPI for two years. If necessary, in subjects with more pronounced complaints of vomiting and reflux, antiemetic drugs were administered for up to two weeks.

After 2 years of follow-up, period endoscopy was performed on the respondents to determine eventual changes in Barrett's length and to perform (C & M) multiple biopsies for microscopic analysis. Endoscopic assessment is carried out by the so-called Prague C and M criteria, representing endoscopic grading of changes in Barrett's oesophagus. This system includes circumferential (C) and the maximum proximal extension (M) of the columnar epithelium over the gastro-oesophagal junction, which in this case is as defined top (proximal) border of the gastric folds present. The distance to determine changes in length measured from the anterior dental arch expressed in centimetres (cm). Short (SSBE) and long (LSBE) segment of Barrett's oesophagus was determined by the length of the segment containing specialised intestinal epithelium (< 3 cm and > 3 cm, respectively). At each endoscopy, the location of the GEJ is defined as a place that the highest gastric fold in the tubular oesophagus meets. The length of Barrett's oesophagus was measured from the site of the highest point of squamo - cylindric transition. A hiatal hernia was diagnosed when the crural impression was separated from the top of the gastric rugal folds by 2 or more centimetres. The size of a hiatal hernia was recorded in centimetres.

Gastro-oesophageal junction (GEJ) is an imaginary line where the oesophagus ends, and the stomach begins. The highest point line with gastric folds was proposed as a marker for GEJ. The difference between the endoscopically located squamocolumnar junction and the endoscopically estimated gastro-oesophagal junction determined the length of Barrett's oesophagus. The positions of endoscopic markings were determined in centimetres from the incisors in documenting the level of esophagitis and squamocolumnar connection, along with the cylindrical extension of the lower oesophagus and the upper limit of gastric folds.

Histological processing was performed in the Institute of Pathology in Prishtina and Skopje. Biopsies were taken from the mucosa changes at intervals of 2cm, and the samples separately from each level were processed in formalin and paraffin. Afterwards, a series of sections were cut and marked with haematoxylin-eosin, alcian- blue and Giemsa-colour. The same experienced expert pathologist reviewed all biopsy specimens. The pathologist was not aware of the treatment applied. The recognition of intestinal metaplasia by biopsy, especially goblet cells, **can be** facilitated with the use of alcian blue stain of pH 2.5.

Calculation of sample size was made based on the data for patients with BE in the GERD group patients, which was 10% in most of the studies. However, for higher security, the sample size was calculated as 15%.

The results were analysed with modern statistical methods. Data processing was performed with Instant 3 statistical package. Statistical parameters were calculated from the index structure, mean, standard deviation, minimum and maximum value. The test data used parametric t-test (for variables with normal distribution, values were shown as a mean and standard deviation, and the differences between them were tested by the arithmetic mean of the differences). The difference was considered significant if $P < 0.05$.

Results

The study included 50 patients with BE. The average age of patients in Group BE was 52.4 years (SD ± 10.8 yrs). In the group of patients with BE, most common age group was 50-59 years. In the group with BE, 78.0% were men.

Average body height of respondents in BE group was 174.8 cm (SD ± 8.2 cm), although 60% of BE patients were smokers. Patients in BE group smoked more cigarettes, in 60% of patients. Patients in BE group had higher Body mass index (29.5).

The duration of symptoms in BE group was 7.8 years. (SD ± 2.16 yrs). In the BE group, 40 (80%) patients had hiatal hernia, of which 29 or 74.4.0% were < 3 cm (SSBE), and 11 or 100% were > 3 cm (LSBE).

Our results for the presence of a hiatal hernia by endoscopic type of BE are presented in the following table. Although without significant difference, the presence of a hiatal hernia was greater in the subgroup with LSBE.

Table 1: The presence of a hiatal hernia by endoscopic type of BE

		A hiatal hernia		Total
		Yes	No	
SSBE	N	29	10	39
	%	74.4	25.6	100.0
LSBE	N	11	-	11
	%	100.0	-	100.0
Total	N	40	10	50
	%	80.0	20.0	100.0
Z-value		Z = 1.87, P = 0.607		

Circumferential extension in BE patients with SSBE before treatment was 1.5 cm (SD ± 0.7 cm), and after treatment was 0.8 cm (SD ± 0.1 cm). The T-test of mediocrity received the significant difference in circumferential spreading the SSBE group before vs after treatment (T = 7.0, P < 0.0001) (Table 2, Fig. 1 and Fig. 2).

Table 2: The effect of PPI treatment by Prague C & M classification in patients with BE

	Circumferential extent Median value ± SD		Maximal proximal extent Median value ± SD	
BE	Before	After	Before	After
SSBE				
n=39	1.5 ± 0.7	0.8 ± 0.1	2.3 ± 1.1	1.1 ± 0.9
T-test paired sample	T = 7.0, P < 0.0001		T = 5.97, P < 0.0001	
LSBE				
n=11	2.5 ± 1.1	1.4 ± 0.8	4.3 ± 1.9	2.3 ± 1.7
T-test paired sample	T = 5.71, P < 0.0001		T = 5.54, P < 0.0001	

Maximum proximal extension with this group before treatment was 2.3 cm (SD ± 1.1 cm), and after treatment 1.1 cm (SD ± 0.9 cm). The T-test of mediocrity received the significant difference in maximum proximal extension in the group before SSBE vs after treatment (t = 5.97, P < 0.0001).

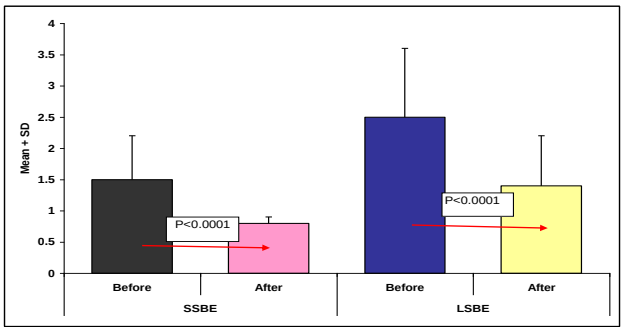

Figure 1: Maximal circumference of BE prior and after treatment with PPI based on Prague C & M

Circumferential extension in BE patients with LSBE before treatment was 2.5 cm (SD ± 1.1 cm), and after treatment was 1.4 cm (SD ± 0.8 cm). The T-test of mediocrity received the significant difference in the stretching group circumferential LSBE before vs after treatment (T = 5.71, P < 0.0001). Maximum proximal extension with this group before treatment was 4.3 cm (SD ± 1.9 cm), and after treatment 2.3 cm (SD ± 1.7 cm). The T-test of mediocrity received the significant difference in maximum proximal extension with LSBE group before vs after treatment (t = 5.54, P < 0.0001).

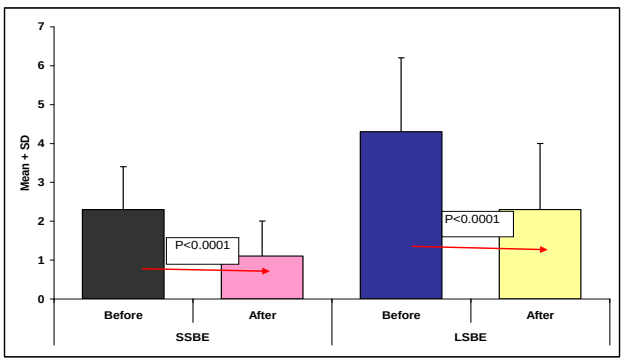

Figure 2: Maximal length of BE prior and after treatment with PPI based on Prague C & M

Discussion

Therapy with PPI showed that long-term safety reduces exposure to acid and prevents the development of dysplasia [8]. The results of our study may be useful to encourage all patients with Barrett's oesophagus to continue therapy with PPI, for long-term, to prevent dysplasia, even if they had symptoms or esophagitis. Furthermore, cases of Barrett's oesophagus can be divided according to the length of the segment of Barrett's oesophagus. BE patients with a short segment is defined as intestinal metaplasia of the distal oesophagus when the length is less than 3 cm. Disease with long segment refers to cases when intestinal metaplasia of the distal oesophagus is measured 3cm or more. Our results show that the BE circumferential changes in patients with SSBE before treatment was 1.5 cm (SD ± 0.7 cm), and after treatment has fallen to 0.8cm (SD ± 0.1 cm) ($P < 0.0001$), circumferential BE extension in patients with LSBE before treatment was 2.5 cm (SD ± 1.1 cm), and after treatment was 1.4 cm (SD ± 0.8 cm) ($P < 0.0001$). Maximum proximal extension in this group before treatment was 4.3 cm (SD ± 1.9 cm), and after treatment 2.3 cm (SD ± 1.7 cm) ($P < 0.0001$).

There are few studies in the literature on this topic. Sampliner [9] studied 27 patients treated for almost 3 years with lansoprazole 60 mg daily. There was no reduction in the segment of Barrett-esophagus, but 62% of patients developed islands in plate epithelium. In one study Sharma et al., [10] followed up 13 patients, for an average period of 5.7 years, who received lansoprazole 30 mg twice daily. There was no reduction of Barrett's oesophagus or complete control of acidity in the oesophagus (documented by pH testing). Extensive study with 47 patients treated with different doses of omeprazole also showed the appearance of islets of plate epithelium, but not completely cut the length of the Barrett-'s segment of the oesophagus. A 12 months study conducted by Malesci et al., [11] showed a total reduction of 4.5 to 2.1 cm with a 12-month suppression of acidity. These data show the most impressive reduction in the length of Barrett's oesophagus to date and they are difficult to replicate.

Recently, Srinivasan et al., [12] reported about 9 patients who had complete control of oesophagal acidity using PPI twice daily, with a histamine blocker, at night. This treatment resulted in a slight decrease in the average length of Barrett's oesophagus, from 7.2 to 5.2 cm reaching statistical value ($P < 0.001$). Indeed, histamine blockers could not produce any islands of epithelium plate or reduced length of Barrett's oesophagus in these studies. In an extensive review of the literature concerning this topic, only 3 patients described complete withdrawal of Barrett's oesophagus following treatment with PPI.

Interestingly, the short-segment disease is at least 3 times more prevalent than long-segment disease [13] [14] [15], and the length of the long segment is associated with greater exposure to acid [16]. But once Barrett's oesophagus is present its overall length does not change, so the disease with a short segment normally remains short [17]. The degree of dysplasia is directly related to the length of the segment [18] [19]. However, variants with a long or short segment of Barrett's oesophagus associated with the development of dysplasia and adenocarcinoma regarding monitoring and endoscopic treatment are both treated similarly [20].

In conclusion, the age group of 50-59 years, male gender, smokers and alcohol consumer are important predictive factors for the development of BE among patients with GERD. Obesity, age, male gender, smoking is also an important factor in the development of the BE, so far as it is represented. A hiatal hernia is confirmed to be an important factor in the occurrence of Barrett's oesophagus since it was present in a high rate in both endoscopic types (74% in SSBE and 100% in LSBE). We confirmed that PPI therapy is useful for long-term safety and the regression of macroscopic changes of BE due to reduced exposure of the distal oesophagus to gastric acid.

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Clinical Science

Influence of the Type and Amount of Liver Resection on the Survival of the Patients with Colorectal Metastases

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Abstract

INTRODUCTION: Colorectal liver metastases have a poor prognosis, and only 2% have an average 5-year survival if left untreated. Despite radical resection, the average five-year survival is between 25% and 44%.

AIM: To explore the experience of the Clinic in the treatment of colorectal liver metastases, comparing it with data from the literature and based on the comparison to determine the influence of the type and extensity of resection survival after radical surgical treatment of patients.

METHODS: This is a retrospective study. The study comprised the period between 01.01.2006 to 31.12.2015. It included a total of 239 cases, of whom: 179 patients underwent radical interventions, 5 palliative and 55 patients underwent explorative interventions due to liver metastases.

RESULTS: Radical resection of liver metastases has the impact of the patient survival, and the survival is the smallest in the patients with left hemihepatectomy and the longest in the patients with bisegmentectomy. But no specific technique and the number of resected segments influenced the survival of patients with colorectal liver metastases.

CONCLUSION: In patients with colorectal liver metastases only resection has potentially curative character. The type and amount of liver resection has no influence of the survival.

Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide after lung cancer and breast cancer [1] [2]. A large percentage of 50-70% of patients develops colorectal liver metastases (CRLM) because of hematogenous dissemination of primary cancer [3] [4] [5] [6] [7]. Synchronous metastases are diagnosed in 15-25% [8] [9] [10] during the primary diagnosis of CRC and in 20-25% [11] [12] [13] [14] [15] in the first five years metachronous metastases develop. They represent the most common cause of death so that 77% of untreated patients die in the first year, and only 14-23% survive more than three years [16] [17] [18] [19]. Surgical resection represents the only curative treatment approach to patients with CRLM; in larger series patients treated with resection have a mean 5-year survival from 25% to 44% [15] [20] [21], but only 15-25% [22] of metastasis of liver are initially resectable. Poor prognosis of the disease is the cause of looking for opportunities to improve postoperative results which correspond with defining determinants of survival.

To explore our experience in the treatment of colorectal liver metastases (CRLM), comparing them with data from the literature, and based on the comparison to determine the influence of type and amount of resection of the survival after radical surgical treatment of patients.

Material and Methods

A retrospective study was conducted at the Clinic of General and Hepato-pancreatic surgery at the University Hospital "Aleksandrovska" – Sofia, Bulgaria. The study comprised the period between 01.01.2006 to 31.12.2015. It included a total of 239 patients, of whom: 179 patients (74.9%) underwent radical interventions (atypical resection - 57, resection of 2 segments - 24, resection of 3 segments - 18, resection of >3 segments - 10, left lobectomy - 15, left hemihepatectomy - 4, right hemihepatectomy - 12, metastasectomy - 20, resection with another procedure - 19, atypical resection and metastasectomy - 9, left lobectomy and atypical resection - 5, atypical resection and alcoholization - 1, atypical resection and thermoablation - 4; and 5 palliative and 55 patients underwent explorative interventions due to liver metastases (biopsy - 55, biopsy and biliary drainage - 2, thermoablation - 1, alcoholization - 2). Also, 119 (49.8%) patients were diagnosed with synchronous metastases, 120 (50.2%) patients with metachronous metastases, including 7 (2.9%) with metachronous metastases with recurrence on the colon.

The follow-up period of the patients operated on for colorectal liver metastases in the Clinic was 5 years after resection of the liver.

The study included all patients with liver metastases from colorectal cancer regardless of their age and gender;

The study included all patients with liver metastases from colorectal cancer: synchronous metastases, metachronous metastases and metastases appearing with local recurrence of cancer;

The endpoints were to determine the following:

1. Survival depending on the type of surgical intervention: radical surgery or the palliative surgery or biopsy;

2. Cumulative overall survival depending on the type of the surgical intervention: radical surgery or the palliative surgery or biopsy;

3. To assess whether the specific type of radical hepatic resection has an influence on mortality (atypical resection, 2 - segment resection, resection of more than 3 segments, left - lobectomy, left hemihepatectomy, right hemihepatectomy, metastasectomy, combined liver surgery) as well as on median survival;

4. To assess whether the specific type of palliative intervention (biopsy, thermoablation, alcoholization) has an influence on mortality as well as on survival;

5. To assess whether the volume of liver resection (small and large resection) has an influence on mortality as well as on median survival.

Statistical analysis of the collected material to determine the factors for survival was done using the SPSS-19 statistical program.

Results

The average survival of patients undergoing radical intervention is about three times longer than that of palliative care. The Logging and Breslow statistical tests, as well as the regression analysis, confirm that the type of operative intervention involved is a significant factor in the survival of patients with this pathology (Figure 1).

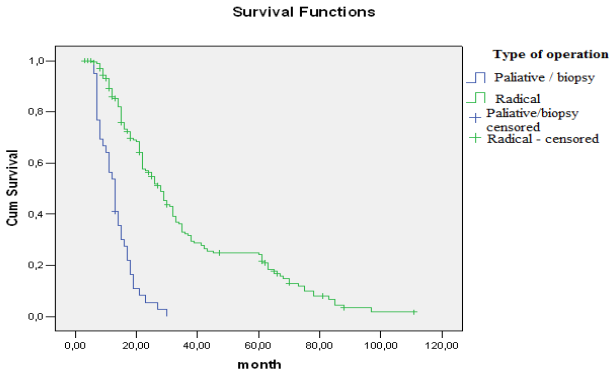

Figure 1: Survival curves depending on the type of surgical intervention; Log Rank (Mantel-Cox) p < 0.0001; Breslow p < 0.0001

Patients undergoing radical surgery are associated with a 79.7% lower risk of lethal outcome than patients with palliative care or patients who underwent biopsy (Table 1, 2).

Table 1: Cumulative overall survival depending on the type of the surgical intervention

Type of operation	Deaths	Cumulative survival % (Std.Error)		
	N (%)	1-year	3-year	5-year
Radical	131 (78.44)	89.2 (0.028)	32.4 (0.04)	24.1 (0.03)
Palliative / biopsy	38 (92.68)	53.8 (0.08)	0	0

To assess whether the specific volume and type of radical hepatic resection influence survival, we analysed the data from the patients we follow.

Table 2: Cox regression analysis according to the applied surgical intervention

	p	Exp (B)	95% CI for Exp (B)
Reference category-palliative intervention/biopsy			
Radical intervention	<0.0001	0.203	0.135-0.306

The distribution of established deaths for patients over the study period treated with radical

surgery depending on the type of resection is as follows: - 64.15% mortality in patients with atypical resection (34/53 patients); - 68.18% in the 2-segment resection group (15/22 patients); - 100% mortality was recorded in resection of more than 3 segments (8/8 patients); - 86.67% of left-lobectomy patients died in the follow-up (13/15 patients); - 100% of those with left hemi-hepatectomy (3/3 patients); - 91.67% of patients with right hemi-hepatectomy (11/12 patients) and 85% of cases with metastasectomy (17/20 patients).

In patients undergoing combined liver surgery, 83.33% of the patients were with fatal outcome.

Survival analysis revealed that median survival was lowest in the left hemi-hepatectomy group (about 14 months), and the highest in patients with two-segment resection (48 months).

Table 3: Mean and median survival based on the type of liver resection applied

Type of resection	Mean and median survival					
	Mean	Std.err	95% CI	Median	Std.err	95% CI
Atypical resection	40.565	4.026	32.675-48.456	35.0	6.525	22.211-47.789
Resection of 2 segments	48.229	7.39	33.744-62.714	36.0	11.38	13.69-58.31
Resection of 3 segments	28.125	5.204	17.924-38.326	22.0	14.0	0.000-49.44
Resection of more than 3 segments	31.125	7.391	16.639-45.611	28.0	11.314	5.825-50.175
Left lobectomy	32.214	5.451	21.53-42.898	26.0	1.852	22.371-29.629
Left hemi-hepatectomy	13.667	1.333	11.053-16.28	15.0	0.0	
Right hemi-hepatectomy	24.476	2.455	19.665-29.287	22.0	3.143	15.839-28.161
Metastasectomy	40.047	7.076	26.178-53.915	32.0	8.876	14.603-49.397
Resection + other procedure	26.063	4.093	18.041-34.085	26.0	2.91	20.297-31.703

This difference is statistically significant (P = 0.004, P = 0.043), but when comparing all methods used, no "best" surgical method for treating CRLMs (Table 3, Figure 2).

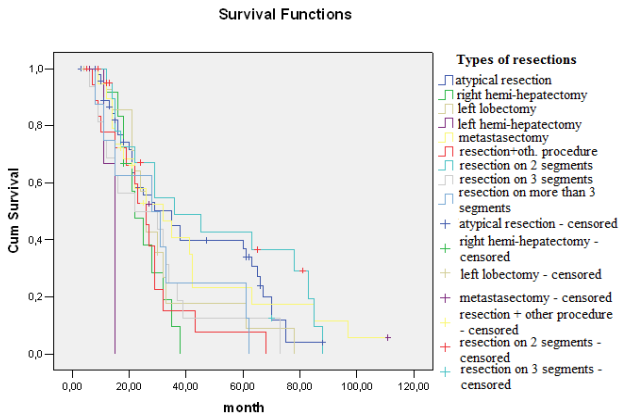

*Figure 2: Survival curves depending on the type of resection; Log Rank (Mantel-Cox) P = 0.004**; Breslow P = 0.043*; *P < 0.05; **P < 0.01*

In regression analysis, none of the operative techniques showed an advantage as a predictor of survival (Table 4).

Table 4: Cox regression analysis according to the type of liver resection applied

Type of resection	p	Exp (B)	95% CI for Exp (B)
Reference category – other types of resection			
Atypical resection	0.237	0.788	0.531-1.169
Resection of 2 segments	0.053	0.583	0.337-1.006
Resection of 3 segments	0.28	1.348	0.784-2.316
Resection of more than 3 segments	0.378	1.383	0.673-2.839
Left lobectomy	0.524	1.206	0.678-2.147
Right hemi-hepatectomy	0.114	1.661	0.885-3.116
metastasectomy	0.299	0.755	0.444-1.284
Resection + other procedure	0.113	1.555	0.901-2.685

The mortality analysis after a palliative intervention or biopsy showed that in this group the mortality was 94.87% of the patients. Only the patient who has been subjected to thermoablation is alive from the group of palliative patients. The Kaplan-Meier method of survival was examined according to the type of palliative intervention performed in figure 3.

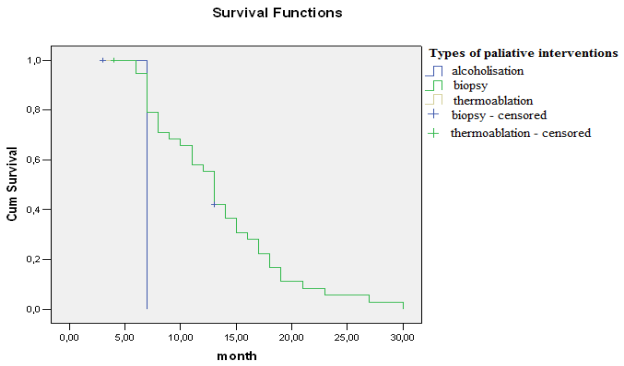

Figure 3: Survival curves depending on the type of palliative intervention

As can be seen from the graph and the additional regression analysis (Table 5), the application of biopsy or palliative intervention due to the inability to perform a radical intervention in CRLM patients significantly degrades long-term treatment outcomes. The fact that adjuvant therapy has been administered in most of these patients, and yet the average survival rate is significantly lower than that of the radically-operated patients, again proves the key role of surgical resection in the treatment of these patients.

Table 5: Cox regression analysis using palliative methods of treatment

Type of palliative operations	p	Exp (B)	95% CI for Exp (B)
Reference category-other palliative interventions			
Biopsy	0.153	0.22	0.027-1.756

Survival analysis was also performed according to the volume of surgery. In the group of patients undergoing large liver resection, 87 people (78.38%) died on long-term follow-up.

Table 6: Mean and median survival based on the volume of liver resection

Volume of the operation	Mean and median survival					
	Mean	Std.err	95% CI	Median	Std.err	95% CI
Big	31.042	2.043	27.037-35.047	26.0	1.84	22.393-29.607
Small	31.283	3.078	25.249-37.316	18.0	1.515	15.03-20.97

A fatal outcome was found in 82 (84.54%) patients with a small volume of hepatic intervention. The average survival in both groups is about 31 months (Table 6, Figure 4).

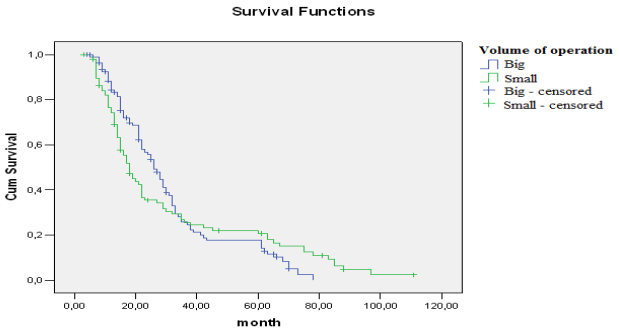

Figure 4: Survival curves depending on the volume of liver resection; Log Rank (Mantel-Cox) P = 0.796; Breslow P = 0.12

The additional analysis of the results (Cox regression) did not establish a significant correlation between the volume of surgical intervention according to the number of resected liver segments and the survival in patients with CRLM (Table 7).

Table 7: Cox regression analysis (Univariate analysis) concerning the volume of liver resection

Volume of operation	p	Exp (B)	95% CI for Exp (B)
Reference category-Big			
small	0.8	1.041	0.762-1.424

Discussion

This retrospective study showed a significant difference in the survival of patients operated radically. Survival of patients OS operated with non-anatomical resection NAR is no different from the survival of patients treated with AR. Also, the difference in patient survival is identified as significantly larger in the group of small resections, but this does not set the number of resected segments as a survival factor. Anatomical resection provides better cleaning between tumor deposits and the liver transaction line and is recommended as a standard procedure for metastatic liver tumor [24]. Non-anatomical resection is useful in small metastases, with little risk of microscopic local invasion [23]. Non-anatomical resection NAR has become more commonly used in view of the possibility of storing a larger volume of hepatic parenchyma, but the NAR compared to AR is associated with a higher incidence of positive resection ranges (R1 resection) [25]. Consensus has been widely accepted that a positive surgical margin is a powerful predictor of patient survival and recurrence [32] [33] [34]. As has been reported, the rate for five-year survival ranges only from 17.1% to 20% for patients with positive margins compared with that ranging from 37% to 63.8% with negative margins [32] [33]. As to median survival, the median length was 23 months for patients with positive margins, less than 45 months with negative margins [33]. Besides, overall recurrence rates were significantly different between patients with positive margins and with negative margins (51.1% and 38.6%, respectively) [33]. Studies show that the type of resection (anatomic or non-anatomic) is irrelevant for postoperative morbidity and mortality, with both negative histological margins (R0 resection) being achieved [25] [26] [27] [28] [29]. The use of non-anatomical resection has certain advantages such as lower blood loss, significant shorter operating times and shorter duration of hospital treatment [25] [26] [28]. As previously reported [35] [36] [37], AR featuring higher level of surgical technique difficulty would often be associated with longer operation duration and more liver parenchyma loss. Bile leakage, wound infection and intra-abdominal collections constituting the major types of complications all show evident preferences to AR group over NAR group. Taken together, AR might promote the incidence of postoperative morbidity. The main cause for the inferiority of AR to NAR in terms of mortality is thought to be its larger loss of liver parenchyma. With more extensive parenchymal resection, AR would consequently carry a more substantial risk. As reported by Lalmahomed ZS [28], postoperative hepatic failure resulting from insufficiency of liver remnant was the primary cause of mortality in AR group. But the type of resection of the liver does not affect the survival of patients with CRLMs.

Zorzi et al., [26] report a 5-year survival rate of 61% in favour of the NAR and 60% in the case of AR. Guzzetti et al., [30] -5-year survival of 29% for the NAR and 27% for AR. Many studies find that the type and extensity of the resection don't influence the survival, but blood loss is crucial as a factor of postoperative morbidity and mortality [31]. Also, other factors of survival such a type, number, size, and localisation correspond with the type and the extensity of resection of liver and also have the implication of long-term survival. To conclude that the patients treated with NAR and AR have similar OS survival of the patients with colorectal liver metastases. Also, the amount of the liver resection sometimes is crucial for the surgical strategy, and it has implications for the postoperative morbidity and mortality, but it isn't a factor of long-term survival.

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Clinical Science

Noninvasive Biomarkers in Assessment of Liver Fibrosis in Patients with HBeAg Negative Chronic Hepatitis B

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Abstract

BACKGROUND: Liver biopsy for evaluation of liver fibrosis has several adverse effects, for which reason noninvasive tests have been developed.

AIM: To evaluate the usefulness of noninvasive biomarkers, qHBsAg and HBV DNA levels in predicting liver fibrosis in patients with hepatitis Be antigen (HBeAg) negative chronic hepatitis B (CHB).

MATERIAL AND METHODS: This prospective study included 50 patients with HBeAg negative CHB. All patients underwent laboratory and serology testing, quantification of HBV DNA and HBs antigen. The liver stiffness was measured with elastography. The patients were analysed for APRI and FIB-4, quantitative hepatitis Bs antigen and HBV DNA.

RESULTS: Logistic regression analysis showed that greatest significance in predicting liver fibrosis has FIB-4 (Wald = 3.24, P = 0.07), followed by HBV DNA ≥ 2 000 IU/ml ≤ 20 000 IU/ml (Wald = 2.86, P = 0.09), qHBsAg (Wald = 2.17, P = 0.14), HBV DNA > 20 000 IU/ml (Wald = 0.58, P = 0.45), APRI (Wald = 0.04, P = 0.84).

CONCLUSION: the FIB-4 index has the greatest value in predicting liver fibrosis while APRI has the lowest; the more advanced liver disease is associated with lower serum level of quantitative HBs antigen. Combination of noninvasive blood biomarkers and imaging tests can provide better diagnostic accuracy and exclude the need for liver biopsy.

Introduction

Chronic infection with hepatitis B virus (HBV) is a global health problem, with over 350 million people worldwide affected by it, remaining the predominant cause of chronic liver disease and liver-related morbidity worldwide. This clinical condition is considered to be the major risk factor for cirrhosis, end-stage liver disease and hepatocellular carcinoma (HCC) [1] [2] [3]. Hepatitis Be antigen (HBeAg) negative chronic hepatitis B (CHB) is characterised by fluctuating levels of hepatitis B virus deoxyribonucleic acid (HBV DNA) and aminotransferases, with temporary remissions during the disease [4]. HBeAg-negative CHB patients with active hepatic necrotic inflammation and persistent viraemia have higher rates of complications in contrast to HBeAg-negative patients with CHB who are inactive carriers. Both forms of CHB have similar laboratory and serologic characteristics and are not always easy to distinguish [5]. Assessment of liver fibrosis and its timely detection is essential for evaluation of liver disease severity. This is of particular importance for decision making and starting antiviral therapy and consequently preventing the development of CHB caused complications [4] [5] [6] [7].

Liver biopsy is the standard gold method for assessing the stage of the liver diseases. It is an invasive procedure, associated with pain and complications, where accurate results rely not only on the tissue sample quality and size but also of the

pathologists' experience [8]. Recently noninvasive methods for predicting liver fibrosis as well as imaging techniques, including transient elastography (TE), ultrasonography (US), computed tomography (CT) and magnetic resonance imaging (MRI) have been developed [4][9]. Serum markers for assessing liver fibrosis and cirrhosis can be used individually, but most of the times are combined to achieve better diagnostic sensitivity and specificity. Direct markers are representative of liver fibro-genesis and include glycoproteins, collagens, collagenases and collagenase inhibitors. Indirect markers reflect liver damage and correlate with liver fibrosis. They include platelet (PLT) count, aspartate transaminase (AST) and alanine transaminase (ALT), globulin level, serum quantitative HBs antigen (qHBsAg), ceruloplasmin, TGF-α, red blood cell distribution width, and serum Golgi protein 73 (GP73) [6].

Fibrosis index (FIB-4) is based on the four factors: evaluation of age, AST, platelets and ALT. Moreover, the FIB-4 index has been used to evaluate significant fibrosis and liver cirrhosis in HBV-infected patients in numerous studies [10] [11]. Concerning aspartate transaminase-to-platelet ratio index (APRI), it was first developed in the study of patients with chronic HCV infection [12], but it has been concluded that has moderate sensitivity and accuracy when it comes to HBV related fibrosis [13] [14]. Evaluation of the level of quantitative hepatitis B surface antigen (qHBsAg) reflects the amount of transcriptional activity of cccDNA and the integrated DNA in the hepatocytes [15] [16] representing one of the main serologic markers in chronic HBV infection; accurately monitoring both disease progression and prognosis as well as response to antiviral therapy [17] [18]. In this context, several studies have observed the correlation between quantitative HBsAg and liver fibrosis indicating their mutual correlation [19] [20] [21] [22].

Moreover, serum HBV DNA levels directly reflect the degree of HBV replication and are considered a strong prognostic indicator for CHB infection. Increasing HBV DNA levels correlate with the higher rate of progression to cirrhosis, the incidence of hepatocellular carcinoma (HCC), and subsequent death from HCC or chronic liver disease [23]. However, high HBV DNA levels do not always predict significant hepatitis [23].

Lastly, transient elastography (TE) is performed to measure the speed of the shear wave which is directly associated with the liver stiffness. TE measures the liver stiffness (LS) which by itself is associated with the degree of fibrosis [24] [25].

In this study, we evaluated the usefulness of noninvasive biomarkers FIB-4, APRI, quantitative hepatitis B antigen and HBV DNA for prediction of liver fibrosis in patients with HBe antigen negative chronic hepatitis B.

Material and Methods

A prospective, non-randomized study was conducted at the University Clinic for infectious diseases and febrile conditions which included fifty patients with hepatitis Be negative antigen CHB. The inclusion criteria were: age over 18 years, patients with serologically confirmed chronic hepatitis B, patients who were hepatitis B e antigen negative. All patients have been examined at least twice, with a minimum follow-up period of at least 6 months. All patients signed informed consent. The exclusion criteria were co-infection with human immunodeficiency virus (HIV), hepatitis A (HAV) and hepatitis C (HCV) as well as other liver diseases with different aetiology. Patients who had received antiviral therapy, patients who are currently on antiviral therapy, patients with hepatocellular carcinoma (HCC) and patients with liver failure were not included in the study.

All patients underwent standard laboratory and serology testing. We evaluated APRI and FIB-4 score as well as the values of aspartate transaminase (AST), alanine transaminase (ALT), qHBsAg, HBV DNA and liver fibrosis.

Relevant clinical variables were age, platelet count, ALT, AST, HBsAg, hepatitis B e antigen, HBV DNA, and fibroscan. The value of ALT, AST, qHBsAg and HBV DNA were expressed in IU/ml.

Quantification of HBV DNA levels in the plasma was performed by real-time polymerase chain reaction (RT-PCR) on COBAS AmpliPrep COBAS TaqMan HBV test and Abbott m 2000 sp/m 2000 rt with a lower detection limit of 10 IU/mL.

The serum level of HBsAg (qHBsAg) was quantified with Architect HBsAg assay (Abbott Laboratories) according to the manufacturers' protocol. The detection level of HBsAg varies from 0.05 to 250 IU/ml. Sera with HBsAg level higher than 250 IU/ml were diluted 1:500.

Abdominal ultrasound, as well as transient elastography, was performed on all patients. The liver stiffness was measured with transient elastography (TE); fibroscan (EchoSens^{R}, Paris, France) and expressed in kilopascals (kPa). The mean value was obtained from 10 performed measures, with success rate more than 60% and interquartile range (IQR) < 0.25.

Aspartate transaminase-to-platelet ratio index (APRI) was calculated with the following formula: ((AST/ULN AST) x100)/Platelets (10^9/L)). APRI score greater than 1.0 has a sensitivity of 76% and specificity of 72% for predicting cirrhosis. APRI score greater than 0.7 has a sensitivity of 77% and specificity of 72% for predicting significant hepatic fibrosis. APRI > 1.5 is the cut-off value for significant fibrosis, whereas a score <0.5 can rule it out [26].

The fibrosis index (FIB-4) is based on the four factors and calculated by the following formula: Age (yr.) xAST (IU/ml)/PLT (x10^9/L) x ALT (IU/ml) 1/2. A FIB-4 score <1.45 has a negative predictive value of 90% in patients with advanced fibrosis. FIB-4 score >3.25 has a 97% specificity and a positive predictive value of 65 % of patients [10] [11].

All data were processed using a statistical computer program Statistica 7.1 for Windows and SPSS Statistics 17.0. For a description of the numerical variables descriptive statistics ((Mean; Std. Deviation; ± 95, 00%CI; Minimum; Maximum) was used, where frequencies and percentages were used for the description of the categorical variables.

To identify the predictive values for FIB-4, APRI, qHBsAg and HBV DNA for fibrosis, logistic regression analysis (Wald, Exp (B), 95, 0% CI for Exp (B), and p) were used. For all analyses, the P-value < 0.05 was considered statistically significant.

Results

A total of 50 chronic hepatitis B treatment naïve HBeAg-negative patients were included in the study. There were 26% female and 74% male patients. The patients' age ranged from 19 to 67 years. The platelet count ranges between the intervals from 104 to 344 x 10^9/L.

The mean values of ALT vary in the interval 44.42 ± 42.63 IU/ml, and AST value varies in the interval of 29.88 ± 18.62 IU/ml. Serum qHBsAg values vary in the interval of 6143.21 ± 9372.24 IU/ml; the level of HBV DNA vary in the interval of 492303.2 ± 1642234 IU/ml (Table 1).

Table 1: Statistical analysis of the patients from the cohort

	Valid N	Mean	Confidence -95.00%	Confidence +95.00%	Minimum	Maximum	Std.Dev.
Age	50	38.820	35.754	41.886	19.0000	67.00	10.787
Tr	50	208.240	194.391	222.089	104.0000	344.00	48.731
ALT	50	44.420	32.304	56.536	10.0000	210.00	42.631
AST	50	29.880	24.589	35.171	13.0000	103.00	18.616
qHBsAg	50	6143.205	3479.644	8806.767	0.0500	55513.63	9372.241
HVD DNA	50	492303.2	25585.48	959020.9	24	10451113	1642234

Abbreviations: Tr: thrombocytes, ALT: alanine aminotransferase, AST: aspartate aminotransferase, qHBsAg: quantitative hepatitis B antigen, HBV DNA: hepatitis B virus deoxyribonucleic acid

Stratification of the patients according to the level of HBV DNA and qHBsAg showed that 50% of the patients had HBV DNA level lower than 2 000 IU/ml, 22% had HBV DNA more or equal to 2 000 IU/ml and lower or equal to 20 000 IU/ml, and in 28% HBV DNA level was higher than 20 000 IU/ml, while 36% patients had qHBsAg level lower or equal to 1 000 IU/ml and in 64% the value of qHBsAg was higher than 1000 IU/ml (Table 2).

Table 2: Frequency table (%) for HBV DNA, qHBsAg, fibroscan, APRI, FIB-4

HBV DNA	Number	Cumulative Number	%	Cumulative %
HBV DNA <2000 IU/ml	25	25	50.00	50.00
HBV DNA ≥2000≤20 000IU/ml	11	36	22.00	72.00
HBV DNA >20 000 IU/ml	14	50	28.00	100.00
Missing	0	50	0.00	100.00
qHBsAg	Number	Cumulative Number	%	Cumulative %
qHBsAg ≤1000 IU/ml	18	18	36.00	36.00
qHBsAg>1000IU/ml	32	50	64.00	100.00
Missing	0	50	0.00	100.00
Fibroscan	Number	Cumulative Number	%	Cumulative %
f0/f1	42	42	84.00	84.00
f2/. f2/f3	5	47	10.00	94.00
f3.f3/f4. f4	3	50	6.00	100.00
Missing	0	50	0.0	100.00
APRI	Number	Cumulative Number	%	Cumulative %
APRI>0.7	4	4	8.00	8.00
APRI<0.7	46	50	92.00	100.00
Missing	0	50	0.00	100.00
FIB-4	Number	Cumulative Number	%	Cumulative %
FIB 4<1.45	44	44	88	88
FIB4>1.45<3.25	5	49	10	90
FIB 4>3.25	1	1	2	100.0
Missing	0	50	0.00	100.00

Abbreviations: HBV DNA: hepatitis B virus deoxyribonucleic acid, qHBsAg: quantitative hepatitis B antigen, APRI: Aspartate transaminase-to-platelet ratio index, FIB-4: Fibrosis index based on the four factors

Liver stiffness measured with transient elastography showed that 84% patients had no fibrosis (f0/f1), 10% had intermediate fibrosis (f2 and f2/f3), while significant fibrosis (f3, f3/f4 and f4) was detected in 6% of the patients (Table 2).

Aspartate transaminase-to-platelet ratio index (APRI score) greater than 0.7 was observed in 8% of the patients, while in 92% of them was lower than 0.7. For detecting significant fibrosis, APRI scores greater than 1.5 was not observed in any patient, while APRI scores lower than 0.5 was found in 42 (84%) of the patients (Table 2).

Fibrosis index (FIB-4) based on the four factors showed that 88% patients included in our study had a FIB-4 score < 1.45, and 2% had a FIB-4 score > 3.25 (Table 2).

The predictive values of APRI, FIB-4, qHBsAg and HBV DNA were evaluated for fibrosis, using the model of discrimination. The global accuracy of this model in predicting fibrosis is 90.00% with a sensitivity of 55.60% and specificity of 97.60% (Table 3).

Table 3: Model of discrimination -Prediction of fibrosis with APRI, FIB-4, HBsQ, HBsQ, HBV DNA

			Predicted		
			Fibrosis		Percentage Correct
	Observed		Absence	Presence	
Step 1	Fibrosis	Presence	40	1	97.6
		Absence	4	5	55.6
	Overall Percentage				90.0

a. The cut value is 500; The global accuracy of this model for predicting liver fibrosis is 86, 00%. Sensitivity is 44.40%, and specificity is 95.10%

Table 4 reports the data obtained through logistic regression analysis which showed that FIB-4 has the greatest significance in this model (Wald = 3.24, P = 0.07), followed by intermediate high level of HBV DNA ≥ 2 000 IU/ml ≤ 20 000 IU/ml (1) (Wald =

2.86, P = 0.09), qHBsAg (Wald = 2.17, P = 0.14), HBV DNA >20 000 IU/ml (1) (Wald = 0.58, P = 0.45), while APRI has the lowest prediction for liver fibrosis, (Wald = 0.04, P = 0.84).

Logistic regression analysis showed that the increase of FIB-4 for one single cut-off value enhances the probability for fibrosis for 9.34 (Exp (B) =9.34)/(834%) insignificant in 95% CI for EXP (B): 0.82-106.54, P > 0.05. Evaluation of the level of HBV DNA showed that patients with intermediate values of HBV DNA level between 2 000 IU/ml and 20 000 IU/ml compared to patients with low values of HBV DNA (< 2 000 IU/ml) have 10 times more probability for liver fibrosis, (Exp(B) = 10.38) 95% CI for EXP (B): 0.69-156.17, P > 0.05. Patients with qHBsAg level more than 1 000 IU/ml compared to patients with qHBsAg level of lower or equal than 1000 IU/ml have 0.15 (Exp(B) = 0.15) times lesser probability for liver fibrosis insignificant in 95% CI for EXP(B): 0.01-1.87, P > 0.05. Patients with high HBV DNA level (>20 000 IU/ml) compared to patients with low HBV DNA level (<2 000 IU/ml) have 3.50 times more probability for liver fibrosis, insignificant for 95% CI for EXP (B): 0.14-88.72, P > 0.05 (Table 4).

Table 4: Assessment of the logistic regression model

								95% CI for EXP(B)	
		B	S.E.	Wald	df	Sig.	Exp(B)	Lower	Upper
Step 1[a]	APRI	-0.46	2.30	0.04	1	0.84	0.63	0.01	57.75
	FIB-4	2.23	1.24	3.24	1	0.07	9.34	0.82	106.54
	qHBsAg	-1.88	1.28	2.17	1	0.14	0.15	0.01	1.87
	HBV DNA ≥2 000 IU/ml ≤ 20 000 IU/ml	2.34	1.38	2.86	1	0.09	10.38	0.69	156.17
	HBV DNA >20 000 IU/ml	1.25	1.65	0.58	1	0.45	3.50	0.14	88.72
	Constant	-3.85	1.23	9.85	1	0.002	0.02		

a. Variable(s) entered on step 1: APRI. FIB-4. qHBs. HBV DNA ≥2 000 IU/ml ≤ 20 000 IU/ml. HBV DNA >20 000 IU/ml; Abbreviations: APRI : Aspartate transaminase-to-platelet ratio index. FIB-4: Fibrosis index based on the four factors. qHBsAg: quantitative hepatitis B antigen. HBV DNA: hepatitis B virus deoxyribonucleic acid.

Analysis of APRI index showed that the increase of APRI score for one single cut-off value decreases the probability for liver fibrosis for 0.63 (Exp (B) = 0.63)/(37%) insignificant for 95% CI for EXP (B): 0.01-57.75, P > 0.05 (Table 4).

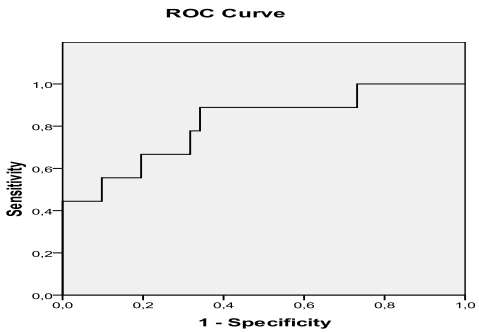

Figure 1: Area in the ROC curve model for fibrosis is 0.840 (95% CI 0.697-0.984), predicting the greater probability for fibrosis in 84% of all possible pairs of patients in which one has fibrosis, and the other does not

In the analysis of the area of the receiver operating curves (ROC) evaluating all four noninvasive biomarkers, the value of 0.840 means that in 84% of all possible pairs of patients where one has fibrosis, and the other pair is without fibrosis, this model will have higher predictive probability for fibrosis (Figure 1).

Discussion

The data from our study indicate that the greatest probability for distinguishing fibrosis in patients with HBeAg-negative CHB has a FIB-4 index. The clinical significance and applicability of this index is based on the following observations: the progression of the liver disease is age-related, and the disease duration is proportional with severe fibrosis; advanced fibrosis leads to mitochondrial injury of the liver cells and greater elevation of AST; more advanced fibrosis is associated with thrombocytopenia due to secondary hypersplenism and decreased production of thrombopoietin by liver cells [12].

Our study revealed that FIB-4 has the greatest significance in predicting liver fibrosis and that the increase of FIB-4 index for one single cut-off value increases the probability for fibrosis for 9 folds. The studies performed by Kim [10], as well as the study of Ma [14] showed that FIB-4 can be suitable for distinguishing significant and extensive fibrosis in patients with chronic hepatitis B. The Kim's study showed that AUROC's area of FIB-4 for predicting significant fibrosis, severe fibrosis and cirrhosis were 0.865, 0.910 and 0.923, respectively. The study conducted by Ma [14] also found that FIB-4 and Lock's model was the most effective models for distinguishing significant fibrosis in patients with chronic hepatitis B. A meta-analysis performed by Yin showed that FIB-4 has relatively high diagnostic value for detecting liver fibrosis in patients with hepatitis B when the diagnostic threshold value was more than 2.0. Similar to these studies, our report shows that patients, who had significant fibrosis, have about 9.3 fold greater chance of being FIB-4 positive (above 1.45) compared to patients without significant fibrosis.

Analysis of the impact of serum level of HBV DNA showed that intermediate high serum level of HBV DNA compared to high HBV DNA viraemia has higher significance in predicting liver fibrosis. Patients with HBV DNA levels ranging between 2 000 IU/ml and 20 000 IU/ml have 10 times more probability for liver fibrosis compared to patients with HBV DNA levels less than 2000 IU/ml, while patients with HBV DNA levels above 20 000 IU/ml have 3.50 times more probability for liver fibrosis compared to patients with HBV DNA level lower than 2000 IU/ml. Comparatively,

the study by Croagh [27] found that HBV DNA level was a predictor of significant fibrosis in HBeAg-negative CHB patients with varying ALT with an OR of 1.3 for every 1 log increment. HBV DNA levels also correlated with advanced fibrosis in HBeAg-negative CHB patients with normal ALT and varying ages as reported in the study of Xiao et al., [28]. In contrast, Shao reported that HBV DNA levels had no significant statistical association with liver histology regardless of HBeAg status [29]. It is known that HBV itself is not directly cytopathic and host immune response plays a pivotal role in HBV-related liver diseases [2] [30]. The role of HBV DNA in correlation with liver histology in HBeAg negative patients remains controversial because different methods and assays have been used in different studies [31]. Zacharakis et al., [32] reported that it is beneficial to follow HBV DNA levels in CHB patients who are HBeAg negative, as the HBV DNA levels correlate with the progression of hepatic damage. According to the data obtained from the patients included in our study, the level of HBV DNA is associated with the progression of fibrosis. In our study, patients with low HBV DNA viraemia have 3.5 and 10 times lower chance for liver fibrosis compared to the patients with intermediate and high HBV DNA level, respectively.

Quantitative Hepatitis B surface antigen represents a marker of CHB related liver damage, and qHBsAg levels are linked with progression of liver disease in HBeAg-negative patients [33]. Our study shows that patients with a qHBsAg level higher than 1 000 IU/ml compared to patients with a qHBsAg level lower or equal to 1000 IU/ml have 0.15 times lower probability for liver fibrosis. Patients who have lower qHBsAg level are associated with a higher probability of liver fibrosis. Our finding is discordant with the findings from several studies which reported that lower HBsAg levels are found in “inactive carrier” patients rather than in HBeAg-negative patients with “active” chronic hepatitis B [34] [35]. It is found that HBsAg production is reflective not only of cccDNA transcriptional activity but also originates from the integrated DNA in hepatocytes [16] [17] [34].

In contrast, it has been shown that the presence of mutations within the pre-S/S region reduces HBsAg production [36]. Patients with more advanced liver disease and liver cirrhosis had more frequent changes in the pre-S/S regions. This could, therefore, explain the lower total levels of qHBsAg in patients with advanced liver disease [34]. The study of Martinot-Peignoux [37] showed that there is a strong correlation between the stage of fibrosis and HBsAg level, but in HBeAg positive patients, while in patients with HBeAg negative CHB, qHBsAg was not found to be associated with any significant liver histologic changes. Unlike Martinot study, our study did not include patients with HBeAg positive CHB. Results from our study show that patients with high level of quantitative HBsAg (> 1000 IU/ml) have insignificant, but still, the lesser probability for liver fibrosis. This can be a result of the pre-S/S region mutation found in patients with CHB genotype D or as a result of decreased liver cell mass associated with more extensive fibrosis. Unfortunately, in our institution, there is no possibility to perform HBV genotyping and detecting mutations.

In patients included in our cohort, the observed APRI score showed that it has the lowest prediction for liver fibrosis. The increase of APRI score for one single cut-off value decreases the probability for liver fibrosis for 0, 63 folds. Our finding is consistent with findings from the other studies which showed that APRI test designed as the “perfect noninvasive model” to evaluate liver fibrosis, has only moderate sensitivity and accuracy for assessing HBV related fibrosis [38].

At present, accurate diagnosis of liver fibrosis is essential for the prevention of disease progression and treatment of chronic liver disease. In our study, we evaluated the association of noninvasive biomarkers FIB-4, APRI, quantitative hepatitis B antigen and serum level of HBV DNA in correlation with transient elastography. The appraised clinical parameters age, platelet count, aspartate transaminase alanine transaminase, ultrasound and fibroscan in detecting liver fibrosis, correlate with noninvasive biomarkers in predicting liver fibrosis. Overall, our study shows that FIB-4 has the greatest predictive value for liver fibrosis in our patients with hepatitis be negative antigen CHB.

In conclusion, a whole plead of noninvasive markers is available for the determination of fibrosis and monitoring the progression and regression of fibrosis in chronic HBV patients. It appears that a combination of blood and imaging tests can provide the highest diagnostic accuracy and exclude the need for liver biopsy. Our study shows that FIB-4 index has the greatest impact in predicting liver fibrosis and that more advanced liver disease is associated with lower serum level of quantitative HBs antigen. It will be a challenge to define the best clinical strategy on how to apply validated noninvasive tests in the management of patients with chronic HBV infection. The drawback of our study was that the sample size may have been too small, our incapacity for HBV genotyping and determining pre-S/S region mutations. Further studies involving a greater number of patients and combination of more noninvasive biomarkers are needed for better evaluation of the applicability of these markers in distinguishing liver fibrosis.

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Clinical Science

Apitherapy as a New Approach in Treatment of Palmoplantar Psoriasis

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Abstract

BACKGROUND: Apitherapy is the medical use of honey bee products, "honey, propolis, royal jelly, bee wax, and bee venom to relieve human ailments. Propolis is one of the most well-documented products derived from the honeybee and has always played an important role in traditional folk medicine.

AIM: The aim was to justify the consideration of Aloe Vera as an effective remedy for the treatment of psoriasis.

METHODS: The study follows (857) patients (354 females, 503 males) with a mean age range from (9 - 62) years, affected with moderate to severe psoriasis in palms and foot soles treated by a combination mixture of propolis and Aloe in the form of an ointment (Aloreed) and Beauty reed cream. The treatment duration was for 12 weeks. Results were evaluated by using clinical, histological and statistical parameters.

RESULTS: After the 12 - week treatment, we observed an 86% overall response rate from which 62% showed excellent results and 24% showed good results, therefore proving the efficiency in the use of the mixture of propolis 50% and aloe vera 3% as topically applied ointment in the treatment of mild to moderate psoriasis

CONCLUSION: Patients who have palmoplantar psoriasis, who were treated with a topically applied mixture of propolis (50%) and aloe vera (3%), have shown noteworthy improvement thus proving the efficiency of propolis and aloe vera in the treatment of mild to moderate psoriasis.

Introduction

Psoriasis is a genetic inflammatory and proliferative skin disease characterised by chronic, sharply bordered, dull - red, scaly plaques appeared on the skin and particularly on the extensor prominences and in the scalp area [1]. On the palms and soles, psoriasis may present as scaly white patches; a fine silvery scale can be evoked by scratching, as less well – defined plaques resembling lichen simplex or hyperkeratotic eczema - or as a pustulosis (chronic palmoplantar pustular psoriasis). A strong association between body mass index and psoriasis was shown [2].

There is no satisfactory or effective cure for psoriasis. However a variety of therapeutic modalities have yielded limited efficacy with frequent side effects. Available treatments include both local and systemic ones. *In vivo* and *in vitro* data prove the effectiveness of cytokines taken in low - doses [3] and antioxidants [4]. Complementary therapy based on psychotherapeutic approaches has also shown some efficacy [5].

Apitherapy is a therapy that uses hive products (e.g. propolis) for medical and pharmacological purposes [6]. It was found that propolis has versatile activities including physiological, antibacterial, antiviral, fungicide, local anaesthetic, antiulcer, immunostimulant and recently discovered

cytostatic activities. The principal components of propolis are flavonoids of group 5 (betulinol, quercetin, isovanillin), cafeic acids, unsaturated aromatic acid, and ferulic acids. Propolis is a very stable substance and has equal composition regardless of the geographic areas where it was produced. The composition of propolis indicates high antioxidant activity, and thus the substance can be investigated for the treatment of psoriasis.

Aloe vera is a plant that grows widely in the Egyptian desert. Its mucilaginous gel has been used in folk medicine for its effectiveness in itching, hair loss and burns. Aloe vera has also been proven to be effective in cutaneous wound healing [7]. Research has shown that active agents have considerable analgesic, antipruritic, wound healing and anti-inflammatory properties [8].

The aim was to justify the consideration of Aloe Vera as an effective remedy for the treatment of psoriasis.

Patients and Methods

The study follows (857) patients (354 females & 503 males) with a mean age range from (9 - 62) years, affected with a clinical diagnosis of mild to moderate psoriasis of palms and soles**.**

The subjects, afferent to the in the centre of dermatology in Heliopolis (Cairo - Egypt), were randomly allocated. Written consent was approved by all patients under this clinical trial, Patient information and progress was thoroughly documented during the study. Both treatment and follow - up was taken place in the centre of dermatology in Heliopolis from July 2006 to August 2008.

During the start and toward the end of the study skin biopsies were taken and stained with hematoxylin and eosin (H&E) for the evaluation of acanthosis, parakeratosis, thinning capillary dilatation, and inflammatory infiltration. A leading pharmaceutical company was responsible for preparation of the mixture (Propolis 50% with Aloe 3%) as an ointment [6].

Pre-treatment biopsy

A punch biopsy was taken before application of the treatment and stained by H&E for histological examination.

Treatment application

Patients were advised to rub the ointment into psoriatic palms and soles twice daily for 4 - 12 weeks.

Follow up

On a weekly basis reexamination and evaluation of the treated areas were done to control any side effects and to analyse the degree of patient satisfaction or inconvenience, follow up lasted for 12 weeks.

Post-treatment biopsy

At the end of the study, a punch biopsy was taken from the treated area to evaluate the efficacy of the treatment.

Post-treatment assessment

Assessment of the trial therapy depended on clinical state, photography and histopathology and was classified as follows:

1. Excellent response: when all psoriatic lesions disappeared, and skin became nearly normal (No erythema, no infiltration or desquamation of skin).

2. Good response: when some of the lesions disappeared.

3. Weak response: when no marked improvement was noted.

4. No response: when no improvement was seen.

For a standard method of clinical assessment of patients, Psoriasis Area and Severity Index (PASI score) was used. This clinical rating system assesses the area of the body affected by the intensity of the main symptoms.

To measure the % reduction of PASI Score, we applied the following equation:

% reduction of PASI score = Baseline PASI x PASI at the end of the program X 100 Baseline PASI

Result

After the 12 - week treatment, we observed an 86% overall response rate from which 62% showed excellent results and 24% showed good results, therefore proving the efficiency in the use of the mixture of propolis 50% and aloe vera 3% as topically applied ointment in the treatment of mild to moderate psoriasis [6].

Table 1: Comparison of the mean effective and non-effective

Variable	Effective Mean ± SD	Non-Effective Mean ± SD	t	P
% -reduction	N = 625 0.73 ± 0.26	N = 232 0.28 ± 0.033	** 5.57	< 0.01

** H. is significant.

Table 2: Comparison of the mean excellent and good effect

Variable	Excellent Effective Mean ± SD N = 531	Good Effective Mean ± SD N = 205	t **	P
% -reduction	0.87	0.38	38.24	< 0.01

** H. is significant.

Punch biopsy was taken before application of the treatment and stained to be examined histologically.

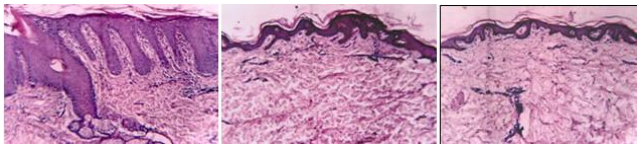

Figure 1: Pre and post Treatment Histology (Biopsy Rt.Sole)

Within the typical plaque, psoriatic epidermis shows marked epidermal acanthosis, hyperkeratosis, and elongation of capillaries, and presence of Munro's microabscesses (to the left).

Table 3: Results of treatment

		Result				
		Excellent	Good	No Response	Weak	Total
Effect	Count %	531 72.1%	206 27.9%	-	-	737 100%
Non Effect	Count %	-	-	68 4%	52 42.9%	120 100%
Total	Count %	531 62%	206 24%	68 4%	52 6%	857 100%

Chi Square Test =857 **; **H sig.

Parakeratosis changed to orthokeratosis, acanthosis decreased, the rete ridges became shorter, and Munro's microabscesses disappeared (to the right).

Table 4: Results of treatment % reduction of the PASI score

%- a reduction of PASI score	Cases	Per cent
Effective 33% ↓↓	737	86%
Non Effective 33% ↑↑	120	14%
Total	857	100%

In conclusion, patients who have palmoplantar psoriasis, who were treated with a topically applied mixture of propolis (50%) and aloe vera (3%), have shown noteworthy improvement thus proving the efficiency of propolis and aloe vera in the treatment of mild to moderate psoriasis.

Table 5: Results of treatment - effectiveness

		Cases	Per cent
Effective	Excellent	531	62%
	Good	206	24%
Non-Effective	Weak	52	6%
	No Response	68	8%
	Total	857	100%

Figure 2: Pre (left) and post (right) 3 months of treatment

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The Effect of Self-Management Educational Program on Pain Intensity in Elderly Patients with Knee Osteoarthritis: A Randomized Clinical Trial

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Keywords: Self-management; Pain; Osteoarthritis; Knee; Elderly; Aged

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Abstract

BACKGROUND: Osteoarthritis is one of the chronic diseases that greatly affect the health and life quality of individuals.

AIM: This study aimed to determine the effect of self-management educational program on the pain intensity of the elderly patients with knee osteoarthritis.

METHODS: In a randomised clinical trial, a total of 82 elderly patients with knee osteoarthritis were randomly divided into intervention and control groups. The intervention group received six sessions of self-management group education, while the control group received only the routine care during this period. In both groups, patients' pain intensity, with a visual analogue scale (VAS), were assessed before, immediately after and eight weeks after the start of the study.

RESULTS: The mean pain intensity scores of the intervention and control groups were not significantly different before the intervention ($P = 0.9$), but after the intervention, the mean pain intensity score in the intervention group (3.61 ± 2.36) was significantly lower than that of the control group (4.93 ± 2.00), ($P < 0.0001$).

CONCLUSION: Implementation of a self-management program for the patients with knee osteoarthritis is useful in reducing their pain intensity and can be used as one of the effective methods for their empowerment.

Introduction

Osteoarthritis is one of the most common joint diseases among older adults. The prevalence of osteoarthritis in the United States is expected to exceed 66% by 2020 [1]. The most common symptom of osteoarthritis is joint pain, which is exacerbated by doing activities. Morning stiffness of the joints limits their daily activities due to their joint pain and tightness [2]. The problems caused by articular diseases are not only limited to a clear reduction in the patients' mobility and daily activities, but can lead to problems such as pain, fatigue, changes in mental self-imagery, and so on [3]. In developed countries, paying attention to osteoarthritis as a cause of pain and disability in the elderly is ever-increasing [4]. Osteoarthritis affects communities through its high prevalence, its effect on the quality of life, and high costs of health care [5]. As osteoarthritis is a chronic and untreatable disorder, caregivers have preferably focused on identifying changeable risk factors that can reduce the effect of the disease [6]. Chronic pain has a negative effect on individuals' physical health. It has

been observed that individuals with chronic pain are more likely to have activity restrictions over time [7]. It was shown that the untreated pain in elderly people could have a general effect on their quality of life and lead to depression, anxiety, social isolation, cognitive impairment, immobility, and sleep disorders [8] [9]. Despite its prevalence and considerable negative impact on patients' daily life, unfortunately, there is no definitive treatment for the osteoarthritis. The therapeutic goals should include reducing pain, improving the range of motion and facilitating the daily activities [10] [11]. Since osteoarthritis is one of the chronic diseases associated with people's habits, behaviors and lifestyles, it is likely that determining appropriate lifestyle might reduce the prevalence of the disease and its complications for the patients and communities [12]. Since drug therapy for osteoarthritis, especially in the elderly, has high costs and, on the other hand, drugs also have side effects, it seems that one of the safest and least costly ways to treat the pain caused by osteoarthritis is pain self-management [13]. Elderly people, especially those with chronic diseases, are incapable of managing their diseases. However, appropriate knowledge and awareness can improve their quality of life [14]. Achieving secure and correct pain management practices in the elderly can improve their performance, enhance their quality of life, increase their comfort and reduce the costs of caring them [8].

Considering the high prevalence of knee osteoarthritis in older adults and its significant pain which can lead to disability and decreased the quality of life in older adults, this study aimed to evaluate the effect of self-management educational program on pain intensity in elderly patients with knee osteoarthritis.

Patients and Methods

This randomised clinical trial conducted on the older adults with knee osteoarthritis who were referred to the elderly care clinic of Imam Reza specialised and subspecialized polyclinic in Shiraz from March 2016 to July 2016. Eighty-two eligible elderly people were selected according to the inclusion criteria. The inclusion criteria for entering the study included the willingness to participate, being 60 years old and over, suffering from knee osteoarthritis according to an expert's final diagnosis (grade one to three), having the ability to do the instructions, having the ability to communicate and not having mental illnesses, lack of life-threatening diseases, and the ability to regularly attend the meetings. The exclusion criteria included two sessions of absenteeism during the educational program, development of disabling illnesses during the interventions that would cause non-identical therapeutic interventions, exacerbations of the symptoms and reluctance of the subjects to continue the treatment. Patients who meet the inclusion criteria were randomly allocated into two equal size group (n = 41) using permuted block randomisation method. In the beginning, and after obtaining informed consent for both the intervention and control groups by the research assistant, a researcher-made questionnaire including age, sex, body mass index (BMI), occupation, degree of suffering, education level, marital status and income level was used to collect the patients' demographic and clinical characteristics. Also, pain intensity of the patients was evaluated using Visual Analogue Scale (VAS). The validity and reliability of the VAS have been previously confirmed by researchers several times (its internal reliability has ranged from 0.85 to 0.95) [15].

The subjects in the control group were emphasised to refer to the clinic three weeks and eleven weeks later for their pain intensity to be re-evaluated. The 41 subjects in the intervention group participated in the educational program for three weeks, two 60-minute sessions a week, and half an hour was allocated for answering the questions raised by the elderly subjects. For better learning, the older adults were divided into three small groups (two groups of 14 and a group of 13) according to their readiness. At the end of three weeks of education, the VAS was completed again for both the control and intervention groups. Upon completion of the educational sessions, the elderly subjects in the intervention group were given eight weeks to implement the education they had received with the help of the researcher at home. To ensure the implementation of the education provided during this period (weekly), the elderly in the intervention group was called and, if necessary, the provided education was reminded. After the eighth week, the VAS was completed again for both the intervention and control groups. It should be noted that one of the subjects in the control group was excluded from the study due to lack of cooperation. To observe ethical considerations and benefit the elderly subjects in the control group, a two-hour intensive educational session similar to what was provided for the intervention group was held for them.

Data were analysed using Statistical Package for the Social Sciences (SPSS) version 23.0 software (SPSS Inc., Chicago, IL). We used the Shapiro-Wilk test to determine whether data were normally distributed. To compare the pain intensity before and immediately after the education and eight weeks after the intervention, the repeated measures analysis of variance (ANOVA) was used. Besides, to do the post hoc tests related to the significant interaction between time factors and study groups, paired and independent t-tests were used. Significance levels were set at $P < 0.05$.

Results

The mean age of the elderly in the intervention group was 65.34 ± 6.19, and it was 64.58 ± 4.67 years in the control group. There was no statistically significant differences between two groups regarding gender, age, BMI, marital status, education level, occupation, the degree of disease and income level according (P > 0.05). At the baseline, no significant difference was observed between the intervention and control groups regarding mean pain intensity, respectively (4.83 ± 2.22 versus 4.78 ± 1.79; df = 79, P = 0.90). The mean and standard deviation of the pain variable for both control and intervention groups are shown in Table 1. The pain intensity score in the intervention group decreased by 2.36% compared to the control group.

Table 1: Table of descriptive statistical indices related to pain intensity in both control and intervention groups before, immediately after and eight weeks after the intervention

Time Period	Control		Intervention	
	Mean	SD	Mean	SD
Before intervention	4.78	1.79	4.83	2.22
Immediately after intervention	4.78	1.79	4.73	2.28
Eight weeks after intervention	4.93	2	3.61	2.36

In order to investigate the effect of self-management education on the pain intensity of the elderly with knee osteoarthritis, a two-group variance analysis with repeated measures of 2 x 3 was performed using the time variable at three levels (before intervention, immediately after intervention, and 8 weeks after intervention) and the intervention variables at two levels (self-management education and routine care) as independent variables, and pain as the dependent variable. The results of variance analysis indicated that the interaction between time and group was significant (P < 0.001); i.e. there was a significant difference between the control and intervention groups in terms of the effectiveness of self-management education programs on severity of the pain associated with osteoarthritis in the elderly people at different times (Table 2).

Table 2: Repeated measured variance analysis results to compare the pain intensity scores in the control and intervention groups at three measurement times

Source of Change	Sum of Squares (SS)	df	Mean Squares	F	P-value	Eta
Group	11.48	1	11.48	0.90	0.34	0.011
Time	0.64	2	2.78	21.47	<0.001	0.35
Time & Group	0.52	2	2.78	35.15	<0.001	0.47

The results of variance analysis showed that the interaction between time and group was significant (P < 0.001); i.e. there was a significant difference between the control and intervention groups in terms of the effectiveness of self-management education programs on severity of the pain associated with osteoarthritis in the elderly people at different times (df = 1.33, F = 34.5, P < 0.001). There was an interaction in the evaluation of pain intensity between time and group. The interaction indicated that the pattern of changes in the pain intensity scores in the two groups depended on the time of its evaluation, and the changes in the pain intensity scores over time in the intervention and control group had a different pattern.

According to the patterns of changes, the mean score of pain intensity before the intervention in the control group was almost the same as that of the intervention group, but at the second time this difference increased, and at the third time it reached the maximum. In other words, the mean pain intensity scores in the intervention group decreased immediately after the intervention and especially eight weeks after the intervention in comparison with the control group.

This means that the self-management educational program had an impact on pain of the older adults with osteoarthritis. Due to the significant interaction of the time and group, the simple effects of the group and time were separately examined. Furthermore, the effect of the group factor was examined separately in each of the three time periods. Table 3 shows the post hoc comparisons of the mean pain intensity scores in the groups separately regarding time. It can be seen that in the control group, there was no significant difference between the pain intensity scores in different time periods.

Also, there was no significant difference in the intervention group between the first period (before the intervention) and the second one (immediately after the intervention), but the mean pain intensity scores in this group were significantly different between the second and third time periods (immediately after and eight weeks after the intervention) Intervention) and the first and third ones (before and eight weeks after the intervention) (P < 0.001).

Table 3: Post hoc comparisons of the mean intensity pain in the groups, separately regarding time

Group	Time (i)	Time (j)	Mean difference (i-j)	Standard error	P-value
	1	2	9	0.055	1
Control	1	3	-0.150	0.115	0.59
	2	3	-0.150	0.120	0.64
	1	2	0.098	0.054	0.23
Intervention	1	3	1.22	0.114	<0.001*
	2	3	1.12	0.119	<0.001*

*The coefficients that have become significant by Bonferroni's correction.

In Table 4, the post hoc comparisons of the mean pain intensity at three time periods are observed separately for the study groups. In the first and second time periods (before and immediately after the intervention) there was no statistically significant difference between the intervention and control groups regarding the mean pain intensity scores. However, at the third period (eight weeks after the intervention), the mean scores were significantly lower in the intervention group (P = 0.009).

Table 4: Post hoc comparisons of mean scores of pain in three time periods, separately, based on the study groups

Time	Group (i)	Group (j)	Mean difference (i-j)	Standard error	P-value
Before intervention	Control	Intervention	-0.054	0.449	0.90
Immediately after intervention	Control	Intervention	0.043	0.456	0.92
8 weeks after intervention	Control	Intervention	1.315	0.488	0.009*

*The coefficients that have become significant by Bonferroni's correction.

Discussion

The results of this study showed that the self-management education program was effective on pain relief in the older adults with knee osteoarthritis. The mean scores of the intensity of the pain caused by osteoarthritis before the intervention were almost the same in the control and intervention groups, but immediately after the intervention (at the end of the third week) the difference increased, and for the third period which was eight weeks after the intervention (at the end of the eleventh week) it reached maximum. The results of this study are consistent with other related studies [16] [17] [18] [19], but they are not consistent with another study conducted on the effect of self-care on arthritis [20]. In a study carried out on 120 patients with osteoarthritis a relatively great decrease of pain was observed in the intervention group, from 11.88 to 1.76 [16]. In our study, too, effective exercises were taught to relieve pain and strengthen the muscles keeping joints. In another study, it was reported that during the twelve weeks of implementing the exercise program, the pain rate decreased from 7.5 at the beginning of the study to 3.5 in the 12th week. That study was carried out on one group as before and after [17], and was consistent with the present study.

Therefore, the self-management arthritis program had played an effective role in improving the pain and motion range of the older adults with knee osteoarthritis [18]. In our study, the self-management education program was also effective in reducing the pain score in the intervention group. In another study, 146 patients with knee osteoarthritis were examined. The intervention group showed pain improvement, physical function, vitality and social functioning in comparison with control group. Therefore, the participants in the self-management program had a significant improvement in their quality of life and their performance eight weeks and six months after the intervention, compared to the control group [19]. It has been reported that the self-management arthritis program reduced anxiety and had a positive effect on self-efficacy of the participants for pain management, but it had no significant effect on their pain and physical functions [20]. The results of their study are not consistent with those of the present study, and the reason can be the differences in the tools used in the studies.

Since osteoarthritis is one of the chronic diseases associated with people's habits, behaviours and lifestyles, it may be possible to reduce the prevalence of this disease and its complications for the patients and the community by determining appropriate lifestyles [10] [15] [19]. As self-management is a protective factor for physical functioning in the patients with osteoarthritis, improving the self-efficacy of the patients with chronic pain can reduce their pain and improve their quality of life [19] [20]. Patient education is an essential part of nursing care, with the aim that the patients will live as independently as possible, take their medications properly, and use their aid supplies correctly [21]. Educating the patients is focused on the type of disorder, the changes caused by the disorder, the prescribed regimen, the side effects of drugs, strategies for maintaining independence and functions of the individuals. On the other hand, in many of these cases, it is possible to reduce many of their limitations and problems by educating the older adults with osteoarthritis regarding activity, rest, taking medications and their complications, and other issues related to lifestyle. Hence, the use of self-management education is the best way to convince the patients of behavioral changes and non-pharmacological treatment to promote their health, prevent the disease and successfully control it.

In conclusion, according to the present study, the self-management education, conducted through six educational sessions, reduced the pain of the elderly patients with osteoarthritis. Therefore, using self-management education is the best way to convince the patients of behavioural changes and non-pharmacological treatments to promote their health, prevent the disease, and successfully control their disease.

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Clinical Science

Third and Fourth Degree Perineal Tear in Four-Year Period at Sestre Milosrdnice University Hospital Center, Zagreb, Croatia

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Keywords: Birth injuries; Perineal tears; OASIS; Obstetric anal sphincter injury; Third-degree tear; Fourth-degree tear

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Abstract

OBJECTIVES: Obstetric anal sphincter injury (OASIS) includes the third and fourth degree of perineal injury. The risk for OASIS is about 1% of all vaginal deliveries. If not recognised and treated properly, obstetric anal sphincter injury can have serious consequences for reproductive age woman.

MATERIAL AND METHODS: We have retrospectively gathered and analysed data on obstetric anal sphincter injury in a four-year period at our department. The control group in this study included vaginal deliveries in 2012.

RESULTS: We recorded 0.34% third and fourth degree of perineal injury in all vaginal deliveries, and 87.9% of those patients were primiparae. Episiotomy was performed in 57.6% of all women with obstetric anal sphincter injury. In 30.3% of cases, newborns were large for gestational age. Gestational diabetes was found in 9.1% of OASIS cases, occipitoposterior position was found in 9.1% of cases. Induced labour took place in 39.4%, and oxytocin infusion was applied in 60.6% of OASIS cases. Vacuum extraction was performed in 12.1% of deliveries with OASIS. The average BMI in 3a and 3b injuries was 29.9. In 3c degree it was 28.0, and in the fourth degree, it was 32.1. In 27.0% of OASIS cases due to the extent of the injury surgeon engagement was necessary. When compared with vaginal deliveries in 2012 we found a significant increase in OASIS in primiparas, large for gestational age, occipitoposterior position, induced labour, vacuum extraction and hypertension ($P < 0.01$). There is also increased incidence of OASIS in episiotomy and oxytocin use group ($P < 0.05$).

CONCLUSION: Low incidence of OASIS in our department is a result of active management of delivery, manual perineal protection and timely episiotomy.

Introduction

Perineal trauma is often observed during the vaginal birth. It can occur spontaneously or secondarily as an extension to an episiotomy. Obstetric anal sphincter injury (OASIS) includes the third and fourth degree of perineal injury. The risk for OASIS is about 1% of all vaginal deliveries [1].

The third degree of perineal rupture is a partial or complete disruption of the anal sphincter and can include both external and internal sphincter. It can be divided in three groups: grade 3a (disruption up to 50% of external anal sphincter), grade 3b (disruption over 50% of external anal sphincter) and grade 3c (any disruption of external anal sphincter with disruption of internal anal sphincter) [1] [2]. The fourth degree includes injury of the perineum, external and internal sphincter and rectum [1]. It also includes "buttonhole" tear, where sphincters are intact but rectal mucosa is injured [1] [2].

Risk factors for OASIS are birth weight over 4000 g, nulliparity, induced labour, prolonged second stage of labour, mothers' age, shoulder dystocia, medial episiotomy, vacuum extraction, posterior occipital position and delivery between 3 a.m. and 6 a.m [1] [3] [4]. Early complications of OASIS are tear disruption and purulent discharge [5] [6], while late complications include incontinence and fistulas [3] [7] [8]. Unrecognized and treated inadequately, OASIS could have serious consequences for women of reproductive age and can influence general health, social and psychological state and sex life [9] [10].

A four-year experience with OASIS at the University Hospital Center "Sestre Milosrdnice", Zagreb, Croatia is presented in this paper.

Material and Methods

This is a retrospective study on OASIS during the period 01.01.2010.-31.12.2013. At the University Hospital Center Sestre Milosrdnice, Zagreb, Croatia. Three groups of patients with OASIS were included in this study. Patients with partial sphincter rupture were included in the first group (grade 3a and 3b), patients with complete sphincter rupture were included in the second group (grade 3c), while the patients with sphincter rupture and rectum injuries were included in the third group (4th degree). Effects of parity, age, episiotomy, newborns weight, gestational diabetes, posterior occipital position, induced labour, infusion of oxytocin, vacuum extraction, body mass index (BMI), weight gain during pregnancy, shoulder dystocia, hypertension (essential or gestational), preterm birth, epidural analgesia, cigarette smoking and surgeon engagement on OASIS cases were analyzed by descriptive statistical methods.

These results were compared with data from all vaginal deliveries in 2012. This group did not include 5 women who had OASIS that year. We had 2416 vaginal deliveries of 3157 in total. The data were analysed by SPSS program.

Results

The total number of deliveries during the 4-year period was 12721, with 9748 vaginal deliveries and 33 cases of OASIS (0.34% of all vaginal deliveries).

Seven patients had partial rupture of the anal sphincter (21.2% of all OASIS cases), 21 women (63.6%) had a complete sphincter injury, while 5 of them had a fourth-degree perineal injury (15.2%). Two patients with fourth-degree perineal injury had “buttonhole” tear injury.

Almost all the patients with OASIS (87.9%) were primiparas (Table 1).

The average age of patients with partial rupture of anal sphincter was 32 years, the average age among patients with a complete rupture of anal sphincter 31 years, while the mean age among the patients with the fourth degree of perineal injury was 28.8 years. The incidence of episiotomies among women with a third and fourth degree of perineal injury was 57.6%. All the performed episiotomies were mediolateral. The incidence of episiotomies among patients with partial anal sphincter injuries had episiotomies was 42.9%, the incidence among patients with complete anal sphincter injuries was 61.9%, while the incidence among women with fourth-degree perineal injuries was 60%.

Table 1: OASIS group compared with vaginal deliveries in 2012

	OASIS	2012	
	N= 33 (%)	N= 2416 (%)	$\chi 2$ test
Primiparas	29 (87.9)	1164 (48.2)	20.44; df=1; P < 0.01
Episiotomy	19 (57.6)	875 (36.2)	6.43; df=1; P < 0.05; P > 0.01
Large for gestational age	10 (30.3)	169 (7)	26.28; df=1; P < 0.01
Gestational diabetes	3 (9.1)	316 (13.1)	0.44; df=1; P > 0.01
Posterior occipital position	3 (9.1)	39 (1.6)	10.93; df=1; P < 0.01
Induced labour	13 (39.4)	449 (18.6)	9.18; df=1; P < 0.01
Oxytocin use	20 (60.6)	1042 (43.7)	3.99; df=1; P < 0.05; P > 0.01
Vacuum extraction	4 (12.1)	51 (2.1)	14.79; df=1; P < 0.01
Shoulder dystocia	0	3 (0.12)	0.04; df=1; P > 0.01
Hypertension	6 (18.2)	67 (2.4)	26.83; df=1; P < 0.01
Preterm labour	1 (3)	114 (4.7)	0.17; df=1; P > 0.01
Epidural analgaesia	6 (18.2)	367 (15.2)	0.22; df=1; P > 0.01

Analyzing the risk factors for OASIS, some interesting results were obtained. About 30.3% of patients with OASIS have had babies large for gestational age. However, there were no newborns weighing more than 4500 grams. Gestational diabetes was found in 9.1% of OASIS cases, same as the posterior occipital position. Induced labour took place in 39.4%, and oxytocin infusion was applied in 60.6% of OASIS patients. Vacuum extraction was performed in 12.1% of deliveries with OASIS (Table 1). The average BMI in 3a and 3b injuries was 29.9.

Furthermore, in grade 3c the average BMI was 28.0, while the average BMI in the fourth degree of perineal injury was 32.07. Weight gain more than 14kg was observed in 57.6% of women with OASIS. However, shoulder dystocia was not recorded. About 6.1% of all the OASIS patients have had a delivery between 3 a.m. and 6 a.m. Hypertension disorder in pregnancy was found in 18.2% of cases, while the preterm birth occurred in 3% of OASIS patients. Epidural analgesia was applied in 18.2% (Table 1). Cigarette smoking was found in 9.1% of the patients with OASIS. The surgeon engagement was necessary in 27.0% of OASIS cases due to the extent of the injury.

When compared with vaginal deliveries in 2012 we found a significant increase in OASIS in primiparas, large for gestational age, occipitoposterior position, induced labour, vacuum extraction and hypertension ($P < 0.01$) (Table1). There is also increased incidence of OASIS in episiotomy and oxytocin use group ($P < 0.05$) (Table 1).

Discussion

Incidence of OASIS is in a wide range of 0.5-8% of vaginal deliveries [4] [5] [11] [12] [13]. Furthermore, there is an increase in OASIS incidence in last few decades [11] [14]. Low incidence (0.34%) in our research is a result of active labour management and practice of classical obstetrics. A special birthing chair for primiparas is not used in our Department, and a timely episiotomy is performed avoiding the medial episiotomy. Oxytocin infusion is

dosed in a way it does not influence the partogram. Line and all have clearly shown in their study that the incidence of OASIS varies in different countries and different obstetric schools. They found the incidence of OASIS in Finland to be only 0.6% and in Sweden 4.2% [11].

Primiparas have OASIS more often as compared to women who have previously given birth. We found that primiparae account for 87.9% of all OASIS cases. In the retrospective study conducted by Rizvi et al., it has been reported that 75% of primiparae were the patients within the OASIS group [12]. Other retrospective and prospective studies report similar results [4] [13].

There are reports that age influences incidence of OASIS especially ages over 35 years [1] [4], but our study failed to confirm these reports.

Medial episiotomy is a known risk factor for OASIS, and mediolateral episiotomy does not prevent anal sphincter injury [4]. In our Department, medial episiotomy is extremely rarely used. We use mediolateral episiotomy for every vacuum extraction for its known protective effect [15] [16]. However, there are some reports questioning the effects of episiotomy even in cases of instrumental delivery [17].

Newborns weight influences the incidence of OASIS [4] [13] [15] [18]. In our study, almost one-third of newborns had a weight greater than 90 percentile for gestational age. Reports suggest that foetal macrosomia is found in 12% of uncomplicated pregnancies [19]. In our study babies large for gestational age increase risk for the third and fourth degree of perineal injuries. Women with gestational diabetes often give birth to macrosomic newborns. Macrosomia increases risk of failure to deliver, and the need for instrumental delivery. If there is suspicion of a foetus weighing more than 5000g in non-diabetic mother or 4500 g in diabetic mother, the elective Caesarean section is recommended [20]. In our Department, the incidence of gestational diabetes does not vary in OASIS from that in general group. Incidence of gestational diabetes varies from 4-17.8% [21].

Although shoulder dystocia is a known risk factor for OASIS, our study failed to confirm these reports. The posterior occipital position was found to be tenfold then usually reported [22].

Labour induction and the use of oxytocin infusion during the labour vary in different countries and even within the country; hence the results of this study have not been compared with the literature. We compared OASIS cases with the representative control group from our Department that consists of all vaginal deliveries in a year 2012, when we had 18.6% of labour induction in vaginal deliveries ($\chi 2 = 10.04$, df = 1, $P < 0.01$) and 43.7% use of oxytocin infusion ($\chi 2 = 4.52$, df = 1, $P < 0.05$). The connection between the labour induction and OASIS is quite obvious.

It has been found that instrumental delivery increases the risk for OASIS in our study, as well as in the literature, especially if the forceps is used [2] [4] [12] [18]. However, forceps is never used in our obstetric Department.

The reports on the effect of increased body weight and BMI on OASIS are not quite clear in the literature [23] [25]. According to World Health Organisation, there are 3 categories of weight: normal weight is with BMI 18.5–24.9, overweight with BMI 25–29.9 and obese with BMI ≥30 [26]. We only found a connection between BMI and fourth degree of perineal injury, but we failed to demonstrate any connection with early morning hour delivery, which was reported in one study [4].

The incidence of gestational hypertension is 5-10% of all pregnant women [27]. We have found the increased percentage of gestational hypertension (18.2%) in OASIS cases. It is known that induced labour is more often among patients with hypertension. This could potentially explain the connection between gestational hypertension and OASIS [28].

Preterm labour occurs in 5-15% of deliveries; however, we have failed to find it as a risk factor for OASIS [29].

Epidural analgesia can prolong the second stage of labour, and this could be the reason for the increased risk of OASIS [30]. In our Department, we tolerate duration of the second stage of labour in parturient women who received the epidural analgesia up to three hours for nulliparous and two hours for multiparous women (one hour longer than the recommended duration of the second delivery stage) [31] [32]. In our study, the use of epidural analgesia is even less frequent than in the literature reports. Hence we did not find the increased risk for OASIS in epidural analgesia cases.

Cigarette smoking is a reason for decreased newborns weight. Hence, it could decrease the risk for OASIS [18] [28]. However, the percentage of cigarette smoking in our study was similar to the rest of Europe [33].

The efforts made to avoid OASIS are understandable considering the repercussions of such injuries. Perineal protection devices are designed to influence the elasticity of perineum. Lavesson et al., in a multicenter, open randomised controlled trial, used this device designed to protect perineum during the labour and did not found a significantly reduced incidence in perineal sphincter injury [34].

In cases when there was OASIS in a previous pregnancy, a careful assessment of the possibility of vaginal delivery is made in our Department. The most important criterion is the newborns' weight. If the newborns' weight is less than 4000 g and all the other criteria are met, a vaginal delivery is recommended.

In case of fourth-degree perineal injury, a Caesarean section is recommended. One retrospective study did not found that previous OASIS can influence the incidence of OASIS in later pregnancies. However, the incidence correlated with newborns' weight and instrumental delivery [35] [36].

Low incidence of OASIS found in our retrospective study is the result of active protection of the perineum and valid episiotomy indications. Lainie et al. found similar results in their study concluding that this could be the reason for the low incidence of OASIS in Finland compared with other Nordic countries [11] and one of the countries with the lower incidence OASIS is Romanian, with only 0.1% [37].

In our Department, a physician is present at every delivery. We practice perineal visualisation during the second stage of labour for a precise moment for an episiotomy. This accompanied by the manual perineal protection is crucial in perineal rupture prevention [13].

In cooperation with surgeons, we always practice primary suturing of the birth canal rupture. Short- and long-term results are better if experienced colorectal surgeon was part of the team [36].

In conclusion, low incidence of OASIS in our maternity unit is the result of the active manual protection of the perineum and valid episiotomy indications. Serious injuries of the birth canal are difficult to predict. It is important to follow the obstetric rules and to perform an episiotomy if indicated. An adequate inspection of the birth canal is necessary to detect third and fourth-degree perineal injury. The consequences for the wellbeing of a young mother with perineal injury can be serious and can affect social and sexual aspects of life. Adequate surgical treatment and postoperative care assure optimal results and prevent long-term complications like fistulas or faecal incontinence.

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Clinical Science

A Study of Nerve Conduction Velocity in Diabetic Patients and its Relationship with Tendon Reflexes (T-Reflex)

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Keywords: Diabetes; Neuropathy; Neurological disorder; Nerve conduction velocity; Tendon reflex

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Abstract

BACKGROUND: Neuropathy is one of the most common complications of diabetes mellitus. Neuropathy can cause the sensory deficit, neurological disorder, limb ulcers, osteomyelitis, and amputation. Therefore, neurological examinations, determining the nerve conduction velocity and performing sensory and motor tests are important for timely diagnosis and treatment.

AIM: The present study aimed to investigate the nerve conduction velocity in diabetic patients and its relationship with tendon reflexes.

MATERIAL AND METHODS: The present study was observational-cross sectional research carried out on 77 diabetic patients who were admitted into the EMG/NCV Department of Shariati Hospital in the academic year 1996-1997. In all patients, the medical history of the patient (age, duration of diabetes, gender and age of onset of diabetes), neurological examination, nerve conduction velocity, heat test, vibration test, tendon reflexes, D.L and Amplitude were examined and recorded. Finally, the raw data obtained were entered into the IBM SPSS Statistics software, and the important relationships between these variables were analysed. Moreover, in the present study, the statistical significance level (P-value) was considered less than 0.05.

RESULTS: The present study was conducted on a population consisting of 48 women and 29 men with diabetes. The age range of participants was 14-70 years old with an average age of 50.506 ± 7.50. The results of present study showed that the participants with clinical neuropathy (11.2 ± 7.2) had a significantly longer duration of diabetes than the normal group and those participants with sub-clinical neuropathy (P-value = 0.12). Statistical analyses indicated that increase in age, increase in the duration of diabetes and the gender of male significantly made the nerve conduction velocity abnormal. The analysis of the response to neural reflexes indicated that the ratio of neurological disorders in the five nerves of the ankle and knee was generally higher in the abnormal group (the patients with nerve conduction disorder) compared to the normal (the patients with normal nerve conduction) and in some cases, such as the ulnar motor nerve of ankle (P-value = 0.010), and the ulnar motor nerve of knee motor (P-value = 0.002) and also in the peroneal motor nerve of knee (P-value = 0.003) and the sural sensory nerve of knee (P-value = 0.003), increase in neurological disorders was significant.

CONCLUSION: Increase in age, increase in the duration of diabetes, and the male gender can significantly increase the risk of abnormal nerve conduction velocity.

Introduction

Diabetes mellitus is one of the most commonly known chronic diseases in the world. Today, in many countries, diabetes has become an epidemic disease [1]. According to World Health Organization (WHO), in 2000, there were about 170 million people with diabetes and this population will increase to 366 million in 2030, if this disease is not treated and prevented [2]. In Iran, the prevalence of diabetes in different regions is more than 5% [3]. Studies have shown that the diabetes is the main cause of blindness and amputation in some countries [4]. One of the most important complications of diabetes is neuropathy or neurological disorder. There are different types of diabetic neuropathy: polyneuropathy, diabetic amyotrophy, autonomic neuropathy, mononeuropathy multiplex, mononeuropathy, and diabetic ophthalmoplegia. Diabetic neuropathy is observed in different forms in patients and corneal sensorimotor neuropathy (about 75%) can be mentioned as its common form [5]. In various studies, the prevalence of neuropathy was reported to be between 1.5% and 100% [6]. Neuropathy has a high prevalence in both IDDM and NIDDM forms [7]. According to the studies in this field,

one of three diabetic patients has diabetic neuropathy [8]. Diabetic neuropathy is diagnosed in 10% of diabetic patients at the time of diagnosis of diabetes, and rest of the patients would suffer neuropathy some years after onset of the diabetes [9]. Some complications of diabetic neuropathy are severe pain, reduced sensation, increased diabetic foot ulcers and amputation [10]. One of the main problems of diabetic neuropathy is the lack of a reliable and agreed clinical scale for grading the severity of neuropathy, so that it can be used in clinical trials to examine the effect of different therapeutic approaches among patients [11]. The most important diagnostic criteria for diabetic neuropathy, which are confirmed by experts, are disturbances in nerve conduction velocity, increased threshold of sensory nerves, and disturbances in autonomic system function tests. One of the diagnostic methods for diabetic neuropathy is to conduct electro-diagnostic tests (nerve conduction velocity determination). According to a study by Dyck, Nerve Conduction Velocity (NCV) determination is not only the most sensitive test for the diagnosis of diabetic neuropathy, but also has some features such as being repeatable. Moreover, it is considered as a specific test for neurological disorder. According to him, the disadvantage of this test is that it does not provide direct information on signs and symptoms of neuropathy [12]. Nerve damages caused by diabetes can be categorized into two groups; myelin and axonal damages categories. The nerve conduction velocity mainly reflects the myelin changes, while the action potential amplitude indicates the axonal changes and the state of the nerve fibers. The action potential amplitude is an estimate of the number of neural fibers activated by electrical stimulation, and its reduction implies an axonal damage. According to studies conducted in this field by researchers and experts, it was found that the nerve conduction velocity is more variable than the action potential amplitude and is more affected by interventions [13].

Given contents mentioned above and in this regard, the present study aimed to investigate the nerve conduction velocity in diabetic patients and its relationship with tendon reflexes.

Material and Methods

The present research was an observational-cross-sectional study. It was conducted on 77 patients with type 2 of diabetic neuropathy who were under the diabetes clinic and admitted into EMG/NCV department of Shariati Hospital in the academic year 1996-1997. After explaining the research process and getting informed consent from patients, the patients voluntarily participated in the present study by medical ethics principals**.** Exclusion criteria were including: being older than 70 years old, the presence of thyroid disease, uremia, autoimmune diseases including Rheumatoid arthritis (RA), nutritional, and toxic disorders, collagen-vascular diseases (CVDs) are a heterogeneous group of autoimmune disorders and history of taking certain drugs. Then, from all the patients selected, serologic tests including thyroid tests, complete blood count (CBC) collagen vascular tests and Rheumatoid factor (RF) were taken, and the patients were excluded from the study if the results of tests were positive. In the next step, the medical history of the patients was recorded, and then, they were placed under physical and neurological examinations. A questionnaire used in present study includes the questions on gender, age, duration of diabetes, the age of onset of diabetes and type of treatment.

Neurological examination includes the examination of cranial nerves, motor system, sensory system (the perception of external sensations (exteroception), including touch, temperature, pressure, vibration, and itch) proprioception, vibration sense and heat sensation), and tendon reflexes. The last part of the study, is devoted to the measurement of the nerve conduction velocity in the median nerves (both sensory and motor branches), ulnar nerve (motor branch), peroneal nerve (motor branch) and the sural nerve (sensory branch) and the rates of NCV (M/S), D.L (s), and amplitude (mv) in the mentioned nerves were recorded and if they were insignificant, they were recorded as detectable in the united nations system. The values of these parameters were evaluated according to the tables presented in the valid electrophysiology books written by researchers such as OH [14] and Chu-Andrews [15] and considering the age of the patient. Finally, they were divided into two groups. The other main variables were the Ankle Jerk (AJ) Reflex and the Knee Jerk (KJ) Reflex. To score the reflexes and according to the age range of patients, just Ankle Jerk (AJ) Reflex could be adjusted.

For this purpose, if the Ankle Jerk (AJ) Reflex was absent and the patient was older than 50, he/she would get the score 1, instead of zero, and if it were reduced, he/she would get the score 2, instead of 1. To assess the vibration sense, the diapason (Hz) was used. First, it was placed on the medial malleolus of the right foot, and the test was considered abnormal when the vibration sense was confirmed by the patient. In the case the vibration sense was not confirmed by the patient, the diapason was immediately moved to the Tuberosity of the tibia. To investigate the heat sensation, two test tubes containing 20°C and 45°C water were used. To examine orthostatic hypotension, greater than 20 mmHg reduction in systolic pressure or 10 mmHg reduction in diastolic pressure while changing the position of the patient from lying down to standing was considered as a positive sign for orthostatic hypotension.

It should be noted that all tests mentioned above, except for vibration and heat test performed by the students at the Research Center of Endocrinology Clinic, were conducted by an expert. Then, the raw data obtained were entered into IBM SPSS Statistics. To analyse the data, T-test, Variance Fisher's Exact Test and Chi-squared test were used. The statistical significance level (P-value) was considered less than 0.05.

Results

In the present study, 77 patients participated of which 49 and 29 patients were female and male, respectively (the female-to-male ratio was 1.6 in the present study). The age range of patients was 14 to 70 years old, and their average age was 50.506 ± 7.50 years the average age of patients at the onset of diabetes was 40.50 ± 10.50 years. Descriptive statistics show that average duration of diabetes was 9.70 ± 6.90 years. Moreover, 41.2% of patients were treated with the pill (oral antidiabetic agents), and 44.2% of patients were treated with insulin. In this study, 66.2%, 11.7% and 2.6% of patients complained of paresthesia, weakness and pain, respectively. According to the examinations, it was found that in 36.5% of patient, heat sensation was impaired and in 32.8% of them, vibration sense was impaired. The patients who participated were divided into three groups based on clinical signs and symptoms:

The first group (clinical neuropathy) was made up of 54 patients with severe neurological symptoms.

The second group (sub-clinical neuropathy) had 20 patients with main disturbances in vibration and heat tests

Third group (normal): consisting of 3 patients with normal test results.

In this study, the duration of the disease was examined with three clinical groups (clinical neuropathy, subclinical neuropathy and normal) (Table 1). The results indicate that there are significant differences between parameters mentioned above (P-value < 0.05).

Table 1: The relationship between clinical symptoms and duration of the disease

		Duration of the disease Mean ± S.D. (year)	P-Value
Group	Clinical neuropathy	11.2 ± 7.2	0.012
	Sub-clinical neuropathy	8.9 ± 8.3	
	Normal	4.41 ± 4.48	

On the type of treatment, in both normal and abnormal groups, no significant difference was observed between parameters mentioned above (P-value = 0.1000). According to NCS results, the patients studied were divided into two groups: normal (normal nerve conduction) and abnormal (disturbance in nerve conduction). There were 7 patients in the normal group, and there were 70 patients in the abnormal group. In Table 2, the average age of the patient, the age of onset of the disease and the duration of the disease in both groups (normal and abnormal) are summarised. Statistical analysis showed that in both groups, there was a significant difference between the age of the patient and the duration of the disease (P-value < 0.05) (each group was examined separately).

Table 2: Relationship between the parameters studied in the two NCS groups

	In NCS group Mean ± S.D.		P-Value
Age (year)	53.3 ± 11.3	42.8 ± 13.8	0.002
Age of the onset of disease (year)	41.07 ± 9.21	35.60 ± 13.37	0.580
Duration of disease (year)	12.27 ± 7.40	4.21 ± 3.74	0.001

The statistical analyses performed in the present study showed a statistically significant difference between gender and nerve conduction velocity (P-value = 0.019). In the present study, tendon reflexes were divided into two groups: Knee-jerk Reflexes (Kj) and Ankle jerk Reflexes (Aj). Each of these groups has been classified into three parts: Absent, Decreased, and Normal. In both normal and abnormal groups, the highest frequency belonged to the Normal part (81.8%), and the lowest frequency belonged to the absent group (1.3%). The researchers have investigated the relationship between nerve conduction velocity and tendon reflexes (Table 3). Statistical analyses showed that there were significant differences among the knee-jerk reflexes and the ulnar motor nerves, peroneal motor nerves and sural sensory nerves (each separately) (P-value < 0.05). It was also found that there is a statistically significant difference between the ankle jerk reflexes and ulnar motor nerves (P-value < 0.05).

Table 3: Relationship between neurological disorders and tendon reflexes

			Nerves (%)				
			Median motor	Median sensory	Ulnar motor	Peroneal motor	Sural sensory
Tendon reflexes	Ankle jerk reflexes	Normal	63.5	63.5	22.2	58.7	41.3
		Abnormal	92.9	92.9	57.1	71.4	71.4
		P-Value	0.050	0.050	0.010	0.560	0.080
	Knee jerk reflexes	Normal	61.9	61.9	20.6	58.7	41.3
		Abnormal	100	100	64.3	71.4	71.4
		P-Value	0.003	0.003	0.002	0.560	0.080

Discussion

There is a direct relationship between the prevalence of neuropathy and the progression of diabetes mellitus [16]. The neuropathy is caused by

the presence of signs and symptoms of peripheral nerve disorders in diabetic patients. Nerve damage in diabetic patients has various features and the change in nerve conduction velocity is one of its symptoms. The position and characteristics of the nerve fibers, the severity of diabetes and demographic characteristics (such as age, duration of disease, and gender) are of the factors playing a key role in the severity of sensorimotor neuropathy. As mentioned earlier, such complications are common in diabetics, and somewhat predictable, but they have a wide range of changes and potency, which have been the subject of research by researchers. For example, Soivers et al (2004) have conducted a research in order to perform the clinical and electroneurographic study of peripheral nerve involvement in diabetic patients.

This study was performed on 103 diabetic patients with a mean age of 52.6 ± 14.00 years old who were randomly selected from patients admitting to endocrinology clinic of Shiraz University of Medical Sciences. It was found that 29.4% of patients had type 1 diabetes and 70.6% had type 2 diabetes. In their study, they stated that there is a direct relationship between the prevalence of neuropathy and the duration of the disease. The most commonly result was the reduced ankle reflexes and reduced vibration sense in the legs [17]. In another study by Andersen et al., (2012), motor dysfunctions in diabetic patients were examined. They argued that neuropathy is a frequent complication of diabetes, and motor system involvement is rarely seen in a clinical examination and can be diagnosed using quantitative techniques (isoquinoline dynamometer, type of diabetes, ankle jerk reflex and knee-jerk reflex). In fact, they believe that muscular weakness depends on the symptoms and severity of diabetic neuropathy in patients. Therefore, it can be said that diabetic neuropathy can reduce muscle strength [18].

The diversity of studies conducted in this field allows us to review the reports on specific cases in a case study. For example, Aaron (2016) has conducted a study on sensorimotor neuropathy in diabetic patients. In this study, it was reported that a 65-year-old woman with a 5-year history of diabetes and the symptoms such as pins-and-needles sensation and pain referred a physician and it was observed that in this case, burning and pinpricks sensation in the knee area, as well as the ability to detect vibration from a tuning fork 128 Hz, have decreased, and she has lost proprioception and sensation to the monofilament 1-g in her toes. Moreover, there was no knee-jerk reflex in her. All of these were due to diabetic sensory and motor neuropathy [19].

Based on the results of our study, it can be concluded that people with clinical neuropathy have a longer duration of diabetes. Moreover, increase in age, increase in the duration of diabetes, and the gender of the male can significantly make the nerve conduction velocity abnormal. The analysis of the response to neural reflexes indicated that the ratio of neurological disorders in nerves of the ankle and knee was generally higher in the abnormal group (the patients with nerve conduction disorder) compared to the normal (the patients with normal nerve conduction).

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Case Report

Comparative Analysis of the Applicability of Island Flap in Primary and Recurrent Basal Cell Carcinomas of Similar Localization

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Keywords: Basal cell carcinoma; Risk factors; Treatment; Island flap; Mohs micrographic surgery

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Abstract

BACKGROUND: Basal cell carcinoma belongs to non-melanoma skin cancers and is the most prevalent neoplasia that shows a tendency to increase over the last few decades. It occurs most often in skin areas exposed to sunlight. It is characterised by slow progression, low tendency to metastasising and good prognosis when the right choice of treatment has been made. The difficulty in the treatment of basal cell carcinomas is determined by their localisation and puts to the test the aesthetic potential of dermatosurgeons. Complete surgical excision is the standard approach in most uncomplicated cases. In relapsing basal cell carcinoma or carcinoma with aggressive or unfavourable histopathological characteristics, the clinician faces the dilemma of identifying the most appropriate method of treatment. To find the decision, help comes from the individualisation of each case and the related risk factors.

CASE REPORT: Two cases of basal cell carcinoma of similar localisation are presented, where the carcinomas are removed using island flaps. In spite of the desire to observe the recommended field of surgical security (by the desire for the ultimate esthetic effect for the patient), one of the tumours was not completely removed, and as an alternative, reoperation was proposed using Mohs micrographic surgery (MMS).

CONCLUSION: The choice of a surgical technique, which would guarantee a better outcome and could be applied depending on the individual risk factor in each patient, is discussed.

Introduction

Basal cell carcinoma is the most common malignant tumour composed of cells resembling those of the basal layer of the epidermis [1]. The disease rate is 200/100.000 people on the average [2] [3]. The affection of patients with white skin, fair hair and blue eyes, as well as male patients, prevails [4] [5]. It is believed that the main reason for its occurrence is the long-term exposure to sunlight over the patient's lifetime or accidental intensive exposure to ultraviolet radiation resulting in skin burns [4] [5]. It usually affects the exposed areas of the skin, i.e. the face, scalp, ears, neck and shoulders [1]. This epidermal tumour rarely metastasises (only certain types), but in the presence of a primary lesion, there is a risk of occurrence of a new lesion at the same site or another part of the body [5] [6]. For this reason, surgical removal of the tumour should be a priority [6].

We at this moment present two cases of basal cell carcinomas with a similar localisation in the area of the face, which are removed by island flap. In one of the patients, the tumour is completely removed, while in the other it is not. The applicability of island flap in various patients is discussed according to their risk profile.

Case Report 1

An 82-year-old woman is presented, with height 156 cm and weight 49 kg, in a good overall condition. There is no evidence of family history, concomitant diseases and medication. The patient was first admitted to the clinic for surgical removal of a neoplasm that occurred approximately 2 years before, which gradually began to increase in the last few months. During the dermatological examination, we found a nodular formation on the left side of the nose, at the border with the nasolabial fold. The lesion had a rigid consistency, nodular, without a pearl edge, located immediately below the nasolabial fold, but at the same time involving the ala nasi on the left (Figure 1a).

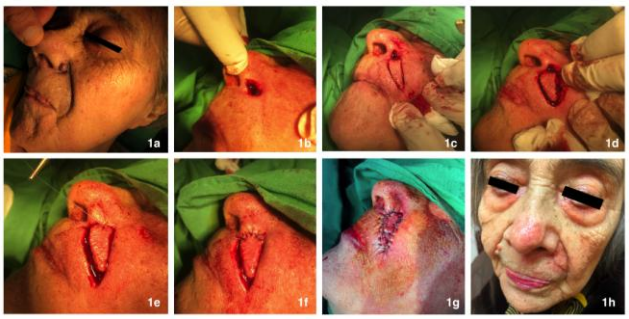

Figure 1: The lesion was removed in the form of the letter O, with a comparatively small field of surgical security of 0.3 cm in all directions. Then contouring of a triangle in the distal direction from the nose was performed, and the contours were gradually prepared to the musculature in depth (1c-d). This was followed by a transposition of the already prepared triangle to the ala nasi and a careful adaptation of the wound edges (1e-g)

There was no clinical evidence of the involvement of the subcutaneous area and the adjacent vascular and nerve bundles. Radical removal was performed under local anaesthesia via island flap (1b-h). The lesion was removed in the form of the letter O, with a comparatively small field of surgical security of 0.3 cm in all directions.

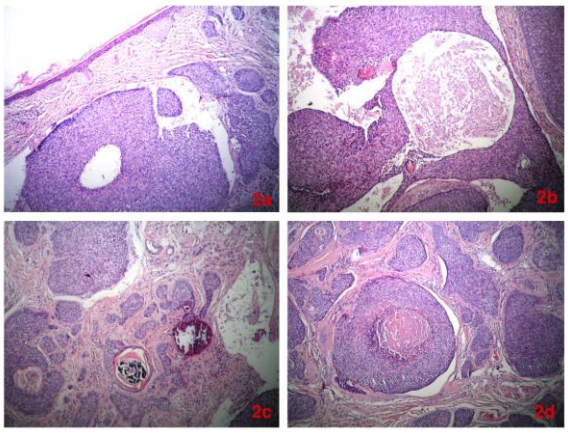

Figure 2: The histological examination confirmed the diagnosis: basal cell carcinoma with free resection edges, size 0.6/0.4 cm (Figure 2a-d). Good cosmetic results were achieved (2h)

Then contouring of a triangle in the distal direction from the nose was performed, and the contours were gradually prepared to the musculature in depth (1c-d).

This was followed by a transposition of the already prepared triangle to the ala nasi and a careful adaptation of the wound edges (1e-g). The histological examination confirmed the diagnosis: basal cell carcinoma with free resection edges, size 0.6/0.4 cm (Figure 2a-d). Good cosmetic results were achieved (2h).

Case Report 2

A 56-year-old man is presented, with height 178 cm and weight 108kg, in a good overall condition. There is no evidence of family history. The patient was first admitted to the clinic for surgical removal of a tumorous formation in the nasal area. In 2006, the patient underwent a primary tumour surgery (a tumour was localised near the described one), and in 2014 there was a relapse, and the patient was operated again using advanced plastic surgery. Histological data showed that the patient had basal cell carcinoma resected in sano. Meanwhile, radiotherapy was also conducted.

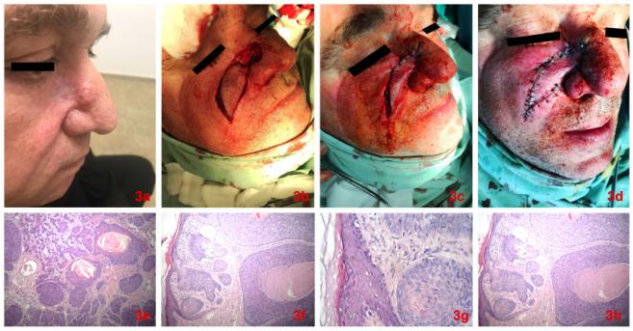

Figure 3: Island flap in patient with recurrent BCC. Similar to case 1 the the tumour lesion was removed, with a comparatively small field of surgical security in all directions. Then contouring of a triangle in the distal direction from the nose was performed, and the contours were gradually prepared to the musculature in depth (3b, 3c). This was followed by a transposition of the already prepared triangle to the ala nasi and a careful adaptation of the wound edges (3d). Histological evidence showed the presence of basal cell carcinoma with multifocal growth, maximum tumor diameter 12mm (Figs 3e-h)

During the dermatological examination, a tumour formation was found, with a solid texture, similar nodular aspect (as in the first female patient described), a clear distinction from the healthy/surrounding tissue, with multiple telangiectasias, located above the ala nasi, in the area of the right nasolabial fold (Figure 3a). Following consultation with an ENT specialist, an involvement of the lateral nasal wall was observed, from a formation adjacent to a cicatrix from a previous intervention,

without adhesion to the underlying cartilage. Island flap was performed similarly to the one described for the first patient (3b-3d). Histological evidence showed the presence of basal cell carcinoma with multifocal growth, maximum tumor diameter 12 mm; tumor infiltration in-depth and along the lateral resection lines (Figure 3e-3h). Tumor remnants were planned for removal using Mohs micrographic surgery.

Discussion

Patients with basal cell carcinoma are susceptible to recurrence of a primary tumour but also to the development of new, not only basal cellular but also other types of carcinomas [7]. Therefore, the identification of additional risk factors is, on the one hand, 1) essential for calculating the individual risk for each patient, and 2) of paramount importance for early stage eradication [2] [7].

Approximately 80% of all basal cell tumours appear in the facial area, with 25-30% of them on the nose [4]. This localisation is related to 2.5 times higher risk of relapse than any other and is considered to be a feature of high-risk carcinoma also because the area is associated with large anatomical features and difficulty in identifying tumour boundaries [4]. Risk factors include: 1) skin phototype, 2) prolonged exposure to ultraviolet radiation, and last but not least 3) genetic predisposition, i.e. xeroderma pigmentosum, albinism, DNA repair deficiencies, family history of cancer, immunosuppression and exposure to carcinogenic substances (arsenic, polycyclic hydrocarbons) [2] [4].

The main goal in the treatment of basal cell carcinomas is the elimination of a tumour with maximum preservation of the normal function and structure of tissues [5]. Therefore, treatment decisions should be individualised according to the specific risk factors for each patient, but should also take into account the respective surgical security zones [5] [6] [7].

What is the most effective treatment approach for small to medium-sized tumours is controversial, and in most cases standard therapy is surgical excision [6][7]? The surgical approach should vary depending on the size, depth and location of the tumour, as well as the individual desire of each patient and his/her physician to be minimally traumatic and at the same time maximally effective [5] [6] [7]. Reconstruction of nasal defects after excision of skin tumours is almost always a challenge due to the complex three-dimensional structure of the nose and the need for the postoperative outcome to be aesthetically acceptable [4] [5].

It is currently believed that MMS or the so-called Mohs micrographic surgery is a “gold standard” for the treatment of advanced and recurrent basal cell carcinomas, where the tumor and the surrounding skin are visualized microscopically, giving the clearest picture of pathological changes in the periphery of the resected tissue [8] [9].

In our two cases, island flap was performed with good results. Mohs micrographic surgery (MMS) should be a priority for tumours that show histologically infiltrative, sclerosing, micronodular or recurrent features [5] [8] [9]. Basal cell tumours measuring over 1 cm, aggressive histology and age of the patient over 80 years are thought to be the main MMS indicators [4] [5] [8] [9]. The clue of MMS is the complete control of the peripheral and deep resection fields, allowing re-excision and microscopic extension of the resection boundaries in a precisely defined surgical zone [8] [9]. This allows avoiding unnecessary removal of additional tissue, which is a common practice in conservative surgical techniques [5] [8] [9]. These properties make MMS a reliable treatment for carcinomas, especially when it comes to delicate areas such as the face [5] [8] [9]. The results from the studies conducted so far have shown that MMS provides the lowest relapse rate between 0 and 2.5%, and therefore, this treatment should also be the first choice for relapsing basal cell carcinomas of the face [4] [5] [8] [9] [10].

In conclusion, island flap should not be a therapeutic option in patients with recurrent basal cell carcinomas and those who have undergone radiotherapy. Mohs micrographic surgery (MMS) in these patients ensures maximum safety, although the aesthetic results are less acceptable than in island flap. In patients with primary, untreated, small to medium basal cell carcinomas, island flap is a good therapeutic solution.

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Case Report

A Saudi Infant with Vici Syndrome: Case Report and Literature Review

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Keywords: Vici syndrome; EPG5 gene; Cardiomyopathy

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Abstract

INTRODUCTION: Vici syndrome, a rare autosomal recessive disorder, was first described in 1988 by Vici et al. Only 78 cases have been reported to date. The syndrome is characterised by agenesis of the corpus callosum, hypopigmentation, cardiomyopathy, progressive failure to thrive, dysmorphic features, immunodeficiency and cataracts. Mutations in the gene epg5 have been identified as the cause of Vici syndrome.

CASE DESCRIPTION: The parents are a consanguineous Saudi couple with two other children diagnosed with Gaucher disease. The patient was born at term and in the first 5 months had many hospital admissions for a recurrent chest infection. Physical examination, investigations and imaging studies revealed that the patient had agenesis of the corpus callosum, cataracts, psychomotor delay, immunodeficiency and hypopigmentation. The initial echocardiogram was normal. At 7 months, genetic testing confirmed the diagnosis of Vici syndrome with a c.3693G>Ap (Gln1231Gln) mutation in the gene *EPG5.* The patient developed a chest infection and was admitted to the pediatric intensive care unit. An echocardiogram was repeated and showed significant left ventricular dilation with a Z-score of 3.1, moderate mitral and tricuspid regurgitation, and depressed ventricular function with a fractional shortening of 17% and ejection fraction 37%. The patient's condition deteriorated, and he died aged 8 months.

CONCLUSION: The symptoms of extensive system involvement in Vici syndrome have been present in the majority of reported cases and should prompt careful evaluation of this syndrome when such symptoms are present in an infant. In confirmed cases, close monitoring of the immune status and cardiac function, the two main causes of death among Vici syndrome patients, is vital to prevent rapid deterioration and improve life expectancy.

Introduction

Vici syndrome (VICIS) (OMIM 242840) is a rare autosomal recessive disease belonging to the group of congenital disorders of autophagy. The syndrome has a wide range of presentations involving various body systems [1] [2] and is caused by a mutation in *epg5*, the gene for ectopic P-granules protein 5 (EPG5) on chromosome 18 [2]. The EPG5 protein is responsible for regulating autophagy activity, a pivotal mechanism for the development and proper functioning of body organs. The first two cases were described by Vici and his colleagues in 1988 when they reported on two siblings with a set of clinical features comprising agenesis of the corpus callosum, cutaneous hypopigmentation, bilateral cataract, cleft lip and palate, and combined immunodeficiency [3].

Since that original description of the disorder, an increasing number of cases have been reported, with almost 78 confirmed cases published to date [3] [4] [5] [6] [7] [8] [9] [10] [11] [12] [13] [14] [15] [16] [17] [18] [19] [20] [21] [22]. The patients have presented, mostly in infancy, with characteristic features of VICIS together with other phenotypic features such as progressive failure to thrive, microcephaly, nystagmus, dysmorphic features, cardiomyopathy, hypotonia, and recurrent pulmonary infection, among others [4]. Features such as hearing loss, lung hypoplasia, and renal tubular necrosis have also been described in isolated cases [6] [7] [8].

In this report, which includes a review of all literature to date, we describe in detail a previously reported case [9] of an 8-month-old male infant diagnosed with VICIS.

Case Description

We report on a boy, the third child of healthy consanguineous first-cousin Saudi parents. His two brothers are confirmed cases of Gaucher disease. The boy is a product of spontaneous vaginal delivery at 36 weeks gestational age with birth weight 3.2 kg. At 10 days of age, he was admitted to the neonatal intensive care unit because of meconium aspiration and group B streptococcus sepsis.

During the first month of life at home, the patient developed poor sucking and choking attacks with each feeding. At the age of 2 months, he was admitted to an outside hospital as a case of aspiration pneumonia and was treated accordingly. Diagnosis of gastroesophageal reflux disease was also made, and he underwent a fundoplication. During that admission, the patient was evaluated for the choking attacks and hypotonia. Chromosomal analysis revealed a normal karyotype (46XY).

At 5 months of age he was admitted to our hospital, for the first time, as a case of pneumonia. On physical examination, he also had profound hypotonia and albinism. Abdominal ultrasound was unremarkable and ruled out any organomegaly for other metabolic syndromes. Visual evoked response (VER) was significant for bilateral P100 prolongation and abnormal NPN configuration. VICIS was suspected as a cause of his multisystem symptoms. Magnetic resonance imaging (MRI) of the brain showed complete agenesis of corpus callosum, hypoplastic pons, and volume loss of white matter (Figure 1). Echocardiographic evaluation showed normal function with only mild left ventricular hypertrophy and fractional shortening of 33.1%. Genetic testing identified a pathogenic variant of *epg5* (c.3693G>A p. (Gln1231Gln) and confirmed the diagnosis of VICIS.

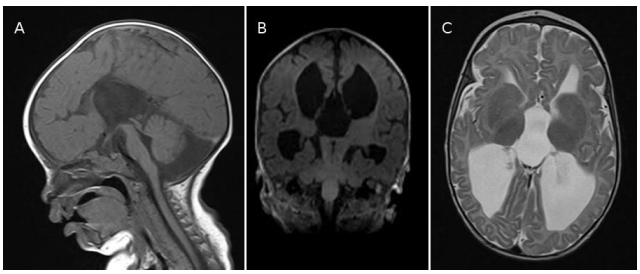

Figure 1: Brain MRI at age 6 months. Medline sagittal T1 weighted sequence (A), showing agenesis of corpus callosum and hypoplastic pons. Coronal T1 (B) and axial T2 (C) showing loss of white matter and dilated ventricular system

Following this admission, the patient presented several times to our hospital with recurrent infections including bronchial pneumonia, urinary tract infection and gastroenteritis. At the age of 7 months he presented to the emergency department following a 3-week episode of bloody diarrhea with severe dehydration. On physical examination, the patient looked pale, cachectic, not oriented, with sunken eyes and anterior fontanelles. His arterial blood gases revealed hypokalemia with metabolic acidosis (K = 2 mmol/L, pH = 7.28, PCO_2 = 37 torr, HCO_3 = 16.8 mmol/L). His weight had dropped 2.1 kg from the admission a month previously. He was admitted to the pediatric intensive care unit for rehydration and monitoring. During the course of this admission, the immunologic workup revealed reduced absolute B and T cell counts; immunoglobulins A, M and G were within the normal range. A few days later, the patient suddenly became apneic and developed respiratory distress. Chest X-ray revealed cardiomegaly. Repeated echocardiography showed dilated cardiomyopathy. His echocardiogram findings were significant for left ventricular dilation with a Z-score of 3.1, moderate mitral and tricuspid regurgitation, and depressed ventricular function with fractional shortening of 17% and ejection fraction 37%. He deteriorated quickly and developed renal impairment, which worsened his electrolyte imbalance and metabolic acidosis. Eventually, the patient went into cardiopulmonary arrest and died.

Discussion

VICIS is a rare disease that affects multiple systems of the body. Since the condition was first described in 1988, a total of 78 cases —including our patient's—have been reported (Table 1). The incidence of this disease is yet to be determined. VICIS was first linked to a recessive mutation in *EPG5* in 2013 [1]. The EPG5 protein is responsible for regulating autophagy activity, which is a survivor-cell mechanism that selectively removes misfolded proteins and organelles and plays a paramount role in ensuring good development of body systems, especially the nervous system. In our patient, genetic testing showed a c.3693G>A p.(Gln1231Gln) mutation in *epg5*, confirming the diagnosis of VICIS.

Table 2 summarizes all the important features that have been associated with VICIS to date. The majority of reported cases were associated with recurrent infections (98.7%), agenesis of corpus callosum (97.4%), profound developmental delay (97.4%), skin involvement (96.2%), immunodeficiency (76.9%), cataract (62.8%) and cardiomyopathy (65.4%) with the hypertrophic type being most commonly reported. We suggest, therefore, that VICIS should be considered to head the list of differential

diagnoses for an infant who presents with all or most of these features.

Table 1: Summary of reported patients with Vici syndrome

Authors, year	Sex	Age (m)	Clinical outcome
Vici et al., 1988 [3]	M	24	Deceased
	M	36	
Del Campo et al., 1999 [4]	M	24	Deceased
	F	11	Deceased
	M	36	Alive
	F	16	Deceased
Chiyonobu et al., 2002 [5]	F	19	Deceased
	M	6	Alive
Miyata et al., 2007 [6]	F	12	Deceased
	M	11	Alive
McClelland et al., 2010 [7]	M	3	Deceased
Al-Owain et al., 2010 [8]	M	9	Deceased
Rogers et al., 2011 [10]	M	96	Deceased
	F	96	Deceased
Said et al., 2012 [11]	F	15	Deceased
Finocchi et al., 2012 [12]	M	24	Alive
Özkale et al., 2012 [13]	F	6	Deceased
Cullup et al., 2013 [1]	F	15	Deceased
	M	3	Alive
	M	48	Alive
	M	36	Alive
	M	9	Alive
	M	36	Alive
	M	24	Alive
	M	24	Alive
	F	120	Alive
Ehmke et al., 2014 [14]	M	3.5	Deceased
Filloux et al., 2014 [15]	F	17	Alive
Tasdemir et al., 2015 [16]	M	9	Deceased
	M	8	
El-Kersh et al., 2015 [17]	F	72	Deceased
Byrne et al., 2016 [2]	NS	NS	NS
Huenerberg et al., 2016 [18]	F	11	Deceased
	F	13	
Maillard et al., 2017 [19]	F	24	Alive
	F	84	Alive
	F	24	Alive
	F	180	Alive
	F	12	Deceased
Hori et al., 2017 [20]	F	48	Alive
	F	24	Alive
	M	168	Deceased
	M	60	Alive
	M	84	Alive
Hedberg-Oldfors et al., 2017 [21]	M	8	Deceased
Elsayed et al., 2018 [22]	M	7	Deceased
This Case	M	8	Deceased

M = male; F = female; NS = not specified; m months.

Our findings support those published in a review of 38 cases [2] of VICIS where agenesis of corpus callosum, profound developmental delay and immune problems were the most common shared features.

Table 2: Common clinical features of 78 cases of Vici syndrome

Feature	Positive	Negative	Not reported	n (%)
Recurrent infections	77	-	1	77/78 (98.7)
Corpus callosum agenesis	76	-	2	76/78 (97.4)
Profound developmental delay	76	1	1	76/78 (97.4)
Cutaneous manifestations	75	3	-	75/78 (96.2)
Immune system involvement	60	16	2	60/78 (76.9)
Cardiomyopathy	51	18	9	51/78 (65.4)
Cataract	49	25	4	49/78 (62.8)
Microcephaly	45	15	18	45/78 (57.7)
Hypotonia	37	-	41	37/78 (47.4)
Seizures	26	16	36	26/78 (33.3)
Growth retardation	24	-	54	24/78 (30.8)

Concurrently with the clinical assessment, there are multiple investigations to help shorten the list of differential diagnoses and assess the extent of organ involvement [23] [24]. Such tests include laboratory investigation, imaging, molecular and other tests. Laboratory investigations will be directed to assess immunodeficiency and the extent of involvement of other organs such as liver and kidneys.

For imaging, a brain MRI is essential for detection of agenesis of corpus callosum and other less specific neuroradiologic abnormalities that have been reported, such as vermis and pons hypoplasia. Chest X-ray and echocardiography are also of benefit for assessing lung and cardiac involvement, respectively. Abdominal ultrasonography helps to confirm laboratory findings, whether abdominal organs are affected or not.

Confirmation of diagnosis requires molecular genetic testing to identify the homogenous or compound heterogenous mutated *epg5*. Other useful tests for VICIS cases are ophthalmologic tests and electroencephalography (EEG), especially if seizures are present. Our patient underwent all these investigations apart from the EEG, as seizures were absent; this helped to confirm the diagnosis and identify the present complications, thus allowing us to tailor our interventions.

VICIS is a progressive disease with poor prognosis. Survival analysis shows that VICIS patients have a median survival time of 24 months (95% confidence interval, 0–39 months) [2]. Thus, therapeutic interventions for VICIS are merely supportive and directed to relieve the symptoms that result from multi-organ involvement and to improve the survival time.

The most common causes of death among all reported patients with VICIS are recurrent infections and cardiomyopathy. Cardiac functions, in particular, can deteriorate from baseline in a short time, as happened in our patient. Immunity status and cardiac function, therefore, need to be regularly monitored at short intervals for early detection of their VICIS-related manifestations, thus allowing timely intervention for a better outcome and prolonged survival.

In conclusion, in VICIS, the symptoms of extensive system involvement (such as agenesis of corpus callosum, profound developmental delay, recurrent infections and immunodeficiency) that present in the majority of cases should prompt a careful evaluation for this syndrome. When the diagnosis is confirmed, close monitoring of the immune status and cardiac function is vital to prevent rapid deterioration and improve life expectancy, as these have been the two main causes of death among VICIS patients in all known cases to date.

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Case Report

One Step Melanoma Surgery (OSMS) Without Using Ultrasonography for Preoperative Tumour Thickness Measurement? - "A Question that Sometimes Drives Me Hazy: Am I or Are the Others Crazy! "

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Keywords: Melanoma surgery; One step; Without tumour thickness measurement; Postoperative results

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Abstract

BACKGROUND: One step melanoma surgery is a new surgical approach by which specific groups of patients with cutaneous melanoma may be operated only by or within a single surgical session. Until now, the Bulgarian Society for Dermatologic Surgery (BULSDS) has presented models of clinical behaviour, in which preoperative measurement of tumour thickness in combination with echographic measurement of the locoregional lymph nodes could lead to the conduct of the so-called one-step melanoma surgery. Although this one step surgery currently does not fit in the recommended guidelines, it ensures compliance of the recommended boundaries of operational security while saving patients a repeated excision and relieves the healthcare institutions or the patients themselves financially.

CASE REPORT: We at this moment present another case from the Bulgarian Society for Dermatologic Surgery (BULSDS) of one step melanoma surgery with a perfect end result, where the tumour thickness was not preoperatively determined by high-frequency echography. Preoperative assessment of tumour thickness was performed based on the clinical picture and dermatoscopy. The histologically established tumour thickness was identical to the preoperative assessment, i.e. <1 mm. Removal of the melanocytic lesion was performed with operational security field of 1cm in all directions, where, as a rule, no further removal of the draining lymph nodes is required.

CONCLUSION: One step melanoma surgery has two significant advantages: 1) it saves a re-excision in certain groups of patients, which in turn is 2) significantly more favourable from a financial point of view. Its applicability in the appropriate groups of patients and the postoperative (although in a limited number of patients) results achieved indicate the need to optimise the current algorithms and direct them individually to each patient. Guidelines may not and should not be unified or set strict limits given the fact that they show a significant level of variability themselves regarding some key moments in the initial surgical treatment of melanoma. More than 10% of the primary melanoma cases refer to thin melanomas, and dermatoscopy and clinics are a sufficient method of optimising the planned surgical excision.

Introduction

Worldwide, melanoma surgery goes through the so-called guidelines [1]. One step melanoma surgery is a new, innovative approach that is still very little discussed in medical circles [2]. It applies to specific groups of patients with cutaneous melanomas and very clearly demonstrates the need for individual preoperative assessment of each clinical case [2]. Through it (based on the experience and individual assessment of clinical, dermatoscopic and ultrasonographic images of lesions and locoregional lymph nodes), an optimal diagnostic evaluation is achieved, as well as a subsequent adequate treatment of cutaneous melanoma [3]. As a major advantage of this method, we may point out the basic fact that the appropriate patient groups are “spared” from the unnecessary, in some cases, repeated surgical intervention [4].

We at this moment present another case from the Bulgarian Society of Dermatological Surgery (BULSDS) of a patient with psoriasis with cutaneous melanoma localised in the area of regio abdominalis

dextra. He underwent one step melanoma surgery technique (without initial echographic measurement of the tumour thickness), other than the established guidelines for treatment of malignant melanoma, with a perfect end result. The applicability of this type of surgery according to the individual characteristics of each patient, as well as the need to optimise the current guidelines, is currently under discussion.

Case Report

A 45-years-old man is presented in good general status and concomitant hypertension controlled with Aspirin 100 mg (0-0-1) and Irbesartan/Hydrochlorothiazide 150 mg/12.5 mg (1-0-0). No family history data is available. The patient was first hospitalised in the clinic for a few months worsening of the clinically diagnosed psoriasis vulgaris. The disease was diagnosed 20 years ago and has not been histologically verified.

During the dermatological examination, it was found that the pathological changes involved the skin of the capillitium, back and gluteal area, represented by erythemosquamous papules, which in some places merged into plaques. Also, there was evidence of maceration and mycotic infection in the inguinal area. In the regio abdominalis dextra, a hyperpigmented macula with uneven pigmentation and uneven edges, which existed for many years, was observed (Figure 1a, 1b). Clinically and dermatoscopically, this finding met the requirements for a malignant melanocytic lesion.

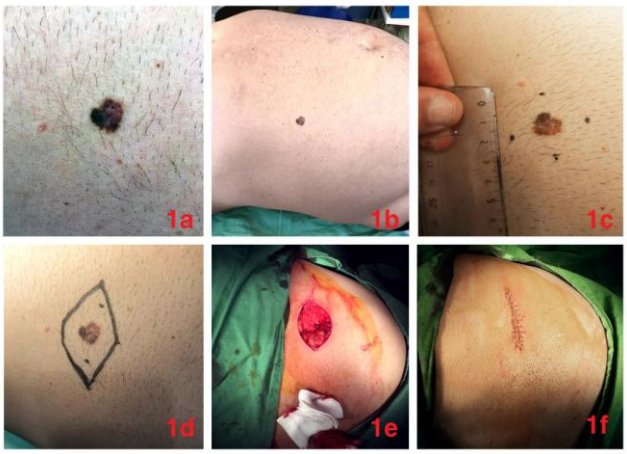

Figure 1: 1a, 1b) Clinical picture of primary cutaneous melanoma located in regio abdominalis dextra. Lesion with uneven pigmentation; 1c, 1d) Outlining the 1 cm operational security boundaries in all directions, preoperative finding; 1e) Intraoperative picture of the lesion removed by elliptical excision; 1d) Postoperative clinical picture of surgical defect closed by single interrupted sutures

During the hospitalisation some examinations were performed, where the paraclinical results were within the normal range, and the panoramic image of the teeth showed evidence of periodontitis chronic granulomatosa 4/5. The rest of the instrumental examinations showed no evidence of abnormal values.

Selective ultraviolet therapy with gradual dose escalation was initiated. Topical therapy included 10% salicylic oil, 10% salicylic ointment and cooling cream. Histology confirmed that it was psoriasis vulgaris. It was recommended to treat the dental focal infection in outpatient conditions and discontinue Aspirin and Hydrochlorothiazide therapy (as possible triggers of psoriasis). After consultation with a cardiologist, Valsartan 160 mg x 1/day therapy was given. For the mycotic infection in the inguinal area, Fluconazole 200 mg x 1/day was administered i.v. Systemically for 3 days, and Clotrimazole cream x 2/day was topically administered.

During the hospitalisation, the pigment lesion, localised in regio abdominalis dextra, suspected for malignant melanoma, was surgically removed. Radical removal was performed under local anaesthesia. Based on the clinical and dermatoscopic evidence of the lesion, the surgery was performed with an operational security field of 1cm in all directions (Figure 1c, 1d, 1e). This was followed by a closure of the resulting defect with stretch plaster (Figure 1f). The histological examination confirmed the diagnosis: Superficial spreading malignant melanoma. Clark level II, Breslow thickness below 1 mm, no ulceration, low mitotic activity, well-expressed lymphocyte stromal reaction, no evidence of spontaneous regression, clear resection lines (p T1aR0). The staging was performed, according to which it was found to be Stage I (T1aN0M0).

Discussion

Malignant melanoma is a diagnosis, which diagnostics and treatment make clinicians think of the progress of medicine and whether we tend to be flexible and innovative [1]. Currently, the American Joint Committee of Cancer recommendations for surgical treatment of primary melanoma is based on Breslow thickness and include resection of 0.5 to 1 cm for melanoma in situ; however, 1.0 cm is also the recommended resection area for melanomas between 1.0 mm to 2 mm?

And according to the recommendation table, this area varies or may vary between 1-2 cm for melanomas with a thickness between 1.01-2.00 mm?

Two cm is also the recommended area for treatment of melanomas with a thickness of 2.01-4 mm [1] [5]. For melanomas with thickness over 4 mm,

resection areas of more than 2 cm in all directions are not recommended [5].

If we are guided by the current guidelines, treatment of melanocytic lesions should start with resection with surgical security margins of 0.4-0.5 cm in all directions, followed by postoperative measurement of the tumour thickness and mandatory re-excision planning within short-term deadlines [4]. Depending on the already established tumour thickness, an assessment should be made at a subsequent or later stage as to the need of re-excision with or without removal of draining lymph nodes [4]. The question arises inevitably whether it would be appropriate to place the various patients under a common denominator or to individualise the therapy, to be innovative and flexible in our decisions? Whether in the modern world of technology, guided to a great extent by the so-called “artificial intellect”, it is better not to act mechanically, or in other words, according to preset algorithms and guidelines, but to think and treat each patient separately and individually? Due to this circumstance, after having observed dozens of patients with melanomas in various phases, we have concluded that the guidelines (in their current version) are not the optimal solution for treatment of melanomas.

Between 1977 and 1980, the idea of excision was adopted for treatment of malignant melanomas, depending on the anatomical features of the area, i.e. such that allows for primary closure of the surgical defect [6]. It varied between 1 and 5 cm even for thin melanomas [7]. From the remoteness of the tumour tissue from the histologically established resection margins (histologically), this method should be more accurate and reliable. In their current version, the guidelines for treatment of melanoma require only an operational security field, which should only be observed clinically and not histologically. It is this very fact that contains a great contradiction and illogicalness.

Over time, the change in the clinician’s thinking has led to optimisation of the recommendations, as well as their gradual change and update. However, they do not give a specific answer to the question why there are again variations in the centimetres of the operational security field (0.5-1 cm for melanoma in situ, 1-2 cm for the thickness of 1 to 2 mm) and often the free interpretation of the guidelines could be reached [1]. What is it that requires these variations in tumours with thickness up to 2 mm? Why is there no exact categorisation of patients’ subgroups according to certain resection boundaries!? It is unclear what is it that requires the variability of these boundaries in thin to medium-thick tumours (find a reference).

It is precisely this that requires guidelines to be redefined and modernised so that they become as personalised as possible. One step melanoma surgery is a new approach that allows us to go beyond the traditional and help the patient as adequately as possible, not to take into consideration the set boundaries. To support early personalisation, which is extremely promising, simple, reliable and easy to apply?

The main diagnostic steps that may be used preoperatively for suspected pigmented or pigment lesions are 1) clinical examination, 2) dermatoscopy, 3) biopsy and histological examination, 4) measurement of tumour thickness by high-frequency ultrasonography, 5) echography and biopsy of lymph nodes [3] [8].

Tumour thickness in malignant melanomas is, in fact, the main indicator determining the action plan, the choice of surgical boundaries, the re-excision, the additional determination of a draining lymph node and the possible lymph dissection [9]. For the patient presented by us, the decision for an initial excision with an operational security field of 1cm in all directions was taken entirely based on 1) the clinical experience accumulated over the years, and 2) the dermatoscopic finding, which undoubtedly indicated melanoma with tumour thickness < 1 mm. The subsequent histological examination of the removed lesion confirmed the initial diagnosis of malignant melanoma with a thickness of less than 1mm, without ulcerations, without increased mitotic activity or angiolymphatic invasion. This clinical case is an example of how both diagnostic determination and subsequent treatment are achieved in a single step. Although it does not meet the recommendations based on European and American guidelines, one step melanoma surgery ensures complete removal of melanoma, meanwhile taking into account the operational security for the respective tumour thickness required or recommended by the guidelines [3]. The difference is that the intervention is only one. Thus, the desired result is achieved within one instead of two operational sessions. This leads to another important difference, i.e. optimisation of the financial coverage of patients or healthcare institutions. The method is only applicable to a limited number of patients (the method without preoperative echographic measurement of the tumour thickness). The question remains, as the guideline authors would say: “Is it worth changing the guidelines, given that these changes will not benefit a large number of patients?” The logical answer would be: “Regardless of the postulates of these guidelines, an increasingly small number of colleagues are guided by them while achieving optimal outcomes for their patients. That is, the guidelines are disregarded and become unnecessary, at least regarding the primary treatment of melanomas!”

Preoperative high-frequency ultrasound diagnostics in cases of thin and medium-thick, as well as thick primary melanocytic lesions, would be extremely useful for determining the operational security boundaries, the indications for lymph node biopsy, and most importantly, the need for re-excision

[9]. However, based on the case presented by us, it should be concluded that ultrasonographic measurement of tumour thickness is not mandatory (in certain groups of patients) (given that it is not even mentioned in the commonly accepted guidelines for treatment of melanoma). Various studies suggest that the tumour thickness measured by ultrasonography corresponds to a very high degree to the histologically established postoperative Breslow thickness [2] [3] [8] [9]. This means that its application (tumour thickness measured by echography) may lead again to avoidance of re-excision and performance of one stage melanoma surgery for patients with melanomas of various tumour thickness [10]. That is, it is a possible addition to the diagnosis of melanomas but is not obligatory. Choosing an optimal approach is clearly in the hands of the clinician.

In thin melanomas with clear clinical and dermatoscopic evidence, dermatoscopic and clinical data on melanoma may be crucial. The clinician should have an individual conversation with the patient within which he/she should provide detailed information on the one-step model of melanoma surgery, its advantages and possible disadvantages. After a signed informed preoperative consent, the procedure should be performed as quickly as possible.

Melanomas referred to in panel 2 (Figure 2a, 2b, 2c, 2d) show a tendency to infiltrative growth and may not be subject to the innovative method of one step melanoma treatment, i.e. without measurement of the tumour thickness by echograph. In such cases, the decision for one step surgery based on clinical and dermatoscopic findings should be defined as high-risk, unreasonable, or rather, a wrong step. In these cases, if we consider the possibility of treatment according to the one stage melanoma surgery method, an initial measurement of tumour thickness should be made by ultrasonography and additionally, by echography of the lymph nodes. Depending on the evidence obtained and the available findings, the approach to the patients should also be varied: 1) for melanomas from 1 mm to 4 mm, resection should be done with an operational security field of 2 cm [11] and dissection of draining lymph node (regardless of whether the node is echographically enlarged) [1] [12] for an established tumour thickness above 4 mm- again a resection with an operational security field of 2 cm in all directions, and it should be remembered that in the event of thickness above 4 mm, there are other two treatment suboptions and at the same time staging of melanoma, namely: 2.1) if there are no enlarged lymph nodes, 2 cm resection is sufficient, together with control/instrumental examinations every two months, 2.2) in the presence of enlarged lymph nodes, it is recommended that they will be removed and examined for the *BRAF V600* mutation [13], followed by computer tomography scan (CT) with contrast or PET scan, the latter at least two months postoperatively. In fact, *BRAF* typing and identification of mutations in the *BRAF* gene helps determine the direction of treatment for patients with malignant melanoma [14]. Depending on the number and localisation of the existing (or additional metastasis), treatment should be individualised. Some units recommend metastasectomy and monitoring for recurrences, which should be tested in turn for *BRAF V600*, but at a later stage. Other units recommend starting an adjuvant Interferon treatment or vaccination, although patients are surgically “cleared” of metastasis/metastases.

Figure 2: 2a, 2b) Clinical view of melanoma showing uneven pigmentation and uneven edges located in the epigastrium; 2c, 2d) Clinical picture of intense black-coloured cutaneous melanoma located in the forehead area

And MEK inhibitors therapy has been shown to significantly improve survival in patients with mutations in these genes [14]. In turn, CT/PET scan images help to better stage melanomas and detect metastases [15]. CT has a sensitivity of about 60% in the detection of systemic metastases [15]. The right approach to the patients should be optimised, and perhaps the solution is to find a balance between the guidelines and one step melanoma surgery. In other words, it is a matter of a point of view or mutual compromise.

In patient number 4 (Figure 3a, 3b) there is clinical and dermatoscopic evidence of melanoma indicating that it is a tumour with a thickness of more than 2 mm. From what has been said so far, if the thickness is above 1mm and less than 4mm, the draining lymph nodes must be removed and examined. Monitoring of the locoregional finding after an initial excision should not be advisable. Determining a draining lymph node in such cases is highly recommended, and if possible, it should be done within one surgical session, along with removing

the primary melanoma, which would be a more sparing solution for the patient (for tumour thickness up to 4 mm).

Lymph nodes dissection depends on the echographic evidence of lymph nodes enlargement, on the one hand, and whether the tumour thickness is more than 4mm, on the other. It is believed that if the evidence is indicative of primaries with a measured tumour thickness above 4 mm, lymph dissection is not so important since then the metastatic spread of a tumour (in the event of intact echographic lymph nodes) is most likely to have occurred. It is possible, however, that the lymph nodes are “bridged”, i.e.: 1) there are accessory parallel lymphatic pathways other than the flow leading to the draining lymph node; or 2) the tumour cells have not been stopped in the draining lymph node and have passed through it, or 3) the tumour cells have already spread haematologically before the involvement of the lymph node.

Figure 3: 3a, 3b) Clinical finding of melanoma located in regio abdominalis sinister showing an ulcerated surface; 3c) Intraoperative status after oval excision; 3d) Postoperative clinical finding

As for our patient number 4 in particular, if the echographic tumour thickness is above 4 mm, surgery should be performed with a 2 cm operative security field in all directions, followed by regular control of the locoregional and distant lymph nodes (if these lymph nodes are not increased/clinically and echographically negative). In other words, we are talking again about single surgical intervention. If, however, we do not use an individual approach and follow the accepted guidelines, this patient should have a primary excision with a 0.5 cm field and a second surgical intervention with another 1.5 cm field in all directions (as it was done). However, in this case, the decision, like in the previous cases in Panel 2, should not be only based on clinical and dermatoscopic data. Such a decision should be defined as a poor assessment, lack of experience, a poorly based approach. If a one-step model of surgery, deviating from the guidelines, is chosen, an echographic measurement of tumour thickness and locoregional lymph nodes here is recommended. The evidence established in these cases should determine the model of clinical behaviour, and the applicability of the new methodology presented.

At this stage, malignant melanoma surgery should not be considered as completely dependent on the accepted guidelines. Any deviation from them must still be logically justified and supported by appropriate documentation. One step melanoma surgery is a bold decision taken by both our colleagues and patients and ultimately, the results achieved may be defined as more than optimal.

This good receptivity, accompanied by maximum adequacy and precision of echographists, dermatoscopists and dermatosurgeons actions approaches innovative and deserving particular attention. The several different cases of melanoma patients described showing that each of them is unique and should be interpreted individually, even if it means getting out of the comfort and security zone provided by the guidelines. One step melanoma surgery should be one solution for each of the above cases.

In conclusion, when clinical and dermatoscopic data indicate the presence of melanoma with a possible predicted tumour thickness of less than 1 mm, one step melanoma surgery is a very good or optimal solution.

Whenever we have doubts about our assessment of the tumour thickness, based on our experience alone (clinical, dermatoscopic), high-frequency ultrasonography is the option that could provide maximum security about the right approach and assurance of the clinician’s actions.

With this clinical case, we present a new algorithm or an option to treat cutaneous melanomas through one surgical intervention. The guidelines for the diagnosis and treatment of melanomas that we use should be individually adapted to the various groups of patients rather than being used universally and often or almost always remain ambiguous. One step melanoma surgery relieves the psychological, physical and financial burden for the patients and this makes it an approach that deserves the attention of the medical college.

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Case Report

Endometrioid Adenocarcinoma Arising in Adenomyoma in a Woman with a Genital Prolapse - Case Report

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Keywords: Endometrial cancer; Uterine leiomyoma; Genital prolapse

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Abstract

BACKGROUND: Endometrial cancer is the third-ranked genital malignancy in women and includes 3% of cancer deaths. There is a 2.8% chance of a woman developing endometrial cancer during her lifetime. Low-grade endometrioid adenocarcinomas are often seen along with endometrial hyperplasia, but high-grade endometrioid adenocarcinomas have more solid sheets of less-differentiated tumour cells, which are no longer organised into glands, often associated with surrounded atrophic endometrium.

CASE REPORT: We present an unusual case of endometrial adenocarcinoma arising in adenomyoma in 74-year old woman presented with genital prolapse, without other clinical symptoms. Ultrasound evaluation revealed endometrium with 4 mm-thickness and atrophic ovaries. The cervical smear was normal. The patient underwent a total vaginal hysterectomy. The histopathology of the anterior uterine wall revealed an intramural adenomyoma of 4 mm in which some endometrial glands with malignant transformation of well-differentiated endometrioid adenocarcinoma without infiltration in surrounding myometrium and lymphovascular invasion were present. The endometrium lining the uterine cavity was predominantly atrophic, and only one focus of simplex and complex hyperplasia was found, with cell-atypia. According to AJCC/FIGO 2010, the tumour was classified: pTNM = pT1B pNX pMX G1 R0 L0 V0 NG1, Stage I. On dismiss, the near-future oncological consultation was recommended.

CONCLUSION: We would like to point out the rare occurrence of such type of malignancy and the importance of meticulous histopathology evaluation, even after reconstructive surgery for genital prolapse.

Introduction

Endometrial cancer is the third-ranked genital malignancy in women after cervical and ovarian cancer and accounted for 3% of all cancer deaths in women. Endometrial adenocarcinoma occurs mostly in the postmenopausal age. There is a 2.8% chance of a woman developing endometrial cancer during her lifetime.

The cancer cells in endometrioid adenocarcinoma grow in patterns evocative of normal endometrium, with new glands arising from columnar epithelium with some abnormal nuclei. Low-grade endometrioid adenocarcinomas are often seen along with endometrial hyperplasia and most often do not invade the myometrium. Higher-grade endometrioid adenocarcinomas are characterised with less well-differentiated cells and have more solid sheets of tumour cells no longer organised into glands, and are associated with an atrophied endometrium. Smooth muscle tumors of the uterus consist of a broad family of tumors.

Leiomyomas are the most common gynaecological neoplasm. In this case, we had an unusual case of endometrial adenocarcinoma arising in adenomyoma. Leiomyoma combined with focal adenocarcinoma of the endometrium is the only rare published article found after the persistent search for the same.

Case report

We present an unusual case of endometrial adenocarcinoma arising in adenomyoma in a 74-year-old woman, presented with genital prolapse and no other clinical symptoms. The gynaecological examinations revealed the normal ultrasonographic appearance of the uterus, endometrial lining and ovaries. PAP smear showed normal findings. Her menopause occurred at the age of 49 years, and she never used hormonal therapy. She has no history of previous medical or surgical illnesses, nor family history of malignancies.

She was admitted to our gynaecological department for operative treatment of the genital prolapse. Pelvic examinations revealed subtotal uterine prolapsed (POPQ2) along with supravaginal cervical elongation and predominant cystocele. The ultrasound examination revealed the normal appearance of the uterus, endometrial lining and ovaries, appropriate for the patient's age. Results of urinalysis and blood chemistry tests were within normal range.

A total vaginal hysterectomy without salpingo-oophorectomy was performed. The operative material was sent for final histopathological evaluation.

The gross pathologic examination revealed an intramural leiomyoma of 4mm in diameter in the outer half of the frontal side of the uterus.

The histological analysis of this tumour revealed the morphology of adenomyoma in which there was a malignant transformation of endometrial glands in a well-differentiated endometrioid adenocarcinoma, without infiltration in the surrounding leiomyoma tissue (Figure 1, 6).

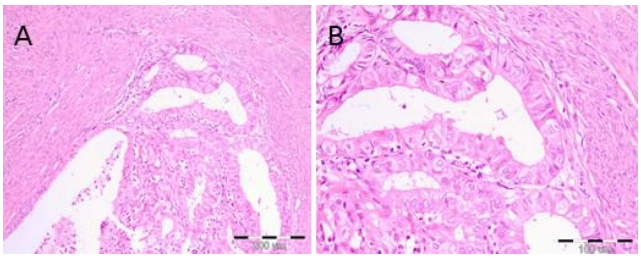

Figure 1: A) G1/NG1 endometrioid adenocarcinoma arising in adenomyoma (HE x100); B) The same lesion on higher magnification (He x200)

The tumour cells stained positive for cytokeratin and progesterone (Figure 2) and the mitotic index examined by the Ki67 immunostaining was about 10% (Figure 3). The lymphovascular invasion was not found.

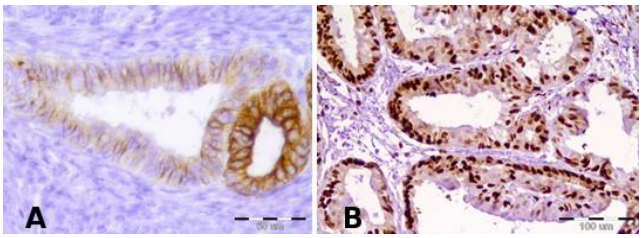

Figure 2: A) Immunostaining with CK7-positive for the epithelial component (x400); B) Immunostaining with Progesterone (Pr x200)

The histopathology also revealed that the endometrium lining uterine cavity was atrophic, except one focus where simplex and complex hyperplasia with cell atypia was found (Figure 4).

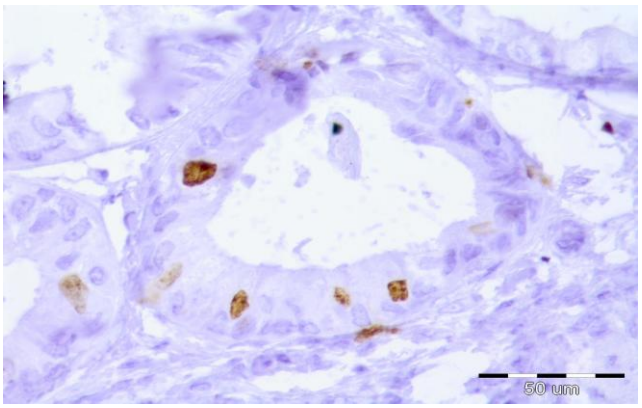

Figure 3: Immunostaining with Ki67 (x400)

Another few areas of adenomyosis in the uterine wall were found with simplex and complex hyperplasia with atypia in the endometrial epithelium.

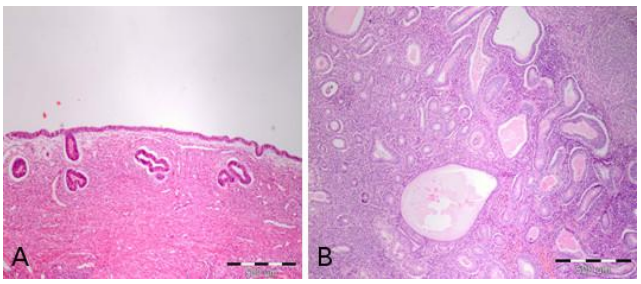

Figure 4: A) Atrophic endometrium (HE x40); B) Hyperplastic endometrium (He x100)

Histologically, the leiomyoma showed well-differentiated tumour cells with abundant eosinophilic cytoplasm, elongated nuclei, and indistinct cell borders. There were no mitotic figures, and the mitotic index examined by Ki67 immunostaining was low. In the silver staining, argyrophilic fibres separated individual cells from each other. The cells stained positive for smooth muscle actin (Figure 5).

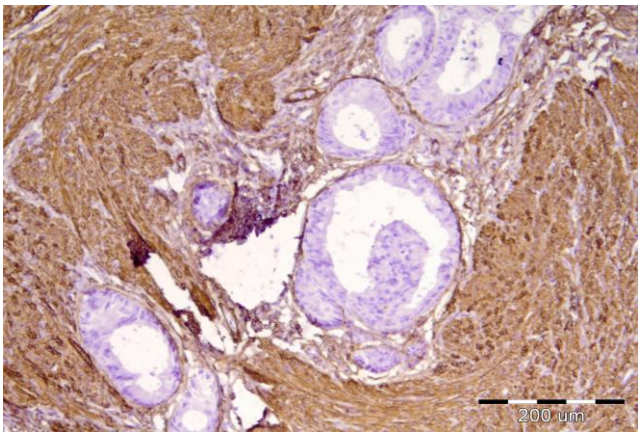

Figure 5: Immunostaining with SMA labelling smooth muscle cells (Smooth Muscle Actin x100)

According to AJCC/FIGO 2010, the tumour is classified: pTNM=pT1B pNX pMX G1 R0 L0 V0 NG1, Stage I.

A topical tumour was composed of epithelial and mesenchymal elements, and the former is completely contained into the latter.

Postoperative period was uneventful, and the patient was discharged 5 days after the operation in satisfactory condition. The patient was referred for oncological consultation.

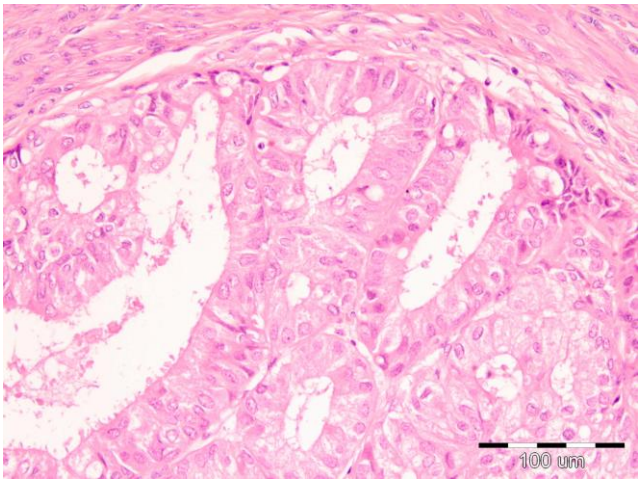

Figure 6: Well differentiated endometrioid adenocarcinoma showing a confluent glandular and cribriform pattern without intervening stroma (HE 10x20)

Discussion

Both, the endometrial adenocarcinoma and adenomyomas are common pathological findings that can develop in the uterus. Adenocarcinoma arising from adenomyoma is a rare entity. There are some cases described with endometrial adenocarcinoma arising from adenomyosis, as well as few reports of adenocarcinoma developing in adenomyotic areas without endometrial involvement, similar to the case that we present. Kurotaki et al., [1] reported a case of adenocarcinoma of the endometrium extending into the leiomyoma of the uterus in a rabbit. Qury et al., [2] reported for Endometrioid carcinoma infiltrating atypical leiomyoma and pointed the necessity of ruling out carcinosarcoma as it has an ominous prognosis. Toshiki et al., [3] reported a similar case of adenocarcinoma arising in adenomyosis without endometrial involvement, but there were already lymph node metastasis and serous uterine involvement by the time of the diagnosis. They made an immunohistochemical analysis of the growth factors, including estrogen receptor (ER), progesterone receptor (PR), p53, bcl-2 protein and Ki-67 antigen, and found no expression of ER, PR or bcl-2 in the carcinoma cells. However, there was a focal expression of p53 and prominent expression of Ki-67. These results suggest that some biological factors, including oncogenes and tumour suppressor genes, may play a role in the carcinogenesis of endometrial carcinoma arising from adenomyosis without surface endometrial changes. In our case, the adenocarcinoma arose from an adenomyoma focus situated in the outer half of the anterior uterine wall, endometrium in the most of its parts was atrophic and only in one focus simplex, and complex hyperplasia with cell-atypia was found.

The histological features of this tumour in our case may be misdiagnosed as a composite mixed Müllerian tumour, which is characterized by an admixture of epithelial and mesenchymal elements and could be categorized as adenofibroma, adenosarcoma, carcinofibroma or carcinosarcoma, depending on whether epithelial and mesenchymal components are benign or malignant. Malignant mixed Mullerian tumours of the uterus are composite tumours and are believed by many, to behave like metaplastic carcinomas. On the other hand, collision tumours are defined by co-existence of two tumors in the same or adjacent organs which are topographically and histologically distinct with minimal or no histological admixture. Collision tumor should be viewed as the occurrence of multiple synchronous tumors in the same organ or two adjacent organs. The components are separated from each other by normal stroma with little or no histological admixture. On the other hand, the composite tumors are characterized by the individual components, which are intimately admixed with each other, and it is not possible to separate them topographically. The two divergent lineages in composite tumors originate from the same neoplastic clonal proliferation. In contrast, the prognosis and management of collision tumors is dictated by the histology of its individual components [4]. Collision tumors of the uterus are rare with only a few such cases reported till date [5]. Most of these are composed of adenocarcinoma (endometrioid or

papillary serous) colliding with a sarcoma (leiomyosarcoma or endometrial stromal sarcoma) [6].

Based on the distinct topographical location and limited areas of tumour admixture of the two tumours, a diagnosis of collision tumour of a uterus comprising of endometrial adenocarcinoma and leiomyoma in our case was made. This case may be misdiagnosed as a carcinofibroma due to an admixture of adenocarcinoma and benign mesenchymal tumour, a leiomyoma. The leiomyoma-cells were well-differentiated and were not relevant to the stromal cells. Collision tumours have been described in various tissues, but they are rare in the female genital tract. Collision is generally used for the tumor where two kinds of malignant tumors collided. In our case, the tumor was composed of two distinct and separated parts and was considered to be a collision of two neoplasms rather than a malignant transformation of the benign mixed mesenchymal tumor.

Nadeem et al., [7] reported a case of a 60-year-old lady who presented with complaints of post-menopausal bleeding. A cervical biopsy was performed which showed a non-keratinizing squamous cell carcinoma of the cervix. In 1986, Woodruff et al., [8] reported two more cases of adenocarcinoma arising in adenomyosis without surface endometrial changes. Both patients were postmenopausal women who underwent a total hysterectomy with bilateral adnexectomy because of persistent atypical cytology, despite negative findings on biopsies obtained by curettage. The accurate diagnosis was made only after the histological examination of the surgical specimens. In our case was the same situation. None suspicion for endometrial carcinoma before the operation was present because of the absence of any clinical symptoms, such as vaginal discharge or abnormal uterine bleeding, as well as ultrasound findings of endometrial thickness>4mm.

In conclusion, collision tumours compound of endometrial adenocarcinoma arising in adenomyosis or leiomyoma are very rare events, and they generally represent pathologic findings of a surgical specimen. Diagnosis is often delayed because of the absence of a lesion in eutopic endometrium and is often made usually when the tumour has grown to involve the endometrium, causing abnormal uterine bleeding or has spread outside of the uterus. Reporting this case, we would like to point out the rare occurrence of such type of malignancy and the importance of strict protocols for histopathologic evaluation, even after reconstructive surgery for genital prolapse.

However, further researches should be made to clarify the pathogenesis of these tumours, namely the genetic abnormalities encountered in those neoplastic areas. Nevertheless, this clinical entity should be kept in mind, especially when these conditions persist after the menopause.

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Case Report

Recycling of Previously Transplanted Hair: A Novel Indication for Follicular Unit Extraction

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Keywords: androgenetic alopecia; Hair transplantation; follicular unit extraction; hair density; efficacy

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Abstract

BACKGROUND: Hair transplantation has enhanced the realm of procedural dermatology. Before the advent of follicular transplantation, androgenetic alopecia was a difficult disease to manage, as there is a limited armamentarium of topical and systemic pharmaceuticals. However, as with other novel surgical procedures, there is a steep learning curve, that may result in poor transplantation or cosmesis.

CASE REPORT: We present a case of androgenetic alopecia, where previously, poorly implanted hairs were recycled by follicular unit extraction to increase hair density at the vertex of the scalp, which resulted in improved cosmesis and patient satisfaction.

CONCLUSION: We have demonstrated that re-transplantation is not only feasible but is effective; therefore redesigning of previous transplantations should be considered as a possible indication follicle unit extraction, particularly in the setting of scarce follicular reserves. The utility of our recycling method may also inspire hope in patients that have undergone failed or unsatisfactory hair transplantations.

Introduction

Androgenetic alopecia is a very common condition in adult men. Hair transplantation is considered an effective treatment. The technique involves harvesting hair grafts from a donor area, mainly the scalp, and relocating the harvested follicles to an area of alopecia (e.g. receding hairline). In recent years, great progress has been made in hair transplantation, with the use of the follicular unit extraction and transplantation method. Follicular unit extraction rapidly becomes a popular technique for hair transplantation as it lacks the major drawbacks of strip harvesting, and can provide highly aesthetic results [1] [2].

Creating a natural and dense hairline is considered one of the greatest surgical challenges [3] [4]. Novice surgical skills and subsequent patient dissatisfaction frequently result in consultation for transplant removal; laser ablation is commonly used for removal. However, low donor hair reserves pose a challenge for re-transplantation. Re-utilizing poorly placed transplanted hair follicles is not presented in the literature as a treatment option for patients that are dissatisfied with their transplantation procedures. We present a case of hairline design dissatisfaction that was referred to our clinic two years after follicular transplantation.

We recycled the previously implanted hair follicles and relocated them to improve cosmesis and achieve patient satisfaction.

Case Report

A 26-year-old man presented to our hair transplant clinic for consultation. The patient had undergone hair transplantation two years prior, for type-seven androgenetic alopecia. He was not satisfied with the results of his transplantation; he was particularly dissatisfied with his transplanted hairline. The patient was originally referred to our clinic for laser hair removal of the transplant.

Figure 1: Extraction of follicles with punches

However, due to the patient's poor follicular reserves, he was offered a revision operation to re-transplant a portion of the previously misplaced hair follicles.

Figure 2: The extracted follicles are recycled and re-implanted in the vertex area of the scalp

After obtaining informed consent, a re-transplantation procedure was performed. The procedure was performed by the clinical standards of follicle unit extraction (F.U.E): 0.9 mm punches were used, and 32 grafts were obtained from the previously transplant area (Fig. 1). The extracted follicles were recycled and re-implanted in the vertex area of the scalp (Fig. 2). Five months after the revision surgery, 26 out of 34 implanted follicles grew successfully, resulting in an appropriate hairline and complete patient satisfaction (Fig. 3).

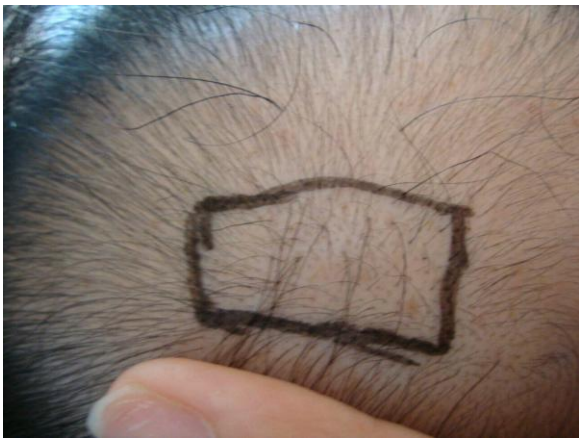

Figure 3: The patient five months after the treatment

Because of the good results, we performed, two other sessions of hair transplant, the first one just after the control and the second one six months later. Each time, we worked in the same modality of the first surgical procedure.

A control five month after the third surgery, showed excellent results regarding hair density (Fig. 4).

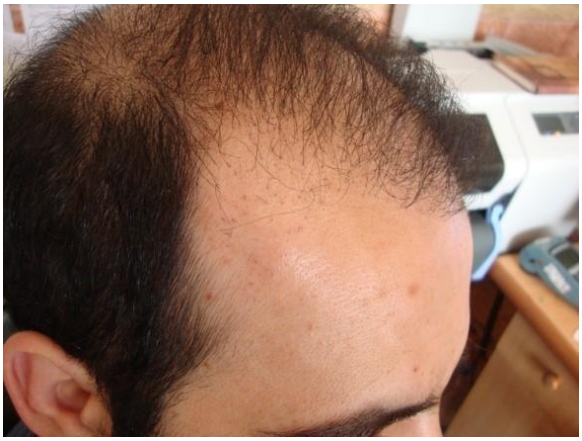

Figure 4: Our patient five months later the third hair transplant

Discussion

Patients and physicians alike are usually pleased with the results of contemporary hair transplantation, so much so that physicians can now recommend the procedure without reservation.

However, this does not guarantee complete patient satisfaction. Therefore, management strategies for dissatisfied patients should be developed and standardised [5]. Frontal hairline cosmesis is most critical in achieving patient satisfaction. A hairline placed too low will result in an unnatural appearance. Many dermatologists use laser hair removal procedures to amend improperly designed hairlines, which results in wastage of viable hair follicles. Herein we describe that transplanted hair follicles can be safely and effectively recycled for re-implantation.

The possibility of complications is associated with any surgical procedure. However, we did not encounter any adverse events in the above presentation. Nonetheless, patients should always be counselled on the potential risks associated with the recycling of pre-implanted follicles [6]. A major concern in recycling transplanted follicles may be that such follicles may lose viability for re-transplantation.

Lower follicular reserve at donor sites is considered to be a challenge, especially in primary hair transplantation, and should be particularly taken into account in the re-implantation setting [7] [8]. We have demonstrated that re-transplantation is not only feasible but is effective; therefore redesigning of previous transplantations should be considered as a possible indication F.U.E., particularly in the setting of scarce follicular reserves. The utility of our recycling method may also inspire hope in patients that have undergone failed or unsatisfactory hair transplantations.

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Case Report

Surgery Resection of a Massive Thymic Carcinoma during Urgent Coronary Artery Bypass Grafting

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Abstract

BACKGROUND: Thymic carcinoma is a very rare tumour. It is classified as thymoma type C according to World Health Organization classification. There are not many publications of simultaneous surgical treatment of thymoma during cardiac surgery interventions.

CASE REPORT: We present a case of simultaneous surgical treatment of incidentally discovered thymic carcinoma during an urgent coronary artery by-pass operation. A 55-year-old man with diagnosis three coronary vessel diseases indicated urgent by-pass surgery. The patient underwent triple coronary bypass surgery. During the intervention, it was discovered incidentally a strong mass 15 x 12 cm located in the right pleural space. A tumour was excised totally, and biopsy referred thymoma type C or thymic carcinoma. The patient did very well early postoperatively. He was referred to oncologist clinicians for further treatment. The patient was clinically very good for at least 1.5 years after surgery.

CONCLUSION: We think that simultaneous surgical treatment of thymoma, whenever it is encountered during cardiac surgery procedures, is the recommended solution.

Introduction

Thymoma is a rare tumour. Its incidence refers to 0.15 to 100 000 persons [1]. They are two main classifications of the thymomas. The Masaoka classification is most widely accepted to determine the treatment strategy of thymomas while the histological classification according to WHO is important especially to distinguish thymomas, thymic carcinomas and thymic carcinoids. Thymic carcinomas are very rare and more aggressive than classical thymomas [2]. The simultaneous treatment of tumours of the thymus during a cardiac surgery procedure is referred to in some presentations, mostly during CABG [3] [4] [5] [6] [7] [8] [9] [10] [11]. We will present below a case of simultaneous surgical treatment of an incidentally detected thymic carcinoma during an urgent coronary artery bypass.

Case presentation

A 55-years old man is hospitalised in our clinic with a diagnosis of severe three-vessel disease with indications for urgent CABG. The patient referred to angina pectoris in low efforts and during rest. Echocardiography showed a normal cardiac function without any other data related to the function and cardiac structures.

Coronarography: Three-vessel disease with very critical stenosis of LAD, LCx and RCA: urgent indication for intervention. Preoperative routine examinations were normal.

Intervention: CABGX3: LIMA-LAD; VSM-OM1, PDA. The interventions were performed under cardiopulmonary by-pass and cardiac arrest. At the moment of hemostasis, occasionally in the right

pleural space attached to the sternum, is discovered a very strong mass. The mass lied from the medial part of the sternum to the right subclavian vein. The rapport with pericardium was clear with no signs of infiltration. The tumour was excised totally. Macroscopically the mass was very strong, and it was about 15 x 12 cm. The mass was incised and had the appearance of the honeycombs (Figure 1).

Figure 1: Photo during the intervention. In the left the moment the mass was excised, in the right, the mass was incised

The mass was sent to the biopsy. The biopsy response consulted by a group anatomopathologists was: lobular neoplasia consisting of solid neoplasms of polygonal epithelial elements (CK19+, CD117-, CD20-, CD5-) mixed with immature T elements (TdT +). There is capsule infiltration. These results support the diagnosis of thymoma type C or thymic carcinoma (Figure 2).

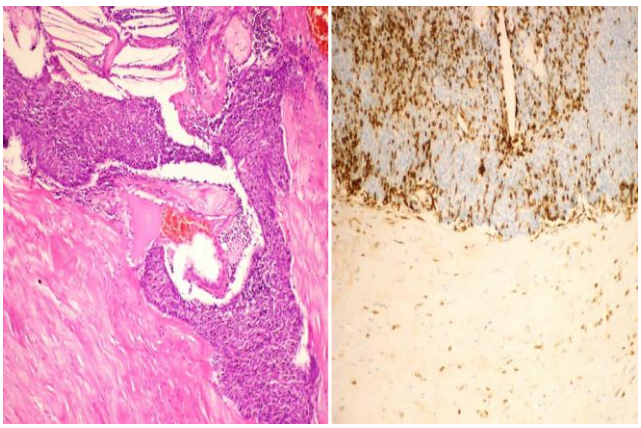

Figure 2: Photos of biopsies

The patient was referred to local oncologist clinicians, and he did not undergo additional treatments. He realised a thoraco-abdominal CT-scan one week and 1.5 years after surgery. Both CT-scans were normal. The patient has been clinically very well.

Discussion

Thymomas are the most common primary tumour in the anterior mediastinum [2]. It is a neoplasm of the thymus that originates in the gland's epithelial tissue. The incidence of thymoma in the United States is approximately 0.15 per 100,000 person-years. Histopathologically thymic carcinomas are also tumours of thymic epithelial cells. The distinction between thymomas and thymic carcinomas is not as clear, because thymic carcinomas have malignant cytologic features, whereas thymomas are generally considered cytologically benign. Patients with thymoma are often asymptomatic, and the tumour is detected incidentally on a chest x-ray or computed tomography. The most common presenting symptoms for thymomas are dyspnea, chest pain, cough, and symptoms of myasthenia gravis. More advanced thymoma present with symptoms related to the involvement of local structures [12]. Thymic carcinomas often cause pericardial and pleural effusion and that and more aggressive than thymomas [2]. Thymomas are classified mostly according to World Health Organization (WHO) and Masaoka staging. The WHO adopted a pathologic classification system that takes in to account both histologic and morphologic features. The WHO classification is also used to differentiate among different histologic types of thymomas. Thymic carcinomas are type C thymomas according to the WHO classification. Masaoka describes thymomas regarding the local extension of a tumour. This staging system is the most widely accepted system for management and determination of prognosis for both thymomas and thymic carcinomas [12].

Our case was a huge thymic carcinoma asymptomatic until was discovered incidentally during an urgent CABG. There are rare cases reported with the coexistence of thymomas with cardiac surgery pathologies and the simultaneous surgical treatment of thymus tumours during a cardiac surgery procedure. The incidence of the coexistence of the thymoma and CABG pathologies is referred 0.2-0.7 % [5] The most of the thymus tumors were incidental findings after the sternotomy is done [5] [6] [7] [8] [10] while a part of them was distinguished prior the cardiac intervention [3] [4] [9] [11]. All cases referred to the localisation of tumour in the anterior mediastinum. Poullis and Punjabi [3] presented a case of resection of thymoma with pure red aplasia during an elective CABG. Agrifoglio referred a case of an occasional finding of thymoma in a patient that was in the theater for mitral valve surgery [7]. Kouzu at al report simultaneous operations performed for angina pectoris, aortic regurgitation and thymoma [11]. In all of them, the tumour of thymus were thymomas [3] [4] [5] [6] [7] [8] [9] [11] while Abdullah et al., [10] refer a very rare case of thymic carcinoid tumour encountered incidentally during a CABG procedure. The case we presented may be the first case of an

incidental finding of thymic carcinoma and treated simultaneously during a CABG. Unusual was the localisation in the right pleural space.

Total thyroidectomy and complete surgical excision of the tumour are the gold standard of treatment and are recommended whenever possible. There are no definitive guidelines about additional modalities of treatment such as radiotherapy or chemotherapy, but the treatment is guided in large consensual documents. Additional radiotherapy, according to National Comprehensive Cancer Network (NCCN), may be considered for the thymic carcinomas. It is recommended an annual CT scan check for the recurrence. The definitive treatment for thymic carcinomas is still undefined. All patients with thymic malignancies should be evaluated by radiation oncologists, surgeons, medical oncologists, diagnostic imaging specialists, and pulmonologists to determine the optimal plan of care before treatment [2] [12]. Surgery is accepted as a mainstay for this type of a tumour while the role of additional therapy is still unclear [13] [14]. We performed total surgical resection of a tumour and referred to the oncologist the patient. The patient underwent some laboratory examinations and CT scan after surgery first immediately after the intervention and 1.5 years after surgery. The patient did not undergo additional therapy and was in very good health conditions at least 1.5 years after surgery.

We think that simultaneous surgical treatment of thymoma, whenever it is encountered during cardiac surgery procedures, is the recommended solution.

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Case Report

An Uncommon Occurrence of Pleomorphic Adenoma in the Submandibular Salivary Gland: A Case Report

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Keywords: Pleomorphic adenoma; Benign mixed tumour; Submandibular salivary gland; Salivary gland; Submandibular gland

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Abstract

BACKGROUND: Pleomorphic adenoma is a salivary gland tumour and mostly found in the parotid gland and quite uncommon in the submandibular gland. Pleomorphic tumours are a mixed tumour (benign mixed tumour) consisted of epithelium, myoepithelium, and mesenchyme and made of a view component variation of it.

CASE REPORT: We reported a fifty-three years old man with pleomorphic adenoma that has been complaining swelling on the left neck for the last twenty years and treated with surgical excision. Computed tomography of the neck showed soft tissue tumour in the left submandibular.

CONCLUSIONS: The best management for pleomorphic adenoma is surgery, the tumour tissue must be removed as a whole because the remaining parts of a tumour can easily become recurrent or turn into a malignant tumour.

Introduction

Pleomorphic adenoma is a salivary gland tumour and mostly found in the parotid gland. A pleomorphic tumour is a mixed tumour (benign mixed tumour) consisted of epithelium, myoepithelium, mesenchyme and made of a view component variation of it [1]. Almost 85% of benign epithelial salivary gland tumours arise from the parotid gland and only 10% in the submandibular gland. Pleomorphic adenoma typically present as a slow growing, unilateral and painless mass of salivary glands, with a predilection for recurrence and risk of malignant transformation (about 1.5% up to 5 years and increases to 9.5% after more than 15 years) [2] [3].

The salivary glands are categorised as major salivary gland and minor salivary gland. There are three kinds of the major salivary gland; they are the parotid gland, submandibular gland, and sublingual gland. The minor salivary glands were lying along upper aerodigestive submucosal such as the palate, lips, pharynx, nasopharynx, larynx, and parapharyngeal. In the major salivary gland, the pleomorphic adenomas are often seen in the parotid gland, while in the minor salivary gland, it is often seen in palate and upper lip [2] [4].

Pleomorphic adenoma can be found among all ages (kids or adults). It shows about 45% to 75% of neoplasm in saliva gland and the incidence of 2-35 cases per 100,000 people. The comparison between woman and man are 2:1. This tumour usually found in the ages of thirties till sixties with the average of 43-46 years old. In America, this tumour was found 80% of all saliva gland benign tumours [5].

B-catenin was a molecule which has a role in invasion and metastatic process of carcinomas in head, neck, oesophagus, gastric, colon, liver, woman genitalia, prostate, vesica urinary, pancreas and also melanoma [6].

Case Report

A patient, 53 years old man referred from Haji General Hospital to the outpatient clinic of Adam Malik General Hospital with the main complain of swelling on the left neck for the last 20 years prior admission. The mass was enlarging slowly. He denied any pain, nasal blocking, headache, diplopia, hoarseness, dysphagia or odynophagia, no history of bleeding and mucous secret.

Figure 1: Anteroposterior view of the neck mass

The physical examination noted the patient's sensorium was compos mentis, pulse 80 beats per minute, respiratory rate 20 times per minute and the temperature was 37^{o}C and also presented a solid, immobile, and no painful left neck mass measuring approximately 6 cm x 4 cm x 3 cm. Ear, nose and throat examination showed no abnormalities.

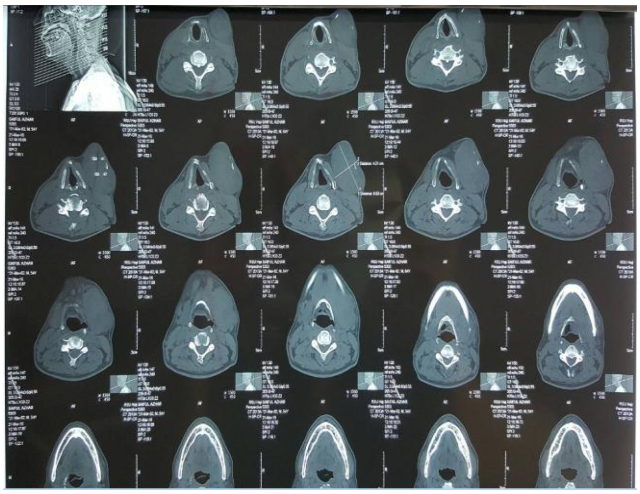

Figure 2: Neck CT Scan (soft tissue tumour size 6.63 x 4.21 cm in the left submandibular)

Fine needle aspiration biopsy found as a pleomorphic adenoma; haematologic laboratory finding was in normal limit. ECG and thorax x-ray were also showed no abnormalities. Then we performed computed tomography of the neck for this patient, and the result showed soft tissue tumour with the size of 6.63 x 4.21cm in the left submandibular.

Figure 3: The tumor size 6 x 4 x 3 cm

We diagnosed the patient with submandibular pleomorphic adenoma and conducted surgical excision. The tumour was removed entirely, and the outcome was good. The histopathologic examination after surgery showed a pleomorphic adenoma.

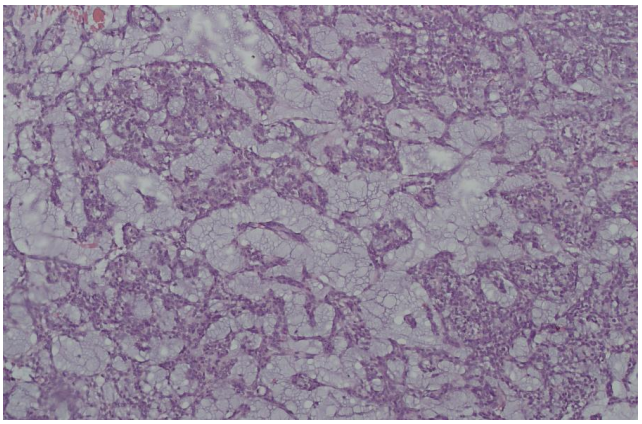

Figure 4: Histopathological examination (x100)

Discussion

Pleomorphic adenoma is the most common benign salivary glands tumour, which is about 65% of all salivary gland tumours. Malignancy in the salivary glands occurs 3-6% of all neoplasms head and neck. White people have a slightly higher risk to suffer pleomorphic adenoma than other races. Women are more dominant with the ratio 3:2.1. Some factors predisposition is suspected of additional exposure to radiation, genetic, users of tobacco, chemicals exposure, and viruses. Patients do not have a history

of smoking or exposure to radiation, but often eating preserved foods such as anchovies and grilled food. The level of accuracy of FNAB in distinguishing malignancy and benign is 79.1%, while the sensitivity of salivary gland neoplasms is about 89.4% [4].

In this case, we found a slow growing, unilateral and painless mass in the submandibular gland.

The aetiology of pleomorphic adenoma in the salivary glands is not known for certain, allegedly because of the involvement of environmental and genetic factors. Radiation exposure associated with the developments of benign tumours and mucoepidermoid malignant carcinoma. One study suggests that the Simian virus (SV 40) plays an important role in the development of pleomorphic adenoma. The Epstein-Barr virus is one of the factors in the development of the tumours of salivary gland lymphoepithelial. Genetic changes, such as allelic loss, monosomy, and polysomy, and realignment of structure [7] [8].

In general, β-catenin plays an important role in the development of pleomorphic adenoma. Not only in the form of malignant change, but also in the regulation of physiological functions. The expression of adhesion molecules in salivary gland neoplasms had been explored. Study now says attempts to clarify the role of cells in oncogenesis and cytodifferentiation pleomorphic adenoma and carcinoma of the salivary glands. The expression of β-catenin is an immunohistochemical test in the lesions and normal salivary gland [6].

Pleomorphic adenoma has clinical features: a single tumour mass, hard, round, mobile, slow growth, painless, single nodule. Although pleomorphic adenoma tumours were classified as benign, it can grow larger and turn into a malignant form of carcinoma. The histopathological examination showed a well-circumscribed and encapsulated mass composed of an admixture of epithelial, myoepithelial and stromal components. One of these components can be found more often than the others, but these three components must be present to make a diagnosis [6] [9]. In this case, from histopathological examination, we found encapsulated mass composed of an admixture of epithelial, myoepithelial and stromal components.

CT picture of pleomorphic adenoma (benign mixed tumour) is a cross-section that is sharp and mostly surrounds homogeneous lesions that have a higher density than the glandular tissue. MRI showed pleomorphic adenoma (benign mixed tumour) with the areas that have relatively low signal intensity (dark area/radiolucent) than glandular tissue. Examination of pleomorphic adenoma with CT and MRI was done by a radiologist. It is done to determine the location and size of the tumors, lesions detection, borderline tumor, aspects of the lesions, the contrast between lesions with surrounding tissue, the image intensity of the lesion, the success of the use of contrast medium, the aspect of the lesion after injection medium contrast, detection of his capsule and bone resorption occurs around the lesion [7] [10].

The best management of pleomorphic adenoma is surgery; the tumour tissue must be removed as a whole because the parts of a remaining tumour are easy to cause recurrence or turn into a malignancy [11].

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Dental Science

Associations between Diet, Dietary and Oral Hygiene Habits with Caries Occurrence and Severity in Children with Autism at Dammam City, Saudi Arabia

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Abstract

AIM: The purpose of the study is to achieve the baseline information of the autistic child's oral health status about the diet, dietary and hygiene habits. The association of these factors with dental caries were assessed.

MATERIAL AND METHODS: The survey was composed of self-administered questionnaires to parents about their children's' demographic data followed by questions related to diet, dietary and hygiene habits. This is later followed by oral examination for estimating the decayed, missing and filled [dmft] scores as per WHO norms. The variables are analysed using t-tests and ANOVA. Pearson's correlation coefficients were calculated for each of the independent variables to examine for autocorrelation.

RESULTS: The mean age for the present study is 5.8 years with more predilections of caries in females. The autistic children prefer soft diet and pouch it in oral cavity resulting in increased caries though not significant. Other foods like nuts and pulses confectioneries and soft drinks resulted in increased caries, and our study shows significant relation. Consumption of sugars between meals and increased quantity of sugar per day also increased dental caries with highly significant results in our study. Hygiene habits also made a difference in the occurrence of caries though, in our study, it's not significant.

CONCLUSIONS: The study suggests that the oral health education programs should be conducted for the parents, caregivers and the teachers about the diet, dietary and the hygiene habits and the role they play in maintaining the oral hygiene.

Introduction

Autism or autistic disorder is a serious epoch of life due to developmental disability which after a profound examination medically, psychologically and neurologically, four definitive criteria were to be fulfilled [1] [2].

1. Severe abnormality in social interaction;
2. Severe abnormality in communication skills even in most frequently speaking language;
3. Very confined, monotonous and stereotyped pattern in his/her behaviour; and
4. Starts very early (before the age of 3 years).

Autistic children are unique in their diet pattern, dietary habits and their hygiene habits leading to a compromised intraoral condition. When diet and oral health is considered, Moynihan stated that "Good diet is essential for the development and maintenance of healthy teeth, but healthy teeth are important in enabling the consumption of a varied and healthy diet throughout the life cycle," thus highlighting the reciprocal relationship between diet and oral health [3]. Parental and informal clinical reports, alongside with a few research investigations, it gives us a hint that children with autism have bizarre dietary patterns [4] [5] [6]. These are regularly depicted as excessively

particular, with antipathies for particular textures, colours, smells, and temperatures and stubborn for particular brands of foods [4].

These children in their daily routine are provided with food rewards mostly in the form of sweets. Restricted diet for these children by making them eat limited types of food or in specific the physician recommended diet like the gluten/casein-free or partially g-tube fed or low acid diet [7] [8] [9] [10].

Different people incline towards foods in their daily life, so there is a part of the individual variation in their dietary habits. These habits play a very consequential role in dental caries with conflicting results from the previous studies. Like for example, the intake of sugar plays a very significant role in dental caries (Gustaffson et al. 1954, Nizel and Papas,1989; Rugg-Gunn 1989) was confirmed long back. But several procedural imperfections built in these studies make it hard to confirm a convinced association between dietary habits and dental caries (Screenby, 1975; Newbrun, 1982) [11]. But its role cannot be taken trivially from the recent studies [12] [13] where it is proved that dietary habits take a very important role.

Oral hygiene practices are voluntary physical activities that have at least two requirements: motivation and manual dexterity [14]. Thus, increase in dental caries would be more in children who are physically or mentally challenged individuals [15]. Like in this study autistic children were taken into consideration to find any association between diet, dietary and hygiene habits with the occurrence of dental caries.

Dental caries is a disorder with a combination of factors, and it is arduous to correlate all these factors at the same time. There have been no studies documented in literature in this part of Saudi Arabia estimating the occurrence of dental caries about a specific diet, dietary and hygiene habits in autistic children. So an attempt was made to correlate these factors in children who have autism in Dammam city. A cross-sectional study was designed for the parents to answer the questionnaire and correlated each of them with the prevalence of dental caries.

The purpose of the study is to assess the association of diet, dietary, and hygiene habits with the occurrence of dental caries.

Material and Methods

After getting this study approved by the institutional review board (IRB), Riyadh Colleges of Dentistry and Pharmacy (Riyadh Elm University), an official letter from the College Board was used for further study in autistic schools in Dammam city, Saudi Arabia. The study was conducted in two steps. Firstly answering the questionnaire by the parent and later oral examination of the child. The sample for our study was taken from three special schools for autistic children in Dammam city, Saudi Arabia.

Study Instrument

The self-administered questionnaire combined from previously reported researchers [12] [13] gathering information about the close-ended questions into four parts as demographic characteristics (age, gender, educational level and income of the parents) followed by 18 questions related to diet (6 question), dietary habits (6 question) and hygiene habits (6 questions).

The consumption of specific diet related to dental caries was evaluated and was assessed with this structured questionnaire. This part of the questionnaire was focused on the consumption of soft diet, confectionaries, soft drinks, nuts and pulses, fruits and vegetables. The response options were regular or occasional. The dietary habits included food rewards, recommended diet, and frequency of sugar intake, fast food and snacks. Oral hygiene habits included here are whether the child brush or not, brushing and the ease of difficulty for the parent. The questionnaire also includes the frequency of dental visit and the reason for their visit. Also asked about by whom the children get their teeth brushed (mother/child himself/caregiver/sibling).

To avoid irrelevant answers by the patients who were not well acquainted with English, an English-Arabic rendition of the survey was defined. The idea of rewording was to obtain an instrument with theoretical similarity. The first English survey was converted into Arabic dialect by a bilingual local Arabic speaker and from that point indiscriminately back-interpreted by another bilingual local Arabic speaker. Through these thorough cycles of interpretation and back interpretation, after getting convinced that the actual meaning of the survey was maintained. A pilot contemplates completed utilising this bilingual instrument to guarantee identicalness, clearness, and understanding. This was done according to Kotha SB et al., [16].

To reduce the bias in answering the questionnaire by the parents, the third person (teacher) distributed and made them answer away from the dentist [17].

Oral examination

The children with Autism were examined at special schools, seated on a comfortable chair, under natural light using sterile portable equipment which included mouth mirror, explorer and cotton pellets. Tell-Show-Do (TSD) technique was used to manage

these children.

Examination of the oral cavity for dmft for the patients was done according to World Health Organization caries diagnostic criteria [18]. The two examiners were selected to examine the children and the method followed was according to that done by Kotha SB et al., [16].

Data collection and statistical analysis

After a brief description of the study to the mothers, they were given two days' time to answer the self-administered questionnaire about diet, dietary and hygiene habits of their autistic children. After getting approval from the parent, it was followed by an oral examination of the child. The data were analysed statistically using SPSS software SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). The variables are analyzed using t-tests and ANOVA. Pearson's correlation coefficients were calculated for each of the independent variables to examine for autocorrelation.

Results

The mean age for the present study is 5.8 years with almost an equal distribution among males and females where in which the females (4.55) found to have more caries in comparison to males (3.42) though not statistically significant. The results of other factors like the mother's (P = 0.346) and father's (P = 0.679) education about dental caries found to be not significant. The caries occurrence is reduced in the group where the parents have a better education. The income of the family (ANOVA) also showed no significance (P = 0.816) though it was found that caries is more in low-income group (Table 1).

Table 1: Sociodemographic characteristics in relation to dental caries

S. No	Demographic data	Groups	dmfs		t	p-value
			Mean	S.D		
	Gender	Male	3.42	3.044	-1.142	0.258
		Female	4.55	2.505		
	Mother's education	School	4.00	3.433	0.944	0.346
		Graduation and more	3.28	2.479		
	Father's education	School	3.85	3.619	0.416	0.679
		Graduation and more	3.52	2.476		
	Income	<5000	4.00	4.472	0.204	0.816
		5000-10000	3.69	2.681		
		10000 and above	3.25	2.745		

Dietary preferences and dental caries

It was observed that autistic children preferred soft diet making them more susceptible to caries though not statistically significant (P = 0.7). Regular consumption of confectionaries increases dental caries (5.08) than children who take them occasionally (1.38) and which seems to be highly significant between two groups with P = 0.000. Similar results are also observed in children who regularly drink soft drinks (5.39) than children who take them occasionally (2.12) with a P value of 0.000. When comparing the other food groups like nuts and pulses, there is a significant correlation between regular (5.09)and occasional consumption (2.74) groups, and it was found to be significant (P < 0.05). Other healthy foods included in our study are fruits and vegetables where in which the caries is increased in children who consume these occasionally compared to a group having it regularly. The results are not significant. These results conclude that regular intake of confectionaries, soft drinks, and nuts and pulses increase the occurrence of dental caries (Table 2).

Table 2: Diet preferences in relation to dental caries

S. No	Dietary preferences	Groups	dmft		t	p-value
			Mean	S.D		
1	Soft diet	Yes	3.80	2.72	0.323	0.748
		No	3.54	3.10		
2	Confectioneries	Regularly	5.08	2.69	5.994	0.000
		Occasionally	1.38	1.71		
3	Nuts and pulses	Regularly	5.09	2.85	3.224	0.002
		Occasionally	2.74	2.69		
4	Soft drinks	Regularly	5.39	2.98	5.111	0.000
		Occasionally	2.12	1.98		
5	Fruits	Regularly	2.92	2.985	-0.958	0.342
		Occasionally	3.81	2.965		
6	Vegetables	Regularly	3.62	2.879	-0.015	0.998
		Occasionally	3.63	3.235		

Dietary habits and dental caries

Food rewards for the autistic children are usually in the form of sweets showed increased susceptibility for dental caries which is not significant (P = 0.2). The physician recommended a diet in specific for autistic children coincidentally reduce dental caries (3.22) compared to the group not consuming this recommended diet (3.69) though not statistically significant (P = 0.6). Sugar intake regarding quantity having two groups in our study, those consuming sugar more than two spoons per day (dmft = 5.5) and less than two spoons per day (dmft = 1.42). The results show that there is high significance (P = 0.000). Similar results are seen in children having sugar in between meals (dmft = 5.19) in comparison to the group not having (dmft = 1.90). Though not significant in the study, unhealthy lifestyle practices like frequent snacking (P = 0.461), regularly having fast foods (P = 0.140) increased dental caries (Table 3).

Table 3: Dietary habits about dental caries

S. No	Dietary habits	Groups	daft		t	p-value
			Mean	S.D		
1	Food rewards	Yes	3.87	3.06	1.207	0.232
		No	2.79	2.51		
2	Recommended diet	Yes	3.22	2.48	-0.436	0.665
		No	3.69	3.05		
3	Sugars/day	> 2 spoons/day	5.05	2.718	5.818	0.000
		1-2 spoons	1.42	1.742		
4	Sugar between meals	Yes	5.19	2.799	5.171	0.000
		No	1.90	2.076		
5	Fast food	Regularly	3.77	3.006	1.495	0.140
		Occasionally	1.50	1.000		
6	Snacking	Regularly	4.00	3.207	0.743	0.461
		Occasionally	3.41	2.844		

Table 4: Hygiene habits about dental caries

S. No	Hygiene Habits	Groups	dmft		t	p-value
			Mean	S.D		
1	Brushing method	Brush and Water	3.72	3.611	0.123	0.885
		Mishwak only	4.25	3.096		
		Brush+paste	3.51	2.694		
2	Brushing	Sometimes	4.21	3.620	0.847	0.400
		Regularly	3.45	2.765		
3	Ease of brushing	Difficulty	3.97	2.658	0.449	0.640
		Moderately difficult	3.25	3.138		
		Easy	3.20	4.324		
4	Dental visit	Never	3.63	2.977	1.922	0.136
		If there is a problem	4.41	3.081		
		Occasionally	1.75	1.832		
		Regularly	1.00			

Hygiene habits and dental caries

Table 4 shows the distribution of parents' responses of how the hygiene habits are maintained of which whether the child is getting brushed regularly or not and the method of brushing about dental caries. The results through ANOVA showed that children using brush and paste (3.51) have fewer caries than compared to other groups who are using either Mishwak (4.25) or brush and water without paste (3.72) to clean the oral cavity. The results shown here in our study are not significant (P = 0.8). The group which brushes regularly have reduced caries compared to the group who does irregular brushing though not significant (P = 0.4). Regarding ease of difficulty, it is understood that brushing an autistic child is a big task and so there is an increase in caries in this group of children (3.97) in comparison to other groups where the parents who feel brushing is moderately difficult (3.25) or easy (3.20). The results here are not significant (P = 0.640).

The results show that only 14.8% of the children were able to brush their teeth on their own, while the remaining 85.2% needed help during tooth brushing. More than half (73.8%) of the children were helped by their mothers in brushing their teeth. 93.4% preferred manual brushing over the use of the electric brush.

Table 5: Pearson correlation coefficients to assess the association between dental caries with diet, dietary and hygiene habits

	Diet	Dietary habits	Hygiene habits
dmfs	-0.591*	-0.057	-0.146

Table 5 showed the Pearson correlation to assess the association between the type of diet, dietary and hygiene habits with dental caries resulted in the diet plays a very significant role about dental caries, and it was significant (P < 0.05).

Discussion

The purpose of the study is because, in Saudi Arabia, very little research is being concentrated on the association of diet, dietary and hygiene habits with dental caries in children with autism. In Saudi Arabia, though autism is more prevalent, the dental care for children with cerebral palsy and Down's syndrome are handled better because these conditions are diagnosed at birth unlike autism where the children are completely normal at birth and is diagnosed later resulting in less direct involvement of the dentists to give instructions and warn about the factors that affect the maintenance of oral hygiene [17].

Before discussing the actual results of the study, the reason for choosing school as the place of study because schools are the best site for the children to cooperate as they are acquainted with this environment, and these children are very dependent on routine and continuity unlike a new place that may evoke a negative behaviour [19].

The caretakers were requested to participate in our study to make the patients open their mouth for oral examination and for some children parental involvement was taken because these children cannot easily make contact with the unfamiliar people. This explained the need for the caretaker and the parent for oral examination [19] [20] [21] [22].

In this study, male predilection is more compared to the females in par with previously reported researches [2] [19] [23] [24] [25] [26] [27] [28] most probably because of high level of fetal testosterone and potential genetic/ chromosomal effects [19] [29].

Numerous researchers demonstrated that various factors are directly responsible for the occurrence of dental caries, but the predictive power of certain factors like mother's and father's education and their income were considered less important. Though the results are not significant (mother's education P-value = 0.3, father's education P-value = 0.6) in this study, the parental education plays a very significant role in dental caries. The parent's education helps the children maintain their oral hygiene as they comprehend the significance of good oral habits and so pass on to their children [30]. Parental income is related to a socioeconomic status where the parents of the autistic children with higher income, will have lower dental caries because the parents can provide good facilities to maintain their oral hygiene [23]. The results also showed that children whose parents income is less showed more caries occurrence though not significant (P = 0.8).

Studies showed that children with autism prefer specific type of food which made us to select specific dietary preferences (confectionaries, soft drinks, nuts and pulses, fruits and vegetables) mentioned in our questionnaire [31] along with certain dietary habits (food rewards, recommended diet, sugars per day, sugar between meals, fast food and snacking).

It is evident that the autistic children prefer

soft food which increases the incidence of dental caries [32] as they prefer to pouch the food in the oral cavity instead of swallowing due to poor tongue coordination leading to increased occurrence of dental caries [29] [33] [34] have similar results though not significant (P-value = 0.7) in the present study.

It was observed that the intake of confectionaries and soft drinks regularly increased dental caries as in our study show highly significant relation (P-value less than 0.001). Children consuming confectionaries four times a day were almost 20 times more chances for the child to develop dental caries [12]. There are various studies supporting the significant relation between caries and soft drinks and are significantly related [12] [35] [36] [37].

The nuts and pulses act bifunctionally about dental caries. These type of foods reduce dental caries because of more fat content (e.g., peanuts) coating the teeth and thus lowers the retention of consumed food. Alternatively, fats may have toxic effects on oral bacteria and thus reduce the sugar solubility [38]. Because of the fibrous content of nuts and pulses increase in saliva production and so the buffering action thereby reduction of caries. The converse results are shown in this study and are significant (P-value = 0.002) probably nuts rich in starch content, reduces the clearance from the oral cavity and autistic children have the habit of pouching food in the mouth increase the chances of caries [38].

Previous researchers [4] [39] [40] [41] [42] showed that autistic children are very sensitive to bitter taste leading to refusal of certain foods and it could be the potential reason for reduced intake of vegetables in par with our study where 68.9% of the children rejected having vegetables. In contrast, According to Diolordi L et al., [39] fruits are preferred by the autistic children may be because of its texture and sweet taste wherein our study, 78.7% of the children preferred having fruits. About dental caries, previous researchers [13] analysed that fruits/vegetables are having a snack replacing the sugar or sweet reduced dental caries. Similar results were achieved but not significant.

The question asked to the child whether he augments appropriate behaviour with food rewards, 23% of the children approved to reinforce but unfortunately the reward was in the form of sugars/sweets increased dental caries. No significant association (P-value = 0.2) was found between dental caries and food rewards as in [7] [43].

Diet for the autistic children is restricted like for example gluten and casein because it was incited with a couple of hypotheses is that the aberrant metabolism of these two proteins may develop an excessive opioid activity in the central nervous system, altering its function [44]. The other reason is that if there is an abnormal gut barrier or intestinal permeability, it results in leaking of gluten, casein, and their metabolites into the bloodstream and to the central nervous system [44]. This combined with the metabolic defect may contribute to developing autistic symptoms. This gives the reason to encourage a gluten-free and casein-free (GFCF) diet for autistic children [44]. According to Avsar et al [45] showed that the regular use of this physician prescribed GFCF diet reduces the occurrence of dental caries which was proved by Acer el in his research that there is low prevalence in the colonization of mutans streptococci (MS) and lactobacillus (LB) [45] and so reduced dental caries. The results of our study show that the caries is reduced in children who are on physician recommended diet but not significant (P value = 0.6).

Our examination did not research the complexities of what kinds of snacks were consumed in between meals (regardless of whether it contained high starch content). However, this present research has mentioned an objective fact that children who were in the propensity for eating in between meals or increase in sugars intake per day had higher dmft scores. This finding would serve as an imperative contribution to educate the children about evading in-between meals eating behavior. Most children have a tendency to go to fast food centers and eat undesirable snacks in-between meals that are generally high in sugar and fat, as many studies [12] [13] [30] [40] pointed that incessant eating of snack, sugar and cooked starch between meals will enhance chances of caries. American Dental Association has suggested that everyone irrespective of age must restrict themselves in eating and drinking in-between meals and when they wanted to snack incline towards nutritious nourishment recognized by the US Department of Agriculture Dietary Guidelines [12]. Our investigation likewise found that there was a very high significant relation of increasing sugars approximately by more than two spoons per day and the habit of eating in-between meals with dental caries (P value < 0.001). No distinction in occurrence of caries when the children have the habit of frequent snacking in fast food centers.

Most of the autistic children because of their physical impairments and poor manual dexterity within them needed help from their parents or caregivers [17]. The mothers' part and eagerness with respect to their disabled childs' oral health was recognized in numerous researches. It is additionally significant that most Saudi families are in the propensity for contracting caretakers, to administer help to their kids and perform family tasks. Though caretakes take the major role, in our study mothers' (73.8%) took the major responsibility in bushing their autistic kids [47]. Window of opportunity to brush for these children is very less so use of electric brush may help but because of the insignificant sound makes the child increase in anxiety [48] end up not brushing so most of the families in our study preferred to use manual brushing (93.4%) over electronic tooth brush. Regarding the dental visit, very interestingly, the participants in our study showed no significant

differences in regards to the reason for visiting the dentist as in previous research [48]. Most of the children (49.2%) among our sample never went to the dentist because of increased anxiety by the child because of the foreign place, acclimatising to new sounds and smell exaggerates sensitivities making the child uncomfortable. These all make the dentist extreme difficulty leading to embarrassment to the parent so try to give extreme care to the child to avoid dental treatment [20] [48] but because of their type of food, dietary and hygiene habits never will the dental care is handled without the dentists' support. That's the reason; many children are taken to the dentist only if they have a problem with 36.1% of our study population attended the dentists.

Limitations: Small sample size; Our study did not explore the complexities of what kinds of snacks were eaten in between meals (whether it contained high sugar content or not); The autistic children not going to any of these special schools are not considered in the study; We didn't define clearly in the questionnaire the definition of regular and occasional in the questionnaire which might be a confusion to the parents while answering.

Further studies are recommended to overcome these limitations

Based on the results, it is concluded that the parents should be educated on how to maintain the good oral hygiene particularly for autistic children which is an integral part of the optimal dental health via proper diet, dietary habits and adequate oral hygiene by regularly visiting the dentist to have a long-term good oral health. Future studies should focus on projects that would encourage the parents or caregivers in improving the oral hygiene of these autistic children.

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Dental Science

Comparative Study Clarifying the Most Suitable Material to Be Used as Partial Denture Clasps

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Keywords: Removable Partial Denture (RPD); Clasps; Direct Retainer; Mechanical properties; Roughness; Retention, Deformation; Cobalt Chromium (CoCr); Thermoplastic Acetal; Versacryl; Thermopress

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Abstract

BACKGROUND: Removable partial denture's clasp is of particular importance as it affects the denture longevity during the function. The key of successful clasp selection is to select a direct retainer that will control tipping and torquing forces on the abutment teeth, provide retention against reasonable dislodging forces and are compatible with both tooth and tissue contour and the aesthetic desire of the patient. In this consideration, different materials employed for the clasp construction were compared mechanically.

AIM: This study aims to compare the most usable esthetic clasps mechanically to clarify the most suitable material to be used as partial denture clasps.

METHODS: Evaluation of surface roughness, retention and deformation has been investigated utilising different in-vitro methods. All these techniques provide valuable information regarding the mechanical properties of the materials tested. However, none of the in-vitro techniques can expose the tested materials to conditions similar to that of the oral environment (in-vivo) such as pH value and temperature variations.

RESULTS: Most commonly, RPD clasps are fabricated from the same alloy of the metal framework, as cobalt-chromium (CoCr) alloy although it is unaesthetic. Other methods consumed to avoid such esthetic mystery have included coating retainers with tooth-coloured resin or introduction of esthetic materials as Thermoplastic Acetal, Versacryl, and Thermopress.

CONCLUSION: It has been concluded that the non-metal Acetal resin retainer reveals superior mechanical properties.

Introduction

The esthetic dental restorations play a great role in neoteric communities not only for females but also for males, due to the assertiveness of physical look. Dental implant succeeded in expanding such scope, yet it is not highly recommended for the tremendous scale of patients, especially those who are suffering from some medical, psychological and financial problems [1].

Esthetic removable partial dentures (RDPs) are considered as the best and most compatible remedy preference for these subjects in replacing their lost teeth with superior esthetics. One of the major problems of RPDs was the display of the clasp assemblies. Etching the retainer's arm and overlaying it with a tooth-coloured resin coat is one of many recent ways employed to solve this issue. Moreover, as the physical appearance of these ethic retainers is of vital essentiality, yet their mechanical properties play a great role in their success and intraoral utilisation [2].

Employing of acrylic resins or resin composite to veneers in metals of RDP faces a difficulty which lies in the diversities between both their potentiality to inflect and coefficients of thermal expansion. Non-noble metals possess durability and resist remarkable flection. However, utmost disfigurement takes place to resins concerning both their physical and thermal

status, as the matrix becomes fragile beyond its elastic borders. The resin composite matrix also tends to be brittle beyond its elastic limit. As a sequel, the capacities of both metals and resins for plastic disfigurement are in a broad conflict. Latest concerns extend to the impact of the intraoral masticatory vigour together with both the adjustability and extra magnitude of the veneered retainers formed by the compiling of the covering matter. Exaggerated declining in the retainer's length and thickness should be averted to secure the stiffness and shorten the fracture of the retainer as well as provide maximum esthetics [3].

One of the different recent modalities utilised to enhance the semblance of metal retainer structures and sour them for outstanding and supreme esthetics is to fabricate the clasps in a tooth-coloured substance as the Thermoplastic resins [4] [5] [6]. However, in literature, few data are obtainable on the long-dated execution of such retainers concerning retention.

Polyoxymethylene (POM) which is well known as Acetal resin, an injection-moulded resin also acts as a standby to the classical PMMA. Fabrication of POM takes place by the polymerisation of formaldehyde. The homopolymer polyoxymethylene is a series of alternating methyl sets united by an oxygen whit. Besides that, it behaves elastically on a wide scale which allows it to be utilised as the suitable material for retainer construction. This is due to its superior proportional limit with the minimal viscous flow [4].

Lately, POM is considered as a highly desirable material for medical employment due to its superior degree of crystallinity as well as it is selected as one of the strongest and stiffest thermoplastic materials. Also, being chemically very stable, resistant to abundant solvents, disinfectants and humidity, together with its lofty tissue compatibility [7].

POM has been consumed globally in dentistry as an offset for both PMMA and metals in tremendous of prosthetic employments since two decades ago. The most commonly functioning appliances were the esthetic clasps of RPD [6][8][9], cast posts and cores [10] as well as brackets [11].

Valaplast is an esthetic retentive retainer utilised in RPDs concerned for cosmetic improvement of teeth since it belongs to the Nylon family. Its retention is noticed on a wide range for being thin, light in weight, resistant to fracture and with a high modulus of elasticity [11].

The thermoplastic resin injection materials are remarkable for their superior merits such as; subsided modulus of elasticity, easily manipulated and esthetically acceptable results. The advantage of such low elastic modulus provokes and facilitates the engagement of more undercut improving the denture retention through these retainers [12].

So, this study aims to compare the most usable esthetic clasps mechanically to clarify the most suitable material to be used as partial denture clasps.

Material and Methods

Ideal model of maxillary partially edentulous case (Kennedy Class III) employed for educational purposes has been selected as a master model replicating the anatomical features of the teeth.

The ideal model was duplicated to make a stone cast with the maxillary premolar and the molar duplicated into wax to be surveyed before casting it into metal. This was carried out to provide mesially (8 mm) and lingual guide planes (6 mm) and create a 0.25 mm undercut area on the distobuccal surface. An occlusal rest seat 2 mm deep was prepared on the mesial occlusal surface for the molar tooth while providing distal (8 mm) and lingual guide planes (6 mm) and to create a 0.25 mm undercut area on the mesiobuccal surface, an occlusal rest seat 2 mm deep was prepared on the distoocclusal surface (Figure 1).

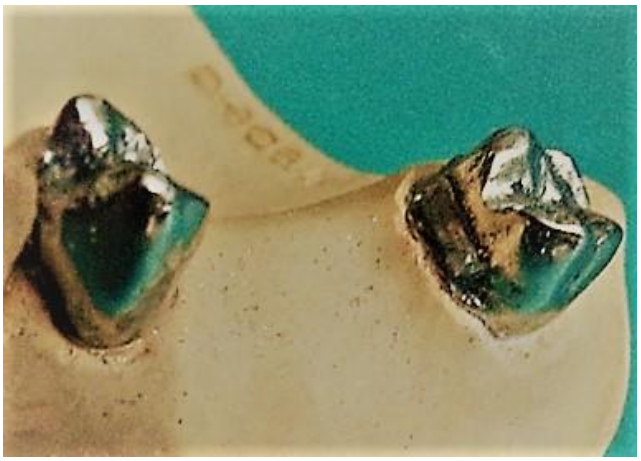

Figure 1: Model of surveyed metal teeth

The specimens included, five premolar clasps with 0.25 mm undercut and five molar ones with 0.50 mm undercut.

The materials of these five retainers for each abutment tooth are Chrome Cobalt (CoCr) metal clasp, Versacryl, Valplast, Acetal resin and Thermopress clasp. Each type of these retainers was fabricated as recommended by the manufacturer attaching to them a wax plate (4 x 7 x 3 mm) which was attached to the minor connector parallel to the path of insertion. The plate was utilised later for maintaining the clasp in the testing machine (Figure 2).

The procedure of testing clasps retention was conducted utilising a specially designed insertion-

removal apparatus (*Festo AG & Co, KG and Istanbul, Turkey*). The apparatus allowed the placement (insertion) of the retainer to its predetermined terminal position and its subsequent removal from the metal model.

Figure 2: The Specimens; A-Thermopress clasps; B-Metal clasps; C-Acetal clasps; D-Versacryl clasps

The retentive force of the retainer (g) was measured during removal (Figure 3). The clasp attached to the testing apparatus was placed on the corresponding abutment metal model fixed on a stainless-steel container. The container was filled with distilled water. Continuous cycles (4380) are starting from baseline till the 3-years of clinical utilisation of placement and removal of the retainer, simulating 3-years of clinical utilisation, were performed along the path of insertion and removal determined by preliminary surveying procedures of the abutment metal model and at each time interval, the maximum load is measured.

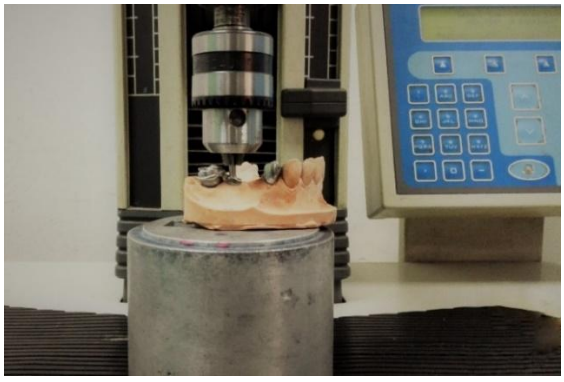

Figure 3: The testing machine with one of the specimens

A tensile load (in Newton) was applied at a crosshead speed of 10 mm per minute to the clasp until it was dislodged. The sensor (*Spider SW; Mettler-Toledo, Inc, Columbus, Ohio.*) connected to the load cell detected the magnitude of the tensile load applied at the moment the retainer was removed from the metal model. The maximum loads required to remove the clasp at 7 different periods of 0, 730, 1460, 2190, 2920, 3650 and 4380 continuous cycles were recorded by the computer (*Inspiron 8600; Dell Inc, Round Rock, Tex.*) connected to the sensor.

Acetal resin clasps and then CoCr ones were tested to avoid any possible surface attrition of the models. After fatigue due to retention testing methods, deformation test was performed; the distance between the tips of the retentive and reciprocal arms of each retainer which were placed in the acrylic resin blocks in the same position as previously described was measured to calculate the amount of deformation happened. The inner surface of each clasp was inspected and was measured with the electron microscope to record the amount of roughness happened (*Toolmaker TM-505; Mitutoyo Ltd.*) and then recorded (Figure 4).

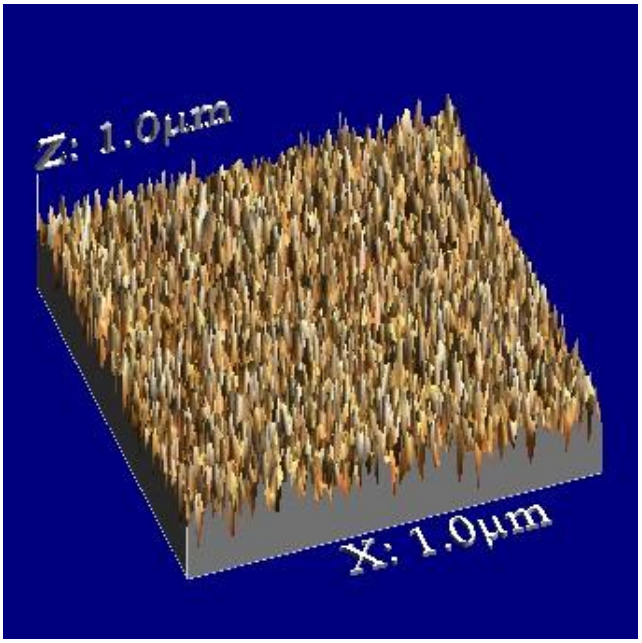

Figure 4: Picture under the electron microscope form the metal specimen

The mean values and SDs of the retentive force magnitudes were recorded for the 7 periods for dislodgement of each clasp (there was no difference between the results from premolar and molar specimens for each group material so, the records from the molar specimen were analyzed as the molar clasps had more surface area to be tested). Comparison of the data was conducted with 3-way analysis of variance (ANOVA) and a least significant difference (LSD) multiple range test (a = 0.05).

Statistical Analysis

Data were presented as the mean and standard deviation (SD). Data were explored for normality using Kolmogorov-Smirnov and Shapiro-Wilk tests. Roughness (Ra) revealed a parametric distribution, so One-Way ANOVA utilised to study the difference between tested Materials on mean

Roughness (Ra) followed by Tukey's posthoc test for pairwise comparison when ANOVA is Significant. Dependent t-test used to compare between Baseline data and each follow-up period data for every material.

Retention and deformation displayed a nonparametric distribution, so Kruskal Wallis used to study the difference between tested Materials on mean Retention and deformation followed by Mann Whitney U-test posthoc test for pairwise comparison when ANOVA is Significant. Wilcoxon Signed Rank test used to compare between Baseline data and each follow-up period data for every material.

The significance level was set at P ≤ 0.05. Statistical analysis was performed with IBM® SPSS® (*SPSS Inc., IBM Corporation, NY, USA*) Statistics Version 22 for Windows.

Results

Difference between Different Tested Materials on Mean Roughness (Ra)

Mean, and standard deviation (SD) for the Roughness (Ra) for different tested Materials were presented in Table 1 and Figure 5.

Metal (0.2549 ± 0.0043) and Acetal (0.2549 ± 0.0043) showed the lowest mean roughness compared to thermo (0.2624 ± 0.0006), Versa (0.2618 ± 0.0014) and Val (0.2626 ± 0.0008) at p ≤ 0.001 at baseline.

Metal (0.2508 ± 0.0027) and thermo (0.2499 ± 0.0019) showed the lowest mean roughness compared to Acetal (0.2633 ± 0.0004), Versa (0.2616±0.0004) and Val (0.2617 ± 0.0004) at p ≤ 0.001 at 1 month.

Metal (0.2493 ± 0.0026) showed the lowest mean roughness followed by Val (0.2609 ± 0.0010) followed by thermo (0.2615 ± 0.0009) and Versa (0.2616 ± 0.0007) followed by Acetal (0.2629 ± 0.0004) and at p ≤ 0.001 at 3 months.

Table 1: Mean and standard deviation (SD) of Roughness (Ra) for different tested materials

	Material										p-value
	Acetal		Thermo		Versa		Val		Metal		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Baseline	.2549a	.0043	.2624b	.0006	.2618b	.0014	.2626b	.0008	.2549a	.0043	≤ 0.001*
1 Month	.2633b	.0004	.2499a	.0019	.2616b	.0003	.2617b	.0004	.2508a	.0027	≤ 0.001*
3 Months	.2629a	.0004	.2615ab	.0009	.2616ab	.0007	.2609b	.0010	.2493c	.0026	≤ 0.001*
6 Months	.2891a	.0302	.2622b	.0016	.2607b	.0011	.2553b	.0047	.2510b	.0031	0.001*
9 Months	.2584ab	.0011	.2602a	.0010	.2593ab	.0010	.2551bc	.0072	.2527c	.0042	0.012*
12 Months	.2579b	.0012	.2592ab	.0017	.2610a	.0010	.2595ab	.0012	.2549c	.0029	≤ 0.001*

Means with the same letter within each row are not significantly different at p=0.05; *= Significant; NS=Non-significant.

Acetal (0.2891 ± 0.0302) showed the highest mean roughness compared to Metal (0.2510 ± 0.0031), thermo (0.2622 ± 0.0016), Versa (0.2607 ± 0.0016) and Val (0.2553 ± 0.0047) at p = 0.001 at 6 months.

Metal (0.2527 ± 0.0042) showed the lowest mean roughness followed by Val (0.2551 ± 0.0072) followed by Versa (0.2610 ± 0.0010) and Acetal (0.2584 ± 0.0011) followed by thermo (0.2602 ± 0.0010) and at p = 0.012 at 9 months.

Metal (0.2549 ± 0.0029) showed the lowest mean roughness followed by Acetal (0.2579 ± 0.0012) followed by Val (0.2595 ± 0.0029) followed by thermo (0.2592 ± 0.0017) and Versa (0.2610 ± 0.0010) and at p ≤ 0.001 at 12 months.

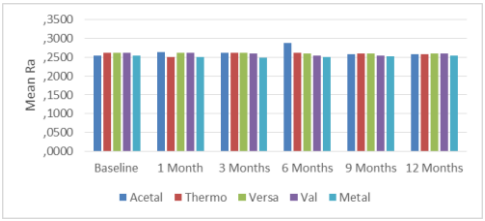

Figure 5: Histogram showing the mean Roughness (Ra) for different tested Materials

Mean Difference and Standard Deviation (SD) Between Baseline and Different Follow-Up Periods for Each Material:

Mean Difference and standard deviation (SD) between baseline and different follow-up periods for each material were presented in Table 2 and Figure 6.

A significant increase on mean Surface roughness after 1 and 3 months; after 6, 9 and 12 months, an insignificant increase on mean roughness for Acetal.

A significant decrease on mean Surface roughness after 1, 3 and 12 months; after 6 and 9 months, an insignificant decrease on mean roughness for Thermo.

Table 2: Mean Difference and standard deviation (SD) between baseline and different follow-up periods for each material

		Paired Differences		t	p-value
		Mean	SD		
Acetal	Baseline - 1 Month	-.0083833	.0044459	-4.619	0.006*
	Baseline - 3 Months	-.0080167	.0045490	-4.317	0.008*
	Baseline - 6 Months	-.0342000	.0333606	-2.511	0.054 NS
	Baseline - 9 Months	-.0035333	.0040128	-2.157	0.084 NS
	Baseline - 12 Months	-.0027400	.0040371	-1.518	0.204 NS
Thermo	Baseline - 1 Month	.0122750	.0027705	8.861	0.003*
	Baseline - 3 Months	.0007000	.0003651	3.834	0.031*
	Baseline - 6 Months	.0009000	.0008042	2.238	0.111 NS
	Baseline - 9 Months	.0021250	.0018264	2.327	0.102 NS
	Baseline - 12 Months	.0023750	.0002062	23.041	≤0.001*
Versa	Baseline - 1 Month	.0002250	.0017727	.254	0.816 NS
	Baseline - 3 Months	.0015000	.0002000	12.990	0.006*
	Baseline - 6 Months	.0015750	.0021329	1.477	0.236 NS
	Baseline - 9 Months	.0021500	.0016823	2.556	0.083 NS
	Baseline - 12 Months	.0003750	.0022780	.329	0.764 NS
Val	Baseline - 1 Month	.0009200	.0010085	2.040	0.111 NS
	Baseline - 3 Months	.0017167	.0009663	4.352	0.007*
	Baseline - 6 Months	.0072667	.0049318	3.609	0.015*
	Baseline - 9 Months	.0074667	.0073650	2.483	0.056 NS
	Baseline - 12 Months	.0030667	.0009730	7.720	0.001*
Metal	Baseline - 1 Month	.0040833	.0051375	1.947	0.109 NS
	Baseline - 3 Months	.0055333	.0042571	3.184	0.024*
	Baseline - 6 Months	.0038833	.0062608	1.519	0.189 NS
	Baseline - 9 Months	.0021667	.0075965	.699	0.516 NS
	Baseline - 12 Months	-.0000667	.0064217	-.025	0.981 NS

** = Significant; NS = Non-significant.*

A significant decrease on mean Surface roughness only after 3 months; after 1, 6, 9 and 12 months an insignificant decrease on mean roughness for Versa.

A significant decrease on mean Surface roughness after 3, 6 and 12 months; after 1 and 9 months, an insignificant decrease on mean roughness for Val. A significant decrease on mean Surface roughness only after 3 months; after 1, 6, 9 and 12 months an insignificant decrease on mean roughness for Metal.

Difference between Different Tested Materials on Mean Retention

Mean and standard deviation (SD) for the Retention for different test. Materials were presented in Table 3 and Figure 6.

Metal (8.02014 ± 2.7228) showed the highest mean Retention followed by to thermo (2.9698 ± 1.2505), Acetal (39527 ± 1.7613) and Val (2.8696 ± 1.3727) and the lowest for Versa (1.7159 ± 0.3434) at P = 0.009 at baseline.

Metal (9.1109 ± 6.4264) and Acetal (3.9527 ± 1.7613) showed the highest mean Retention followed by to thermo (2.2149 ± 0.6867) and Val (2.3340 ± 1.5755) and the lowest for Versa (1.3805 ± 0.4561) at P = 0.003 at 1 month.

Table 3: Mean and standard deviation (SD) of Retention for different tested materials

	Material										p-value
	Acetal		Thermo		Versa		Val		Metal		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Baseline	3.9527^{ab}	1.761	2.9698^{ab}	1.250	1.7159^{b}	0.343	2.8696^{ab}	1.372	8.2014^{a}	2.722	0.009*
1 Month	8.2350^{a}	1.940	2.2149^{ab}	.686	1.3805^{b}	0.456	2.3340^{ab}	1.575	9.1109^{a}	6.426	0.003*
3 Months	3.3129^{ab}	0.712	3.0995^{ab}	1.553	1.8504^{b}	0.875	2.4068^{ab}	0.274	9.8193^{a}	4.889	0.012*
6 Months	2.3163^{b}	0.364	8.4239^{a}	1.805	2.1581^{b}	1.024	1.4516^{b}	0.409	14.5209^{a}	2.546	0.001*
9 Months	2.6660^{b}	0.439	8.6016^{a}	5.094	3.2561^{b}	1.257	4.6698^{b}	2.343	10.1663^{a}	4.264	0.039*
12 Months	2.2418^{b}	1.968	4.1404^{b}	1.540	1.3633^{b}	1.177	2.0611^{b}	0.660	13.0802^{a}	8.668	0.037*

Means with the same letter within each row are not significantly different at P = 0.05; = Significant; NS = Non-significant.*

Metal (9.8193 ± 4.8893) showed the highest mean Retention followed by to thermo (3.0995 ± 1.5530), Acetal (3.3129 ± 0.7124) and Val (2.4068 ± 0.2746) and the lowest for Versa (1.8504 ± 0.8753) at P = 0.012 at 3 months.

Metal (14.5209 ± 2.5468) and thermo (8.4239 ± 1.8059) showed the highest mean Retention followed by to Acetal (2.3163 ± 0.3646), Val (1.4516 ± 0.4099) and Versa (2.1581 ± 1.0241) at P = 0.001 at 6 months. Metal (10.1663 ± 4.2645) showed the highest mean Retention followed by to thermo (8.6016 ± 5.0946), Acetal (2.6660 ± 0.4394), Val (4.6698 ± 2.3431) and Versa (3.2561 ± 1.2570) at P = 0.039 at 9 months.

Metal (13.0802 ± 8.6684) showed the highest mean Retention followed by Acetal (2.2418 ± 1.9687), Val (2.0611 ± 0.66), thermo (4.1404 ± 1.5407) and Versa (1.3633 ± 1.1774) and at P = 0.037 at 12 months.

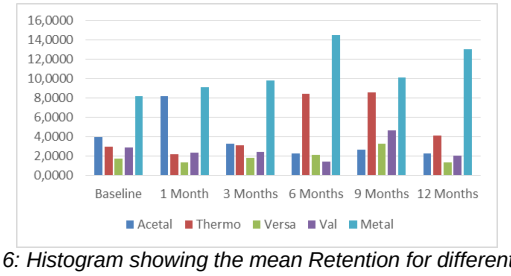

Figure 6: Histogram showing the mean Retention for different tested Materials

Mean Difference and Standard Deviation (SD) Between Baseline and Different Follow-Up Periods for Each Material

Mean Difference and standard deviation (SD) between baseline and different follow-up periods for each material were presented in Table 4 and Figure 7.

An insignificant difference after different evaluation periods for all materials except for after 1 months for ACETAL which showed a significant increase in mean retention at P = 0.043. And Thermo after 6 months at P = 0.043.

Table 4: Mean Difference and standard deviation (SD) between baseline and different follow-up periods for each material

		Paired Differences		Z	p-value
		Mean	Std. Deviation		
Acetal	Baseline - 1 Month	-4.2823930	2.0530038	-2.023	0.043*
	Baseline - 3 Months	.1614410	1.9629010	0	1.00 NS
	Baseline - 6 Months	1.1579828	1.6436127	-1.461	0.144 NS
	Baseline - 9 Months	1.2866424	1.5967715	-1.214	0.225 NS
	Baseline - 12 Months	1.2325433	1.6628911	-1.461	0.144 NS
Thermo	Baseline - 1 Month	.7549448	1.8965472	-0.405	0.686 NS
	Baseline - 3 Months	-.1297194	1.8294536	-0.405	0.686 NS
	Baseline - 6 Months	-5.4541192	2.7634226	-2.023	0.043*
	Baseline - 9 Months	-5.0927237	3.6755167	-1.604	0.109 NS
	Baseline - 12 Months	-1.0003463	2.8312038	-0.73	0.465 NS
Versa	Baseline - 1 Month	.3353628	.5346009	-0.944	0.345 NS
	Baseline - 3 Months	-.1345190	1.1697202	-0.405	0.686 NS
	Baseline - 6 Months	-.4421946	1.2282727	-0.674	0.5 NS
	Baseline - 9 Months	-1.5402088	1.3595830	-1.753	0.08 NS
	Baseline - 12 Months	.4449913	1.4839723	-0.365	0.715 NS
Val	Baseline - 1 Month	.5355882	1.7906744	-0.943	0.345 NS
	Baseline - 3 Months	.1601983	1.6170592	-0.365	0.715 NS
	Baseline - 6 Months	1.1153513	1.4992394	-1.095	0.273 NS
	Baseline - 9 Months	-2.1028110	2.9035494	-1.461	0.144 NS
	Baseline - 12 Months	.9795627	1.6585333	-1.069	0.285 NS
Metal	Baseline - 1 Month	-1.4141635	11.7759675	-0.447	0.655 NS
	Baseline - 3 Months	.1090153	6.4564111	0	1.00 NS
	Baseline - 6 Months	-5.6249250	7.3720054	-1.342	0.180 NS
	Baseline - 9 Months	-1.9649557	6.5059171	-0.535	0.593 NS
	Baseline - 12 Months	-4.8787943	10.0146186	-0.535	0.593 NS

** = Significant; NS = Non-significant.*

Difference between Different Tested Materials on Mean Deformation:

Mean, and standard deviation (SD) for the Deformation for different tested Materials were presented in Table 5 and Figure 7.

Table 5: Mean and standard deviation (SD) of Deformation for different tested materials

	Material										p-value
	Acetal		Thermo		Versa		Val		Metal		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Baseline	.55	.43	1.66	2.23	3.09	2.39	.73	.50	1.23	.71	0.348 NS
1 Month	.50	.31	2.66	2.15	2.58	2.48	5.07	.11	.66	.41	0.173 NS
3 Months	1.39	1.10	4.57	.60	2.21	2.44	2.97	2.34	.85	.61	0.354 NS
6 Months	.66	.52	2.82	1.89	3.75	2.50	.97	.98	.64	.43	0.363 NS
9 Months	.93	.46	1.00	.67	.89	.76	.97	.98	1.44	1.14	0.778 NS
12 Months	2.08	2.55	.98	.30	1.19	1.68	1.89	.67	1.35	.24	0.579 NS

*Means with the same letter within each row are not significantly different at P = 0.05; * = Significant; NS = Non-significant.*

An insignificant difference between tested materials for all different evaluation periods.

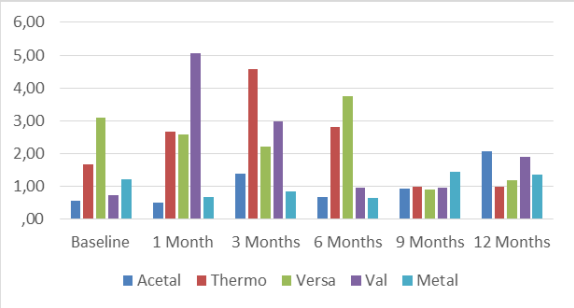

Figure 7: Histogram showing the mean Deformation for different tested Materials

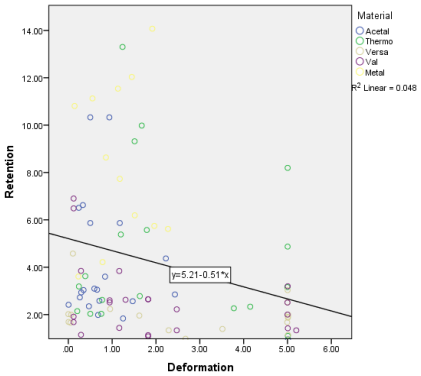

Figure 8: Scattered plot for the Correlation between Retention and Deformation

Mean Difference and Standard Deviation (SD) Between Baseline and Different Follow-Up Periods for Each Material:

Mean Difference and standard deviation (SD) between baseline and different follow-up periods for each material were presented in Table 6 and Figure 8.

An insignificant difference after different evaluation periods for all materials.

Table 6: Mean Difference and standard deviation (SD) between baseline and different follow-up periods for each material

		Paired Differences		Z	p-value
		Mean	Std. Deviation		
Acetal	Baseline - 1 Month	.05687	.60921	-0.365	0.715 NS
	Baseline - 3 Months	-.82736	1.53201	-0.73	0.465 NS
	Baseline - 6 Months	-.03126	.65298	0	1.00 NS
	Baseline - 9 Months	-.30465	1.03263	0	1.00 NS
	Baseline - 12 Months	-1.45135	3.07872	0	1.00 NS
Thermo	Baseline - 1 Month	-.99450	1.47213	-1.095	0.173 NS
	Baseline - 3 Months	-1.82127	3.78217	-0.447	0.655 NS
	Baseline - 6 Months	-.85927	3.87533	-0.535	0.593 NS
	Baseline - 9 Months	.96685	2.52155	-0.535	0.593 NS
	Baseline - 12 Months	-.54021	.21998	-1.342	0.180 NS
Versa	Baseline - 1 Month	-.12240	2.53189	0	1.00 NS
	Baseline - 3 Months	.88350	4.59077	-0.73	0.465 NS
	Baseline - 6 Months	-.65704	4.28074	0	1.00 NS
	Baseline - 9 Months	1.56253	3.23707	-0.535	0.593 NS
	Baseline - 12 Months	1.34495	5.18134	-0.447	0.655 NS
Val	Baseline - 1 Month	-4.19957	.45768	-1.604	0.109 NS
	Baseline - 3 Months	-2.24534	1.83802	-1.857	0.063 NS
	Baseline - 6 Months	-.24545	.47691	-0.743	0.458 NS
	Baseline - 9 Months	-.24545	.47691	-0.743	0.458 NS
	Baseline - 12 Months	-1.16345	.16988	-1.857	0.063 NS
Metal	Baseline - 1 Month	.56813	1.04913	-0.535	0.593 NS
	Baseline - 3 Months	.38367	.69116	-1.069	0.285 NS
	Baseline - 6 Months	.59026	1.09239	-1.069	0.285 NS
	Baseline - 9 Months	-.21568	1.82849	0	1.00 NS
	Baseline - 12 Months	-.48644	.67592	-1.342	0.180 NS

** = Significant; NS = Non-significant.*

There was negative significant correlation between the retention and deformation; r = -0.218; P = 0.032

Table 7: Pearson Correlation between the retention and deformation

		Deformation
Retention	Pearson Correlation	-0.218
	Sig. (2-tailed)	0.032*
	N	97

** = Significant; NS = Non-significant.*

Discussion

The removable partial denture's direct retainer is of particular importance as it affects the denture longevity during the function. In the current consideration, different materials employed as a clasp were compared mechanically to reach the decision of ideal clasp material for denture immortality.

The surface roughness of denture bases promotes adhesion of microorganisms and plaque accumulation. It is mainly stimulated by the material's deep-rooted countenances, polishing method and the operator's manual expertise.

It has been revealed in the current contemplate that CoCr clasps were of least roughness, this could be attributed to its superior resistance to corrosion, microhardness and modulus of elasticity as well as low density [13].

Another addition to the metal-free removable partial denture (RPD) market is polyoxymethylene (Acetal resins). In the present contemplate Acetal showed different roughness behaviour within the different follow-up periods but least non-metal roughness after one year of follow-up. This could be simply clarified as all Acetal resins are characterised by having superior abrasion resistance, limit water sorption and exhibit lower creep. Moreover, Acetal's resin superior solidity favours the imitative retainer's layout, connectors and other components with several restitutions desired [14].

Acetal resin proved to be superior in flexibility, strength as well as resistance to wear and fracture. Moreover, it is remarkable for its high creep resistance, fatigue endurance as well as its hydrophobic nature. Acetal resin is free from micro porosities or with rarely few ones reduces the accumulation of biological materials as plaque, which in turn resists odour and stains [15].

A broad extension to the metal-free RPD

emporium has been the polyamide (nylon) removable partial denture (Valplast). The current trial revealed both the worst and highest roughness in all the follow-up periods. On employing the polyamide base resin (Valplast), it revealed that both its surface roughness and difficulty that takes place during polishing leads to bacterial and fungal colonisation on its surface which is considered as its negative aspect as was reported by a previous study [16].

Regarding retention, in this trial, both the CoCr and Acetal clasps revealed the highest mean retention with the one-year follow-up period. The experimental design of this consideration tested a single-retainer system and documented that retention with CoCr alloy clasps is remarkably greater. It should be clarified that tremendous factors have a great impact on RPD's retention. Appropriate guiding plane on the proximal planes of abutment teeth is one of the retention enhancers. Clinical experience indicates that ineffective reciprocation may result in lack of both retention and stability [17] [18].

Acetal resins are highly versatile engineering polymers that bridge the gap between metals and ordinary plastics. Since they combine between both metal's strength and plastic's pleating and relief, they provide an ideal substance for the construction of dental prostheses specifically retainers [19]. It has been clarified that the Acetal's retention was the highest within the one-year follow-up period, this might be attributed to its combatively elevated proportional extent with the slight flow of viscosity allowing it to proceed elastically over abroad area to be employed as a favourable matter for providing retention [20].

Ulterior to three months follow up period it was revealed that, although retention declined in all parties, yet Acetal's clasp retention is still significantly superior ($p \leq 0.05$) than the cobalt chromium one. This coincides with results of another trial which mentioned that Acetal resin as a thermoplastic clasp enhancing they're positioning deeper into undercuts for preferable settlement and retention with minimal bulk which is also easily adjusted [21].

Abutment's teeth number and allocation, wax's block-out bulk and framework's fit are auxiliary agents that impress the degree of retention achieved. This contemplates employed an experimental design for single retainer system. Later on, Acetal resin retainers might be more adequate for clinical employment, when two or three Acetal resin retainers are utilised in RPD construction regarding all the factors above [20].

Acetal resin clasps can be expended in retaining Kennedy's class III RPD. However when utilised on molars it is recommended either to maximise the thickness of the retentive clasp arm or use deeper under the cut. On consuming Acetal as a direct retainer, it provides more retention qualities on premolars than on molar teeth, and this was followed in the current contemplate as premolar teeth were utilised for direct retention [22].

Aiming to adequate function of RPDs, it has been suggested that a retentive force of 5 N is desired. Moreover, a contemplate mentioned that the mean retentive force for the 1.0 mm thick thermoplastic resin retainers at the end of the cycling test ranged from 1.7 N to 3.7 N, while that for the 1.5 mm thick ones from 5.4 N to 10.8 N. Such outcomes revealed that thermoplastic resins could be utilized in generating RDPs' retainers, since they supply sufficient retention even in accordance with a decade of simulated employment [23] [24].

Custom made Acetal resin retainer provides a great difference between this contemplate and other studies since it is more adaptable and highly fitting to the undercut which consequently improves the retention. This is coinciding with another trial which described that Acetal resin as an injection moulding substance is suitable for RPDs with flexible esthetic retainers [18].

Thermoelastic resins (Versacryl) in this trial revealed the lowest mean retention within the same follow-up period, this was attributed to their viscoelastic properties as they are fabricated with emanant pliability, which is about ten times as that of the metal retainers, and they return to their current magnitudes in accordance with stretching as recommended by other considerations [28]. Moreover, a latter paramount merit for the thermoplastic retentive arm is remarkable for having a domestic remembrance to revert to its main posture as compared to the casted retainer which ordinarily in accordance with 500 times of insertion and removal becomes fatigued which is considered as an additional merit for Versacryl as reported by previous trials [26] [27].

There was the insignificant difference between all the different materials utilised regarding the deformation. This was simply explained as retainers afford both permanent deformation and fatigue which allows it to shatter following repeated flexures initiated by both denture insertion and removal as well as chewing [28] [29] [30] [31]. The fatigue life of CoCr proved to be the highest of all the casted clasps as those made of commercially-pure titanium and gold alloy clasps [32]. Permanent deformation and fatigue fracture result from the overwork that took place in the retainer [33] [34]. Alloy's modulus of elasticity, retainer's sizes and curvature [35] [36], and both the amount and direction of deflection about the abutment undercut are the main agents upon which load distribution depends on [37] [38]. The CoCr alloy's stiffness makes them unsuitable for their placement in deep undercuts, as they can induce stresses on the abutment teeth or may result in permanent deformation of the class [39].

It was concluded that though the flexural strength and modulus of elasticity were relatively low

in the thermoplastic resins, they demonstrated great toughness and resistance to fracture; thermoplastic resins could afford forces till a considerable deflection limit which remarks to their adequate longevity for multiple intraoral insertions and removals [40]. Another contemplate mentioned that, if all other variables were equal, a 15 mm long CoCr clasp of one mm diameter would exhibit the same stiffness as an Acetal resin clasp of five mm in length and 1.4 mm in diameter which justifies that, thicker Acetal resin retainers were utilized for comparison in the current contemplate [22].

After one month of insertion, retention of both CoCr and Acetal declined, this was by results of other considerations as it proved that there was no deformation for the Acetal resin clasps after 36 months of simulated clinical employment, unlike the CoCr ones which presented an increase in the distance between its tips. Due to permanent deformation of CoCr retainers, the retentive force was lost within 730 cycles of placement and removal and continued to lose its retention during the remaining testing period [20]. In general, Polyamide resins utilised in dentistry exhibit superior flexibility, physical strength, heat and chemical resistance. On the other hand, all nylons revealed superior water sorption and crept than most dental polymers [41].

From the outcomes of this in-vitro study, it can be accomplished that Acetal resin proved to be the non-metallic material of choice due to its superior properties regarding roughness, retention and deformation, while Valplast is the lowest at the end of one year follow up period.

Recommendations: This contemplate recommends utilising Acetal resin as the best non-metallic partial denture clasp, while the Cr Co can be used as the metallic one.

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Retention of Approximal Guiding Plane Surfaces in Removable Partial Skeletal Prosthesis

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Keywords: Retention; Partial dentures; Clasp; Retention force; Guiding surface

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Abstract

BACKGROUND: The morphology of the retention tooth often does not correspond with the required design; hence there is often an indication for enamel recontouring or other restorative procedures.

AIM: The study aimed to determine the impact of changing the path of insertion of the prosthesis by reshaping the anatomical and morphological structures of the natural teeth predetermined for the retention of the prosthesis.

MATERIAL AND METHODS: The group of 40 patients with Class II, Subclass 1 according to Kennedy was formed, and 120 approximal surfaces of retention teeth were obtained. Two different types of prostheses were made on the models: one group in the zero point position of the model, and another group in the zero position of the model, with changing of the direction of input at an angle of 2˚.

RESULTS: The difference between the established and theoretical normal distribution of frequencies was tested with the Kolmogorov-Smirnov and Lilliefors tests ($r < 0.10$; $r < 0.01$). The first group showed a retention force of 0.08 N. In the second group the retention force was 0.94 N.

CONCLUSION: It could be concluded that the change in the path of insertion of the dental prosthesis with conservative restorations as composite inlays, as well as the accurate extension of the prosthesis onto guiding plane surfaces, will undoubtedly increase the retention force of the prosthesis.

Introduction

Creating a successful partial skeletal prosthesis (PSP) by a general practitioner or a specialist prosthodontist is always a tough requirement. The prosthesis should fulfil the basic prosthetic principles of patient aesthetics, speech or phonation, function and even distribution of pressure, stability and retention [1].

To achieve these characteristics, the prosthesis must be carefully planned, designed and constructed. The basis of these processes is the parallelometry surveying that allows a dimensional analysis of the ratio between oral soft tissue and oral hard tissue. Parallelometry is accomplished by the collaboration of a therapist and a technician depending on the choice of retention teeth and the location of the occlusal rests. An analysis of the dimensional ratio between the anatomical characteristics must be completed before a final decision on the design of the PSP is made by a therapist. Parallelometry and dental surveying is an essential component of PSP therapy [2] [3].

However, in spite of the advantages of a well-made prosthesis, some studies have pointed out to the problems that arise when wearing the prosthesis, such as caries, gingival inflammation, increased sulcus depth and loss of retention [4]. The deviation from fabrication standards that later leads to unwanted consequences regarding pressure distribution, are one of the main reasons why patients are not satisfied with their prostheses [5].

The basic idea of this study was to examine the effects of modifying the guiding plane surfaces on the retention of PSP. Guiding plane surfaces are

vertical parallel surfaces of retention teeth and abutments of dental implants, so oriented that they contribute to the determination of the path of placement and the path of removing the PSP [6]. The guiding surfaces are usually formed on the approximal surfaces of the retention teeth that look toward the edentulous ridge. It is generally accepted that the guiding surfaces cover about two-thirds of the width between the buccal and the lingual tubers [7].

The functions of guiding surfaces on a single desired path of insertion of the prosthesis are equal retention of all abutment teeth, protecting from horizontal masticatory forces, minimising forces that may destabilise the partial prosthesis and allow the prosthesis to be removed without premature contact and damaging effect on retention teeth. They also direct the forces to act on the longitudinal axis of the teeth and provide frictional retention of the parallel guiding plane surfaces [8].

Changing the path of placement for equalisation of the undercut spaces on the teeth often occurs as a need for planning and surveying of the diagnostic cast of the prosthesis. In a very small number of cases, the zero point position will allow preparation of the prosthesis without additional alterations of the retention teeth [9].

One of the factors influencing the proper planning of the guiding surfaces and their modification is the correct transmission of the analysis of the path of placement of the prosthesis by a dentist on a model in the dental laboratory. This problem was solved using a surgical operating microscope combined with coaxial illumination [10]. A special technique for fixing pins and the cylinder device, described by NaBadalung *et al*., [11] ensured proper positioning and easy handling in the design planning process. Advantages and increased function of guiding surfaces can be achieved by reshaping the approximate guiding surfaces of retention teeth. Alfonso C. *et al*., [12] in their clinical trials showed a simple method that made contact between the skeleton and retention teeth closer, which increased the retention of the prosthesis.

When testing the value of the guiding surfaces of the PSP, Ahmad and Waters used the ratio between the direction of the guiding surfaces and their inclination angle. They have found that the increase in retention occurs when the incline of the surveyor table grows to 22º with an inclination of guiding surfaces up to 12º [13]. Multiple methods and devices for parallelising retention teeth were created to determine the guiding surfaces of the definitive model and precisely transpose them into the patient's mouth. However, these techniques are quite expensive and complicated for practical application [14].

The preparation of the guiding surfaces mostly depends on the experience and ability of the dentist [15]. Friction retention or retention created from contact on guiding surfaces is an excellent source of retention force in the prosthesis, but often cannot be accomplished, because the leading surfaces must always be parallel. Since the creation of a guiding plane surfaces is difficult and often impossible, the friction force is not always a reliable source of retention. One of the circumstances in which the friction force is used is when the crowns on retention teeth are used, and the approximal and lingual surfaces are parallelised in a laboratory. In his research, Uemura *et al*. compared the techniques of equalising leading surfaces, showing that the intra-oral method was superior to other techniques [16].

Today, modern technology provides unlimited possibilities for performing morphological interventions on teeth, with non-invasive or less invasive techniques. This can be used in optimal interventions on the teeth, depending on the needs indicated, which will replace the preparation of the crowns. All of these, as well as other problems, should be properly observed with a thoughtful solution from the first contact with the patient to the finally provided function of the prosthesis in the patient's mouth due to its long-term use.

The paper aimed to determine the force of retention on the approximate guiding surfaces at the zero point position of the model and to determine the force of retention by remodelling the approximate guiding surfaces that occupy an angle of 2º concerning the model base.

Material and Methods

The investigation was performed at the University Clinic for Dental Prosthetics at PHI Dental Clinical Center "St. Panteleimon " - Skopje, during a specified period. The patients were with the partially edentulous ridge, an endless one-sided terminal and one inserted saddle or class II, sub-class 1 according to Kennedy. A group of 40 examinees was created, which resulted in 120 approximal surfaces of retention teeth for further analysis. The parallel-meter, model AF 30 (NOUVAG, Switzerland) was used in the test. This parallel-meter possesses the possibility for adjustment of the head when milling the inclination angle of 2º, 4º and 6º, about the vertical axis. The formation of the guiding surfaces according to the principles of the purpose-built inlays in the test was made with the material Grandio (VOCO). For fabrication of the removable partial denture framework, a Co-Cr-Mo alloy metal (Dentaurum, Remanium GM 380+), with the following chemical composition: Co 64.6%, Cr 29%, and Mo 4.5% was used.

The dynamometer that measures the strength of the denture retention consists of a power sensor, a

DO663i model, a CoachLab II interface, a table kit for fixing models and a computer. The first three sections are produced by CMA (Center for Microcomputer Applications) Amsterdam, Netherlands. The sensor was connected to a computer through the CoachLab II interface. It was a multifunctional interface that offered many possibilities for computerised measurement and control of devices. It was supplied with its microchip and its embedded system.

Anatomic impressions with a two-phase method were taken from the selected patients. The first impression was made with the heavy body silicone impression material (Optosil, Heraeus Kulzer) and the second with the light body material (Xantopren L, Heraeus Kulzer). The impressions were cast with super hard stone (Polident, Polish one type IV). Two types of dentures were made on the master cast, and the strength of retention force was measured. In the first case, the model was set to zero points, and the planning of the framework of the prosthesis was made in that position. In the second case, the model was also set to zero point position.

Inlays were made in the cavities on the approximal surfaces of retention teeth, oriented towards the free space, and the nanocomposite material was applied. The guiding surfaces of the three retention teeth were modelled by a cylindrical milling cutter placed in the parallel-meter head. The milling cutter was placed in an anteroposterior position, at an angle of 2º, whereby, with the help of milling, a remodelled, the artificial guiding surface was obtained (Figure 1).

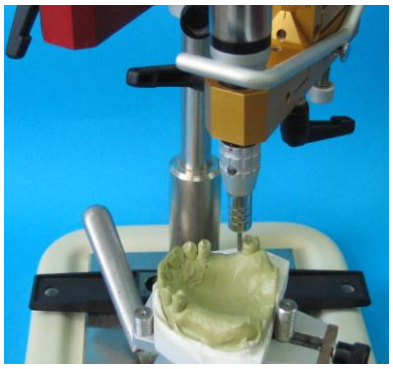

Figure 1: Formation of guiding surfaces

We made a framework for removable partial prosthesis on the master casts prepared according to the standard procedure. Thus, as a result of the test, a modification was made in the fabrication of the framework, and the retention clasps, the occlusal rests and the stabilisation elements were omitted. To enable the measurement of the total retention force, three rings were placed on the same level, at the area of the guiding surfaces. This allowed attaining tension evenly and deployed the tensile force on all parts of the prosthesis (Figure 2).

Figure 2: The framework with three rings

The process of measuring the retention power was made as follows: the model with the framework of the prosthesis was fixed on the table that was able to move in a vertical direction, and then it was put under the clasp that was connected to a power sensor (Figure 3).

Figure 3: Measurement of retention force

The retention force or the tension between the clasp and the model was accomplished through the rings on the framework and the strings that connected them to the clasp. The power of tension was caused by lowering the carrier table vertically downwards (Figure 4). Its intensity was automatically read and registered on the computer screen. Each model was measured in five series, and the obtained data were statistically processed with descriptive statistical analysis.

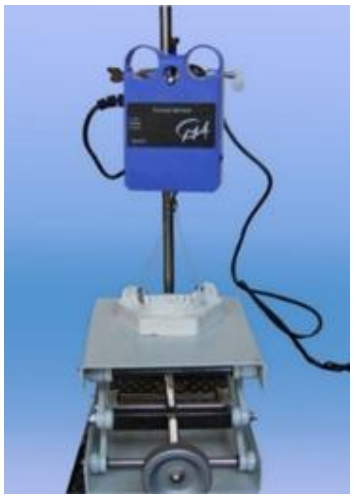

Figure 4: Moving the carrying table downwards for tension force

Results

Descriptive statistical indicators of measured data for the group of unprepared retention teeth or zero position of the model are given in Table 1. Distribution of the data for the variable "unprepared retention teeth-zero angle" is shown by using a histogram, as well as by expected distribution of frequencies.

Table 1: Mean size of retention force in prostheses without preparation of retention teeth

N	$\bar{X}$ (N)	SD(N)	SE_{x} (N)	$SE_{\bar{x}}$ (N)	V_x	P(N)	I_v (N)
40	0.08	±0.037	±0.038	±0.008	0.46	-0.07-0.081	0.0-0.13

The difference between the established and theoretical normal distribution of frequencies was tested with the Kolmogorov-Smirnov and Lilliefors tests (r<0.20; r<0.01). The established distribution was significantly different from the theoretical one (Figure 5).

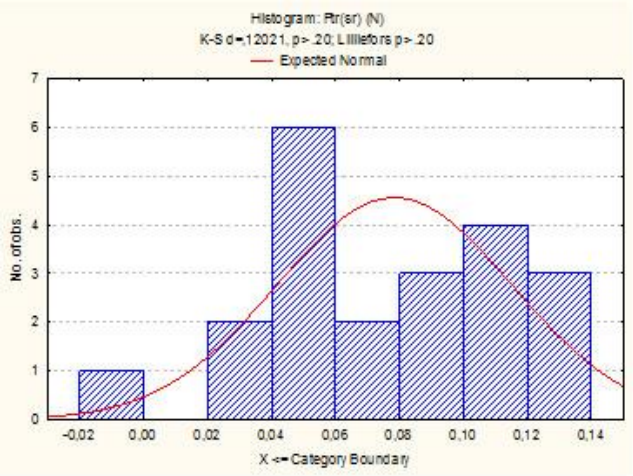

Figure 5: Histogram of the distribution of the variable retention force in unprepared retention teeth

Distribution of data on the variable "prepared guiding surfaces of retention teeth, at an angle of 2º" is shown by using a histogram. At the same time, the theoretical (expected) distribution of frequencies is also shown. The difference between the established and theoretical normal distribution of frequencies was tested with the Kolmogorov-Smirnov and Lilliefors tests (r<0.10; r<0.01). Determined distribution was significantly different from the theoretical normal, but after several measurements, it came close to the normal one (Figure 6).

Table 2: Mean size of the retention force in prostheses with prepared retention teeth

N	$\bar{X}$ (N)	SD(N)	SE_{x} (N)	$SE_{\bar{x}}$ (N)	V_x	P(N)	I_v (N)
40	0.94	±1.43	±1.467	±0.328	±1.52	0.297-1.583	0.075-6.51

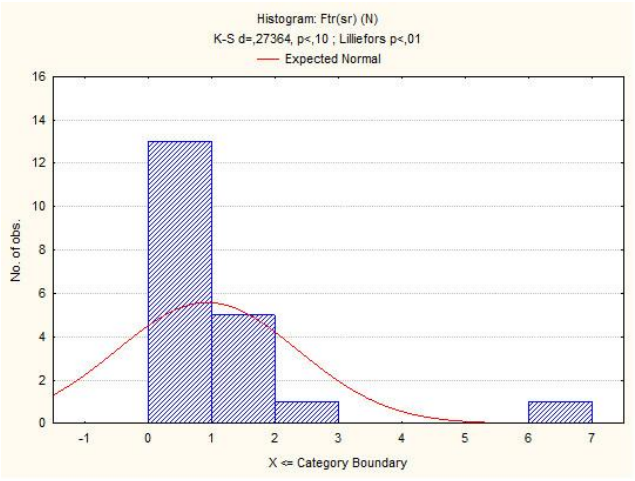

Figure 6: Histogram of the distribution normality of the retention variable strength in prostheses with prepared guiding surfaces

Discussion

The value of the retention force is a determining factor and a measure of the success of planning and implementation, as well as of the functional capabilities of the PSP. The term “retention force” of the prosthesis presents the size of the resistance of the retention elements to the forces that tend to remove the prosthesis from the basis. In the particular case, the resistance/retention arises from the friction between the parts of the prosthesis and the remaining teeth, which is achieved on the contact surface on which they accurately lie down. It is a result of a frictional force between retention teeth and prosthesis, as well as the mechanical resistance from the restrained retention tooth.

In this study, to make a comparison, two groups of subjects were treated: the first group of models - without preparation of the retention teeth, and the second set of models with planned and prepared to guide surfaces at an angle of 2º. The following condition was determined: in the first group, the retention was very small and varied from 0.0 to 0.13 N, with the medium value of 0.08 N. This value does not satisfy the stability requirements and functionality of the PSP. In fact, the measured values were close to zero, as a constant size, with a narrow confidence interval P = -0.07-0.081 N, and with an interval of variation Iv = 0.0-0.13 N.

In the second group with planning and preparation of the guiding surfaces and by providing the angle of retention of 2º, the size of the retention force significantly increased. It varied from 0.075 N to 6.51 N, with the medium value of 0.94 N. These results showed that with the increase in the angle of undercut, the strength of retention increases.

These results are consistent with those obtained by Walter [17], who, as a disadvantage of the vertical path of placement and removal of the prosthesis, assumes that it coincides with the path of denture displacement when sticky foods are chewed. The same author in his analysis of retention has suggested that when the guiding surfaces are chosen or formed so that the path of placement is different from the path of maximum displacement, other parts of the prosthesis, such as the base, acrylate wings or minor connectors can be modelled as guiding surfaces.

In a very small number of cases, the current position of retention teeth will satisfy the necessary undercutting. Although in some cases the approximal surfaces of retention teeth are assessed as favourable and able to serve as guiding surfaces, however, due to the filling of the undercut spaces, the possibility of retention is lost. In this study, we decided to increase the influence of the guiding surfaces on the retention force of the prosthesis by modifying the guiding approximal surfaces on retention teeth. This decision is based on the fact that higher inclination changes the position of the prosthetic equator, and the possibility of applying stabilisation elements and retention clamps in precisely determined areas of a tooth is disabled.

Wulfes [18] came to a similar conclusion, which confirms the fact that, very often with minor corrections through reshaping, ideal conditions are created in cases where there are no natural guiding surfaces. With this inclination, although minimal, we are changing the path of placement and removal of the prosthesis, which has also been supported by Bates [19], Borges [20], Krikos [21] and other authors. The inclination of the model in the parallelogram and the change in the direction of the prosthesis with the formation of guiding surfaces has also been accepted by Garcia, Bohnenkamp [22].

In conclusion, by implementing the procedure with the reshaping of guiding surfaces and creating the undercut areas, the prosthesis lied better on the retention teeth.

1. It can be concluded that due to the wide variability of the angle of the undercutting, it is difficult to ensure a unique path of the PSP and its proper function without reshaping of the potential retention teeth.

2. With the formation of artificial approximate guiding surfaces of retention teeth from nanocomposite inlays, conditions have been created for placement and removal of the PSP into a single direction, which is not perpendicular to the occlusal plane resulting in the appropriate size of the retention force and satisfying other expected functions.

3. Further research is necessary to achieve a significant frequency of occurrence of each potential retention tooth and studying the dependence, defining a diagram of the change in the size of the force of retention, depending on the increase in the angle of guiding artificial surfaces.

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Dental Science

Evaluation of the Masticatory Efficiency at the Patients with New Complete Dentures

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Abstract

BACKGROUND: There are a lot of factors influencing the efficiency of mastication; therefore there are also a lot of methods for testing this efficiency.

OBJECTIVE: The study aimed to test the efficiency of mastication and evaluate it in the function of time, based on previous experience with the complete dentures.

METHODS: A total of 88 patients (42 female, 46 male, mean age 52.2, SD = 5.76), complete dentures wearers, participated in this study. Masticatory functions were investigated by using the method of electromyography (EMG), analyzing electromyomasticatiogram. For testing the masticator efficiency, the further parameters of the masticatiogram were used: duration of the Standard Masticatory Task (SMT) (t), number of the masticatory cycles within the masticator arch (F) and maximal amplitude within the masticatory arch (F). All data were statistically analysed by using standard software package BMDP (bio-medical Statistical package). Parametric data have been tested using One Way Repeated Measurement of ANOVA test.

RESULTS: The changes of the relation A/t are evident in different time intervals in both measured sides: F-DS-time of the measurement = 34.86, P = 0.0000; F-NDS- time of the measurement = 26.1, P = 0.0000. There are also differences of the masticatory index A/t between sexes in both, dominant and non-dominant side: F-DS-gender = 237.05, P = 0.0000; F-NDS-gender = 12.90, P = 0.004. Masticatory index (A/F) varies in time, from first to the sixth measurement in both sides: F-DS-time of the measurement = 32.36, P = 0.0000; F-NDS-time of the measurement = 30.53, P = 0.0000. The interaction of the time and gender was also important: F-DS-interaction = 6.95, P = 0.0000; F-NDS-interaction = 14.9, P = 0.0000.

CONCLUSION: Masticator indexes are A/F, and A/t are a very important indicator of the masticatory efficiency and the level of the functional adaptation on complete dentures. Both masticator indexes show the same dynamics (reaching the stationary condition after the 15th week after getting new dentures). After the observing period, all examinee express the same degree of masticator efficiency known as functional adaptation. Patients with previous experience with complete dentures reach faster the stationary condition, compared with those non-experienced.

Introduction

During the masticating process, the food is crushed and ground by placing between the teeth, by the cheek and tongue. During the chewing process, the food is reduced in small size by 10-40 masticator cycles, forming a bolus which can be swallowed easier [1] [2] [3].

Masticatory efficiency is defined as the number of strokes needed to achieve a certain particle size reduction [4].

There are a lot of factors influencing the efficiency of mastication: teeth condition, the number of antagonists left, the size of the chewing surfaces of the teeth, chewing force, masticatory movements and masticatory tempo, factors pertaining with a prosthetic work, food consistency and regular distribution of the bolus during mastication. In the natural dentition bolus size and its preparation are adapted to swallowing capacity [5] [6].

Nagasawa T et al., [7] showed that reduced masticatory efficiency could be associated with a relation of small contacts between the teeth and

reduced activity of masseter muscle and temporalis muscle. A lot of researchers showed that complete denture wearers, compensate reduced efficiency of mastication with a larger amount of bolus and not with extended chewing [3] [6] [8] [9].

The problems associated to the relation between the masticatory efficiency and tests of the food grinding, evaluated by patients, are shown from a lot of authors as Agerberg and Carlsson [10], Gunne J. et al., [11], Carlsson and Ericson [12].

After getting new dentures, geriatric patients have low indexes of the masticatory efficiency.

After special treatments (like dentures over the implants), the efficiency of mastication increases [13] [14] [15]. The period of adaptation to new complete dentures is longer in geriatric patients than it is in other patients [16].

A lot of methods are in use for investigating the masticatory efficiency as Fluctuation of the food particles, tested by the system of nets (strainers) with different diameters; Photocolorimetry; Electromyo-graphy; Ultrasonography [17].

The study aimed to evaluate the efficiency of mastication at different time periods, based on patient's previous experience with complete dentures; also, to indicate the period needed for adaptation to the new complete dentures.

Material and Methods

A total of 88 patients (42 female, 46 male, mean age 52.2, SD = 5.76), complete dentures wearers, participated in this study. The research was carried out at the University of Prishtina, Dental School, Department of Prosthetic Dentistry. The research has been accepted and approved by the Institutional Ethics Committee (Medical Faculty, School of Dentistry, University of Prishtina). All participants were first clinically examined, and after they were informed about investigation procedures, they signed a written consent.

The main criteria for including in this study were: patients with complete dentures, eugnathic jaw relation and uniform reduction of the alveolar ridge. Excluding criteria were**:** age over 70 years, jaw relation anomalies in the horizontal and sagittal plane, dysfunctions of the masticatory system and high rate of the resorption of the alveolar ridge (negative ridge).

All examinees were selected into two experimental groups: Group 1 was composed of new complete denture wearers, and Group 2 was composed of the patients who were already complete denture wearers.

All examinees were followed for 6 months. During this period, the result of the prosthetic therapy was expected after insertion of new dentures. During the observation period, each examinee was tested six times by the same examiner, to reduce inter-observer error. The first test was done one week after inserting new dentures, following other tests every 5^{th} week.

In this study, masticatory functions were investigated by using the method of electromyography (EMG), analysing electromyomasticatiogram. This method enables: direct analyze of bioelectric activity of the masticatory muscles in vivo, during realization of SMT; analyze of the synchronized action of homologous pair of the muscles; expression of the regularity of masticator scheme; expression of the dynamics of the masticatory cycle and its determinants (duration, frequency and amplitude of the masticatory cycle) and finally enables documentation of the obtained results. Standard masticatory task (SMT) depends on the type of food (hard, soft and impulsive food)

A different kind of food can be used as a test food (natural and artificial) [18]. For this research, a peanut was used as a test food (hard food), as this food enables analyse of masticatory efficiency without initial contacts of the teeth and in the same time affects the changing of the load level of the neuromuscular control of the mastication. Peanuts are non-synthetic, relatively hard, popular, swallowable, and represent test food in previous studies of mastication as well [13].

Masticatory efficiency test consists in chewing the food of constant quality and quantity. Patients were suggested to chew spontaneously from one side to another.

For testing the masticatory efficiency, the further parameters of the masticatiogram were used: duration of the SMT (t), number of the masticatory cycles within the masticatory arch (F) and maximal amplitude within the masticatory arch (F).

For this purpose, two masticatory indexes were used:

Masticatory index: muscular activity in relation with time (A/t) presents the relation between the maximal amplitude of bioelectric oscillations of the tested muscle (A) (masseter muscle and temporalis muscle) and duration (t) of the standard masticatory task in electromasticatiogram. This relation is expressed by nV/sec.

Masticatory index: muscular frequency activity (A/F) presents the relation between the maximal amplitude of bioelectric oscillations of the tested muscle (A) and frequency of the masticatory cycles (F) during registering of the standard masticatory task in electromasticatiogram. This relation is expressed by nV/Hz.

Masticatory indexes strictly define the standard masticatory task. The duration of the

standard masticatory task (t) is determined by appearing of deflection in the electromasticatiogram.

EMG registered SMT as an electromasticatiogram on a paper of Dynograph. (EMG-Dynograph R-511 A) (Figure 1).

Figure 1: EMG-Dynograph R-511 A

During the measurements, the side of the jaw that showed higher masticatory force was named as a dominant side (DS) while the opposite side was named as a non-dominant side (NDS).

All data were statistically analysed using standard software package BMDP (bio-medical Statistical package), dedicated to research in the biomedical sciences. This package includes all methods of statistical procedures, (Dixon, 62.). Parametric data have been tested using One Way Repeated Measurement of ANOVA test.

Results

Of 88 patients, 45 belong to the new complete denture wearers while 43 were experienced wearers of complete dentures (Table 1).

Table 1: Comparison of gender, age and non – experienced/experienced group

	Gender		Non–experienced/experienced group with complete dentures	
	Female	Male	Non – experienced	Experienced
N	42	46	45	43
X	54.6	55.7	52.7	57.8
DS	5.4	6.1	5.7	4.1
X max	66	68	65	68
X min	42	44	42	49

Dynamics of the index of masticatory efficiency: Masticatory index was analysed according to the duration: using the index of muscular activity in time (A/t) and according to the frequency, using indexes of muscular frequency during masticator cycles in SMT (A/F).

Dynamics of the masticatory index (A/t) is presented in Table 2.

Table 2: The values of the masticatory index during the realisation of SMT in 6 time intervals, in DS and NDS, in both sexes with and without experience with complete dentures

Measurement	Gender				Experience				Total	
	F		M		E		NE			
	DS	NDS	DS	NDS	DS	NDS	DS	NDS	DS	NDS
N	42	42	46	46	45	45	43	43	88	88
1x	12.9	10.8	12.4	14.8	13.6	14.6	11.9	11.2	12.7	12.9
DS	0.3	0.74	0.83	0.76	0.78	0.48	0.61	0.46	0.53	0.6
2x	25.3	19.5	16.3	23.1	20.9	22.1	20.3	20.7	20.6	21.4
DS	0.62	0.81	0.74	1.16	0.98	1.14	0.95	0.68	0.74	0.69
3x	33.9	29.6	19.5	18.4	27.9	24.9	24.7	22.5	26.3	23.7
DS	1.89	1.74	0.77	0.73	1.85	1.65	1.65	1.40	1.25	1.1
4x	24.2	25.8	20.8	21.0	23.0	25.1	21.9	21.4	22.5	23.5
DS	0.8	1.55	0.90	1.0	0.92	1.45	0.90	1.17	0.64	0.94
5x	28.4	20.7	20.0	220.1	24.8	22.1	23.2	18.7	24.0	20.4
DS	1.0	1.14	0.89	0.78	1.225	1.0	0.99	0.80	0.80	0.68
6x	33.6	26.8	18.6	22.1	25.8	26.1	25.8	22.5	25.8	24.3
DS	1.11	1.31	0.87	1.10	1.58	1.43	1.36	0.93	1.0	0.88

The changes of the relation A/t are evident in different time intervals in both measured sides: F-DS-time of the measurement = 34.86, P = 0.0000; F-NDS-time of the measurement = 26.1, P = 0.0000.

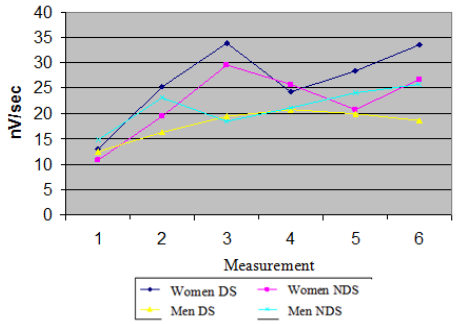

Figure 2: MI A/t during the realisation of SMT according to gender

In DS is evident increasing from 13 nV/sec. In the first two months after getting new dentures, to the stationary value of approximately 24 nV/sec. In the fourth month.

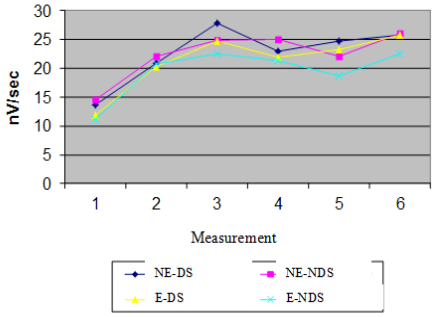

Figure 3: MI A/t during the realisation of SMT according to the experience with complete dentures

In NDS the stationary condition is reached in the second measurement 22 nV/sec.

There are also differences of the masticatory index A/t between sexes in both, dominant and non-dominant side: F-DS-gender = 237.05, P = 0.0000; F-NDS-gender = 12.90, P = 0.004.

In the DS the values of the masticatory index

A/t are higher in women then in man. Interaction of the gender and time shows also significant differences: F-DS-interaction = 18.52, P = 0.0000; F-NDS-interaction = 14.14, P = 0.000.

In the DS there is no significance of the index values F-DS-experience = 3.75, P = 0.0535 while in the NDS there is a significance of the index values: F-NDS-experience = 20.12, P = 0.0000.

In NDS the values of the masticatory index are higher in patients with no experience with complete dentures then in those who have some experience.

The dynamics of the masticatory index A/F is presented in Table 3.

Table 3: The values of the masticatory index (A/F) during the realisation of SMT in certain intervals, in DS and NDS, in both sexes with and without experience with complete dentures

Measurement	Gender				Experience				Total	
	F		M		E		NE			
	DS	NDS	DS	NDS	DS	NDS	DS	NDS	DS	NDS
N	42	42	46	46	45	45	43	43	88	88
1x	9.8	7.7	8.3	10.2	9.2	9.9	8.8	8.0	9.0	8.9
DS	0.3	0.4	0.5	0.4	0.5	0.4	0.4	0.4	0.3	0.3
2x	16.9	14.1	10.7	15.1	13.5	15.0	13.9	14.2	13.7	14.6
DS	0.5	0.7	0.6	0.6	0.7	0.7	0.7	0.7	0.5	0.5
3x	24.4	20.5	14.5	12.2	20.2	16.3	18.3	15.9	17.0	17.3
DS	1.1	1.2	1.8	0.4	2.0	1.1	1.2	1.1	1.2	0.8
4x	18.6	18.6	15.5	16.1	17.4	18.7	18.7	16.5	17.0	17.3
DS	0.8	1.2	0.7	0.6	0.9	0.7	0.7	0.6	0.5	0.5
5x	21.4	16.0	15.5	15.9	18.6	16.8	18.0	14.9	18.3	15.9
DS	0.7	0.8	0.7	0.6	0.9	0.7	0.7	0.6	0.6	0.5
6x	22.6	19.9	14.1	14.9	18.0	18.2	18.3	16.4	18.2	17.3
DS	0.6	0.9	0.6	0.7	0.9	0.9	0.9	0.9	0.7	0.6

As it is noticed in the table, masticatory index (A/F) varies in time, from first to the sixth measurement in both sides: F-DS-time of the measurement = 32.36, P = 0.0000; F-NDS-time of the measurement = 30.53, P = 0.0000.

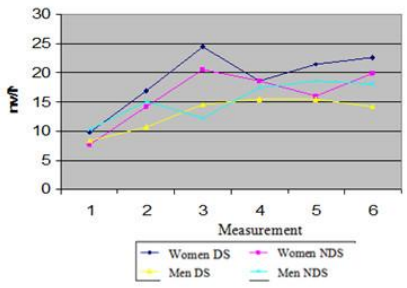

Figure 4: MI A/F during the realisation of SMT according to gender

The stationary condition is reached after the third measurement, in DS from 9nV/Hz to 19nV/Hz. In the NDS this condition is reached on the third measurement.

A/F index is also different in different gender, on both measured sides. The interaction of the time and gender was also important: F-DS-interaction = 6.95, P = 0.0000; F-NDS- interaction = 14.9, P = 0.0000.

In the DS there is no sign of the A/F index as far as the previous experience in complete dentures concerned: F-AD-experience = 0.86, P = 0.3530 while in the NDS there is a significance: F-AJD-experience = 11.88, P = 0.0006.

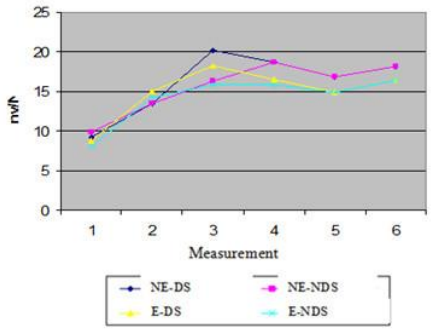

Figure 5: MI A/F during the realisation of SMT according to the experience with complete dentures

The examinees with no previous experience with complete dentures have had higher average values of the masticatory index in the NDS then examinees with some experience with complete dentures.

Discussion

The main task of this research was an investigation of the masticatory efficiency in patients with complete dentures. Muscular activity is improved by getting new dentures; however, a muscle adaptation to the new prosthesis is still needed [19].

Goiato MC et al., [17] concluded that after getting new dentures, the muscular balance of the masticatory system happens, by neuromuscular reprogramming.

The first parameter that prescribes the adaptation of the examinees with the new dentures is time needed for realisation of SMT. On average, from initial values of 29 seconds, this parameter decreases continuously reaching the time of 21 seconds finally. The variation of these values is relatively small what means that in general, the group is homogenous about measured time. The stationary condition is reached relatively fast (after 10 weeks from getting new dentures. According to Goiato, after getting new complete dentures, lowered muscular capacity and ability reduced [20].

Initial values of 29 seconds were same for both sexes decreasing in the end at 17 sec. In women and 24 sec. In man. The reason for this can be that women react more intensively in the beginning while a man needs more time for the functional adaptation.

In the DS the values of the masticatory index, A/t is higher in women than in man. In NDS the values

of masticator index are higher in patients with no experience with complete dentures then in those who have some experience.

In the DS there is no significance of the A/F index as far as the previous experience in complete dentures concerned. The examinees with no previous experience with complete dentures have had higher average values of the masticatory index in the NDS then examinees with some experience with complete dentures.

The examinees non experienced on complete dentures, in the beginning, have had a shorter duration of SMT comparing to the examinees experienced on complete dentures, but in the end, the values of the final duration of SMT were the same (NE and E group). This can be explained by neurophysiologic models of mastication since they provide a lot of information on the functioning of the masticatory organ in human [21].

Masticatory index A/t defines the bioelectric activity of the tested muscles (Masseter muscle and Temporalis muscle) in time. It was expected that higher values of the amplitude would be followed by shorter duration of the SMT and vice versa. In the same way the second masticatory index A/F too, brings in a connection the amplitude and the frequency of the masticatory cycles during the realisation of the SMT. Efficient SMT can be performed in a shorter time with a smaller number of masticatory cycles but with a higher maximal amplitude of the masticatory arch. According to Kijak E et al. [21], the consistency of the food products had a decisive impact on the frequency of mastication and the number of cycles necessary to grind the food.

In this investigation, the SMT was used as an index for measuring the masticatory efficiency while other authors have used other indexes. Ferreira et al. used MSI (masticatory stability index) as an efficient method to measure the stability of the masticatory cycles [22].

The method of analysing of the masticatiogram, with EMG is objective since the evaluation of the masticatory index is not taken due to the referent values as it is done in the classical investigations.

In conclusion, masticatory indexes, A/F and A/t are a very important indicator of the masticatory efficiency and the level of the functional adaptation on complete dentures. Both masticatory indexes show the same dynamics (getting in the stationary condition after the 15th week after getting new dentures). After the observing period, all examinees express the same degree of masticatory efficiency known as ideal or functional adaptation. Patients with previous experience with complete dentures reach faster the stationary condition, compared with those non-experienced.

Based on the results of the investigation, it is concluded that after getting new dentures, the stationary condition is reached after about 10 weeks. This fact can help doctors and patients for better understanding the period of adaptation and easier facing with new dentures.

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Dental Science

The Effect of Orthodontic Intervention on Mental Health and Body Image

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Keywords: Mental Health; Orthodontics; Body Image

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Abstract

AIM: Physical health especially oral and dental health can play a leading role in individuals' mental health status. Therefore, determining the relationship between orthodontics, mental health, and body image can provide solutions for the development of treatment services and provision of greater accessibility of communities to them.

METHODS: The present study was natural experimental research conducted to determine the relationship between orthodontic treatment, mental health, and body image. To conduct this research, individuals referring to orthodontic clinics in the city of Kermanshah and meeting the inclusion criteria in the study were selected using convenience sampling method and then they were assessed before and after undergoing orthodontics via the standardised Goldberg's General Health Questionnaire (GHQ) and Multidimensional Body-Self Relations Questionnaire (MBSRQ).

RESULTS: The mean scores of mental health problems in the study participants reduced and such a difference calculated by Wilcoxon signed-rank test with a 95% confidence level was significant (P = 0.001). Moreover, all the subscales of GHQ in the study participants showed a significant decrease after orthodontics compared to the stage before that (P < 0.05). The mean score of MBSRQ before orthodontics was equal to 171.78 and this value reached 172.51 after that, indicating individuals' increased scores and their high levels of satisfaction with their body image. Nevertheless, the given difference was trivial, and it was not statistically significant (P = 0.751).

CONCLUSIONS: The results of the present study showed that orthodontic treatment could significantly increase mental health status in the clients and also improve multidimensional attitudes towards body image.

Introduction

The World Health Organization (WHO) has introduced the issue of mental health as one of the main health problems in the world [1]. Today, biological, mental, and social dimensions are of utmost importance in the field of dentistry [2]. In this regard, the effects and complications of oral and dental diseases can have their own psychological and social aspects contributing to mental health in individuals, which have been less considered in previous decades [3].

Health and particularly oral health, the appearance, shape, and position of the teeth, as well as treatments of these disorders are among the significant factors that can affect mental health and well-being in individuals [4]. Malocclusion is considered as an inappropriate condition, grasped by many people as a deviation from the beauty norms [5]. People who have a higher level of personal satisfaction on their face are also endowed with a higher degree of self-esteem. Besides, the total appearance of the teeth and the face can have an impact on an individual's mental image of one's body, and such an image can contribute to personal satisfaction [6].

Teeth and jaw can be treated using orthodontic tools. Making coordination between the

upper and lower teeth, orthodontic intervention can improve the performance of chewing and crushing and, consequently, prevent indigestion in the long run. Together with tooth alignment, orthodontics can improve the health of the teeth and the gums [7]. Since orthodontic treatments can directly change clients' appearance and face, there is a need for orthodontic experts to recognize the underlying issues of social psychology and facial attractiveness theory [8]. Besides, studies have revealed that orthodontics can significantly influence the quality of life in children and adolescents although no impacts on their daily activities particularly their mental-psychological ones can be observed [3].

In addition to the structural and anatomical factors of the face, personal satisfaction with one's face is under the impact of self-assessment. When there is a slight difference between one's current face and their ideal one, the level of personal satisfaction is assumed to be high; but whenever such a difference is big, one's attitude toward his/her face will be at low levels. In other words, the beauty of the face in the eyes of an individual is a set of personal attitudes to one's face accompanied by social and mental acceptability [9]. For this reason, one of the most important benefits of orthodontic treatments and cosmetic surgeries is improving the beauty of the teeth and the face, which is accompanied by social and mental satisfaction [2].

In this regard, aesthetic beliefs are divided into traditional and new ones. Considering changes in aesthetic beliefs of the youth from traditional to new ones, dissatisfaction among this age group toward body image has raised, and they have greater desire to manipulate their body to fulfil their aspirations of this image [10]. Moreover, according to studies in this field, it seems that the stimulus of orthodontic intervention is associated with clients' perception and understanding of how far their dentofacial appearance is from sociocultural norms [6]. Therefore, efforts made to deal with this situation in communities are growing [5] because the need to receive treatments in clients is a feeling that can raise necessary motivation and the ability to get the mentioned services [3]. Today, orthodontic intervention is considered a successful and appropriate treatment to improve the position of the teeth and to increase physical attractiveness within communities [11].

According to the related studies, given that orthodontic services are costly and people with high socioeconomic backgrounds are practically looking for such services, and considering the results obtained by the given researchers, the present study was conducted to find the relationship between orthodontic treatment, mental health, and body image of individuals referring to orthodontic clinics in the city of Kermanshah in Iran in 2017.

Material and Methods

The present study is natural experimental research conducted to determine the relationship between fix orthodontic treatment, mental health, and body image. To carry out this study, 110 participants meeting the inclusion criteria were selected among the individuals referring to orthodontic clinics in the city of Kermanshah using convenience sampling method. Before receiving orthodontic services, the participants were assessed via a demographic characteristics form, standardized General Health Questionnaire (GHQ), and Multidimensional Body-Self Relations Questionnaire (MBSRQ). Six months after the end of fixed orthodontic treatments, the patients were re-assessed through the GHQ and the MBSRQ and the data obtained were analyzed. The inclusion and exclusion criteria of this work were as follows:

Inclusion Criteria: at least 14 years of age; patients with dental malocclusion that need to be fixed orthodontic treatment only; no skeletal discrepancy in jaw relationship or facial cleft and any craniofacial syndrome; the absence of body dysmorphic syndrome.

Exclusion Criteria: withdrawal from continuing orthodontic intervention during the study; completion rate of the questionnaires below 80%.

The statistical population of this study included all the clients referring to orthodontic clinics and performing this treatment in the city of Kermanshah, who were selected using convenience sampling method. The sampling context was private orthodontic clinics within this city. The sample size was determined by a minimum number of 58 individuals considering 95% confidence level. Moreover, body image values before and after the treatment were 7.72 and 5.41, respectively [8]. Given the probability of 30% sample attrition, the sample size was calculated about 90 people. To this end, 110 clients were selected and assessed. Finally, only 102 questionnaires were evaluated due to the presence of some incomplete ones.

The data collection instruments included a researcher-designed demographic characteristics form along with two specific questionnaires evaluating mental health (GHQ) and body image (MBSRQ) because they are reliable and valid tools for Iranian population [12] [13] [14].

Goldberg's General Health Questionnaire (GHQ)

This questionnaire included four subscales:

1) Physical symptoms including cases of individuals' feelings about their health status and their fatigue associated with physical symptoms from items 1 to 7;

2) Anxiety and sleep disorder symptoms

comprised of causes related to sleeplessness and anxiety from items 8 to 14;

3) Social functioning as ability in individuals to deal with professional demands and daily life issues as well as revealing how people feel about coping with common life situations from items 15 to 21; and

4) Depression symptoms including chronic depression cases and suicidal orientations from items 22 to 28. Moreover, there was a score for each subscale, and a score was also assigned to the total score of individuals [12]. This questionnaire had been already used in Iran, and its internal consistency using Cronbach's alpha method had been reported [13]. Also, this scale has been standardised for the Iranians [12].

Multidimensional Body-Self Relations Questionnaire (MBSRQ)

This questionnaire includes 69 self-report items designed to evaluate individuals' attitudes toward different dimensions of body image including evaluation, recognition, behaviour, as well as an individual's orientation of one's health status or sense of illness [15]. Three physical dimensions dominant in the BSRQ were the appearance of the body, physical fitness, and health. Also, each of these sections contained two domains of evaluation and orientation: appearance evaluation and appearance orientation, fitness evaluation and fitness orientation, and health evaluation and health orientation. Other subscales of MBSRQ are illness orientation, body areas satisfaction, overweight preoccupation, and self-classified weight [16]. Each of these domains had 5 scores; so, score 1 was assigned to disagree strongly and score 5 was considered for strongly agree [15]. This scale has been standardized for the Iranians, as well [14].

Data extraction and analysis were conducted using the SPSS Software (Version 25). This analysis was performed via descriptive statistics (tables of frequency distribution and calculation of concentration and distribution indices) and analytical methods. To perform statistical tests in the analytical section, first, all distance variables (including the GHQ scores and its subscales before and after the intervention, as well as the mean scores of the MBSRQ and its subscales before and after the treatment) were evaluated by Kolmogorov-Smirnov test for normal distribution. The results revealed that only the mean scores of the MBSRQ and the subscale of fitness orientation before and after the intervention were following a normal distribution. To compare the scores before and after the intervention, paired t-test was used, and other variables were found not to follow a normal distribution, which inevitably led to using its nonparametric equivalent; i.e. Wilcoxon signed-rank test.

Ethics approval and consent to participate

After obtaining the letter of permission from Kermanshah University of Medical Science Ethical Committee (KUMS.REC.1396.60), a written informed consent form was prepared and then was completed and signed by the researchers and the participants after providing brief and clear explanations about the given form.

Results

The mean age of the participants was 20.03 ± 6.29 years with a minimum of 14 and a maximum of 46 years old. The average monthly household income was 391 ± 200$; ranging from a minimum of 180$ to a maximum of 1500$. Other sociodemographic data of the subjects are presented in Table 1.

Table 1: Participant's demographic characters

Variable		N (%)
Sex	Female	81 (79.4)
	Male	21 (20.6)
Married State	Single	92 (90.2)
	Married	10 (9.8)
Graduate level	Elementary	11 (10.8)
	High School	47 (46.1)
	University	44 (43.1)
Job	Housewife	15 (14.7)
	Worker	7 (6.8)
	Employee	12 (11.8)
	Student	68 (66.7)
Residence	City	96 (94.1)
	Village	6 (5.9)
Fathers job state	Worker	20 (19.6)
	Employee	48 (47.1)
	Missing Data	34 (33.3)
Fathers graduate level	Under diploma	9 (8.8)
	Diploma	26 (25.5)
	Upper diploma	32 (31.4)
	Missing Data	35 (34.3)
Mothers job state	Housewife	35 (52)
	Employee	15 (14.7)
	Missing Data	34 (33.3)
Mothers graduate level	Under diploma	17 (16.7)
	Diploma	25 (24.5)
	Upper diploma	26 (25.5)
	Missing Data	34 (33.3)

As can be seen, the mean scores of the GHQ obtained by the study participants were reduced from 20.68 before the orthodontics to 17.78 after this treatment, suggesting a statistically significant difference considering Wilcoxon signed-rank test at the 95% confidence level (P = 0.001).

Likewise, all the subscales of the GHQ as physical symptoms, anxiety and sleep disorder symptoms, social functioning, and depression symptoms (Table 2) showed a significant decrease in the study participants after orthodontic intervention compared to those before dentofacial orthopaedics (P < 0.05). Therefore, it was found that orthodontic intervention had reduced mental health problems and consequently enhanced mental health status among the clients.

Table 2: GHQ and subscale's score in participants for pre and post-orthodontic interventions

Variable	Pre-orthodontic intervention		Post-orthodontic intervention			
	Mean	SD	Mean	SD	t	Sig
GH problems	20.68	10.897	17.78	8.72	-3.41	0.001
Physical symptoms	4.84	3.74	3.98	2.87	-2.74	0.006
Anxiety and sleep disorder symptoms	4.96	3.82	4.26	3.12	-2.15	0.031
Social functioning	7.07	2.79	6.08	3.05	-3.69	0.001
Depression symptoms	4.25	4.48	3.1	3.598	-3.22	0.001

According to the guidelines of this research instrument, obtaining higher scores suggests higher levels of satisfaction with body image. As shown in Table 3, the mean scores of the MBSRQ before orthodontics was 171.78 and it reached 172.51 after this treatment, showing an increase in scores and an individual's satisfaction with their body image. It should be noted that such a difference was negligible and was not statistically significant (P = 0.751).

Table 3: MBRSQ and subscales' scores in participants for pre and post-orthodontic interventions

Variable	Pre-orthodontic intervention		Post-orthodontic intervention			
	Mean	SD	Mean	SD	T / Z	Sig
MBSRQ*	171.78	23.07	172.51	18.57	-0.319	0.751
Appearance evaluation	17.23	3.78	18.87	2.59	-3.51	0.0001
appearance orientation	29.07	5.43	28.34	6.43	-1.69	0.091
fitness evaluation	6.96	2.28	7.77	1.82	-3.45	0.001
fitness orientation*	31.08	4.7	33.83	6.56	-4.002	0.0001
health evaluation	16.42	2.41	14.65	3.06	-5.096	0.0001
health orientation	20.27	4.4	20.72	3.75	-1.36	0.171
illness orientation	13.98	2.69	17.34	3.35	-6.22	0.001
body areas satisfaction	5.1	1.18	5.53	1.15	-3.24	0.001
subjective weight	2.46	0.65	2.33	1.22	-3.25	0.001
weight preoccupation	5.73	0.83	2.83	0.48	-8.29	0.0001

*Paired T-test.

In relation to the MBSRQ subscales illustrated in Table 3, the scores of the subscales of appearance evaluation, fitness evaluation, fitness orientation, fitness orientation, illness orientation, and body areas satisfaction before orthodontics demonstrated a significant rise compared to that before this treatment, which was significant in most cases (P < 0.05) except for the subscales of health and appearance orientation. Therefore, considering the mentioned subscales, individuals' levels of satisfaction had been boosted.

As shown in the table, scores obtained by the study participants were reduced after orthodontics considering the subscales of to have appearance orientation, health evaluation, subjective weight, and weight preoccupation compared to those after this treatment. In other words, the levels of satisfaction in the study participants were lowered, and such a descending trend was not statistically significant for the subscale of to have appearance orientation (P > 0.05), and they were significantly reduced in the three other subscales (P < 0.05).

Discussion

This study was conducted to determine the relationship between mental health, body image, and orthodontics using a natural experimental research design.

The results showed that orthodontic intervention had resulted in increased levels of mental health in the study participants. Many investigations have also highlighted the relationship between mental health and orthodontics. The results of related studies have shown that self-confidence can significantly be increased by performing orthodontics and correcting the teeth [17]. The results of the present study indicated that mental health subscales of GHQ were promoted after the orthodontics. Additionally, the findings revealed improved physical health, decreased anxiety, and depression, and increased social work. These results are in line with those of many other previous works. In numerous investigations, the results suggested an increase in health-related quality of life after this treatment [18]. Also, orthodontics could significantly be contributed to the quality of life of children in Mehrzarom School in Turkey [3]. Minghui et al., (2017) also found that orthodontic interventions could increase not only self-awareness but also had a positive impact on adolescent mental health [19].

Johal et al., (2014) also observed no statistically significant difference after 6 months of orthodontic intervention in the quality of life of the clients [17]. In this study, the mental health status of the participants was examined, and it was found that orthodontics caused a significant increase in this variable, which positively affected individuals' quality of life. Other investigations showed an increase in the mental health status related to the quality of life [20]. This difference could be associated with the study population, study time, and even the results of the orthodontic effect. As can be seen, all related studied emphasize the positive impacts of orthodontic services on mental health status.

The results of this study showed that the symptoms of depression in the subjects were decreased after the orthodontic intervention. Studies have also highlighted the existence of degrees of depression in the problems of periodontal [21] [22]. Topcuoglu et al., conclude that the extraoral appliance therapy has negative effects on the mental health of individuals, and their levels of depression and anxiety are increased [23]. This difference can result from the use of orthodontic intervention method.

The results showed the development of social functioning in subjects after the orthodontic intervention. In this regard, Tamme et al., (2017) concluded that the combined orthodontic and maxillofacial surgical treatment increased the quality of life and improved their social functioning [24]. Other

studies found that orthodontic intervention leads to an increase in the self-esteem of adolescent girls and their influence on their social functioning [25][26]. These results show the direct effects of orthodontic interventions on the external situation, self-esteem, and ultimately the behavioural functioning of clients, which confirm the results of our study and stresses the importance of the impact of orthodontic interventions on social activities.

In general, the results of this study showed that multidimensional body-self relations were increased after orthodontic intervention, but such a rising trend was not statistically significant. Results of various studies were to some extent consistent with the findings of this investigation. In this regard, Varela and Garcia-Camba (1995) showed that orthodontic services in the short term from 1 to 4 weeks after this treatment had no effects on self-esteem in adolescents; however, such services led to an increase among this age group within 6 months [27]. In another study published in China in 2018, various aspects of life-related psychology such as self-esteem and body image after the orthodontic intervention were improved and a decline were observed regarding negative effects after orthodontics [20]. Moreover, another study by Cifter and Cura (2016) showed that orthodontic intervention for clients with cerebral palsy and spastic quadriplegia raised their self-esteem and self-confidence [28]. In the study by Alanko et al., (2017), the clients were also examined within one year before orthodontics and one year after that, and the results revealed a decline in the quality of life and body image and an increase in symptoms of psychiatry after one year [29]. This difference in the results would be due to small sample size (752 individuals in the given study) as well as follow-up period of 6 months. In the present study, the number of samples was low, and the duration of the follow-up was 6 months after the start of orthodontics. Perhaps, if the duration of the follow-up was longer, the results could be different; thus, a short follow-up period and the small number of samples could be considered as the limitations of the present study.

The results of this study showed that the subscales of the MBSRQ were changed after orthodontic services, wherein appearance evaluation, fitness orientation, and illness orientation among the clients were significantly improved. However, a significant decline was observed in the subscales of health evaluation, body areas satisfaction, subjective weight, and weight preoccupation among the study participants. Furthermore, the mean age of the clients was 20 years old, and these people needed special attention to body weight and fitness in this age group. Accordingly, performing orthodontics due to treatment and follow-up problems could reduce the time of exercise and physical activity of such individuals and challenge their concerns for weight, health evaluation, and health orientation. In this regard, Bremen et al., (2016) concluded that once the orthodontic intervention was finished, the clients could be affected with an increase in their body mass index (BMI), which was also considered as a risk factor for such individuals. It was suggested that the longer the treatment, the higher the weight and ultimately the BMI, which should be addressed in treatments [30]. This study was consistent with the findings of the present investigation and could reasonably be accounted for preoccupations, mental health problems, and attention to physical well-being in the clients.

This study was completed within a year. Restrictions on sampling and no willingness among the samples to cooperate were the most important limitations of this study, which inevitably led to a 6-month follow-up after the orthodontic intervention to avoid further sample attrition. Also, the study samples were in the age range of 14 to 46 years with the mean age of 20 years, suggesting younger study participants that could have an impact on the results. Furthermore, the questionnaires were completed by the samples, and the co-researcher provided samples with information if needed; thus, the answers in this study were assumed accurate. Although this study achieved significant results, more accurate and reliable results could be obtained if more studies were conducted with larger sample size and longer follow-ups.

In general, the results showed that orthodontic treatments could increase mental health and its subscales in clients undergoing orthodontic intervention and also bring about a non-significant increase in multidimensional body-self relations. Considering the subscales associated with health evaluation, subjective weight, and weight preoccupation, we identified that orthodontic services not only could not lead to an improvement but also reduce the clients' status which needed more attention to these subscales in this domain.

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Dental Science - Review

Recent Advances in Material and Geometrical Modelling in Dental Applications

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Abstract

This article touched, in brief, the recent advances in dental materials and geometric modelling in dental applications. Most common categories of dental materials as metallic alloys, composites, ceramics and nanomaterials were briefly demonstrated. Nanotechnology improved the quality of dental biomaterials. This new technology improves many existing materials properties, also, to introduce new materials with superior properties that covered a wide range of applications in dentistry. Geometric modelling was discussed as a concept and examples within this article. The geometric modelling with engineering Computer-Aided-Design (CAD) system(s) is highly satisfactory for further analysis or Computer-Aided-Manufacturing (CAM) processes. The geometric modelling extracted from Computed-Tomography (CT) images (or its similar techniques) for the sake of CAM also reached a sufficient level of accuracy, while, obtaining efficient solid modelling without huge efforts on body surfaces, faces, and gaps healing is still doubtable. This article is merely a compilation of knowledge learned from lectures, workshops, books, and journal articles, articles from the internet, dental forum, and scientific groups' discussions.

Introduction

Dental sciences started thousands of years ago in ancient Egypt by using precious materials like gold for restorations. By the time new materials were utilised in dentistry as new treatment methods appeared. Also, new technologies help dentists to introduce new treatment techniques.

The new imaging, modelling, and analysis methods lead to great improvement in treatments quality and success rates.

Therefore, dental materials are considered the cornerstone of all advances in oral and dental medicine. Furthermore, geometric modelling and stress analysis of the restorative materials, prosthesis, and new dental tools are used as a preliminary step before commercial usage.

Dental materials

Rigid polymers, elastomers, metals, alloys, ceramics, inorganic salts and composite materials are commonly used within a variety of dental materials as demonstrated in Figure 1.

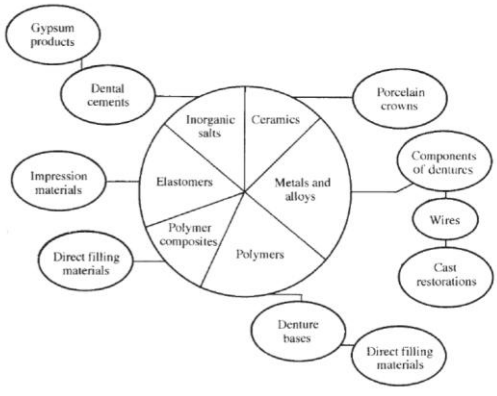

Figure 1: Variety of materials used in dentistry and some of their applications [1]

The physical properties and biocompatibility govern the dental material selection. Some dental materials should be sufficiently strong to withstand biting forces without fracture. Others should be rigid enough to maintain their shape under load, while these materials' properties are typically characterised by the stress-strain relationship. Additionally, chemical and electrical tests may be required for more properties as corrosion and conductivity.

Optimal dental material selection based on Finite Element Analysis

Stress analysis by finite element packages might be necessary due to its accuracy, low cost, and less time-consuming than experimental research. In many dental applications, Finite Element Analysis (FEA) can provide information would be difficult or impossible to be obtained by experimental observations, while, FEA cannot be performed without experimental inputs and validation [2]. The following examples demonstrate validated FEAs that focus on material selection:

1. The effect of post materials on stress distribution on endodontically treated lower first premolar was studied by FEA [3], and resulted in criteria for post material selection. Where, stiffer post materials are preferred to transfer less stresses to the root dentine with less displacement.

2. Reciprocating endodontic files material were investigated by FEA [4], that resulted in using stainless steel, is not suitable for manufacturing rotary or reciprocating instruments. NiTi is suitable for reciprocating instruments although it has short lifespan, thus one file per tooth is usually recommended.

3. Crown and implant-abutment materials combinations were studied [5] in details by FEA to find that using more rigid material for implant-abutment complex is preferred for low density bones to have better stress distributions. Using crown material with lower modulus of elasticity reduces the stresses generated on mandible, that it absorbs more energy from the applied load, and transfers less energy to the following parts.

4. Luting cement material type and its layer thickness were also studied [6] to conclude that increasing the cement layer thickness (ranged 20-60μm) ensures a longer lifetime of the restoration, and slightly increase the total deformation induced on the cement layer. Regardless of the cement type, thicker cement layer is preferred to reduce cortical bone stresses by about 6.5%. While spongy bone is insensitive to cement type or its layer thickness.

Geometric modelling in dental applications

Two major applications depend on geometric modelling: stress analysis, and production by computer-aided manufacturing (CAM). As the human teeth and bone have very complicated geometries, thus, it is extremely difficult to use conventional graphics and/or engineering modelling techniques in generating an accurate three dimensional (3D) geometric model(s) for bio-tissues.

Engineering CAD and graphics packages produce 2D and 3D geometric models in what is called "vector graphics/image" format. That, build a geometry with few numbers of points and correlate them by lines, areas, and …etc. Which generated by a set of mathematical equations. The resulting file size is relatively small (kilobytes) for huge or complicated geometry. On the other hand, the actual data from digitising (3D scanning like a laser, blue ray or contact probes ones), computed tomography (CT), cone-beam computed tomography (CBCT), Spiral Tomography, and other techniques can be taken as a base for the 3D geometric model(s). Such techniques produce set(s) of "raster graphics/image" formats. The resultant file size is very large (Mega/Gigabytes) that containing a huge number of points' coordinates (cloud of points). These points are not related to each other, but they are too close to each other and may be common in one coordinate as a result of fixed step by the scanning machine.

As, laser or blue ray, or contact probes scanners usually used for modelling inanimate objects. Laser and contact scanners (Figure 2) produce moderate file size that containing data of the outer surface only. The idea of how it works is to move the sensor within a certain domain steadily and in specific directions from an origin to record coordinates of any point are located on the scanned part when it interferes with the sensor movement.

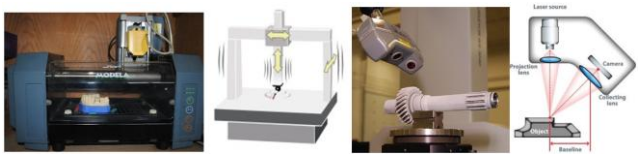

Figure 2: Contact probe and laser scanners pictures & schematics [8]

On the other hand, all other tomography systems are capable of producing images containing the entire body details, as schematic illustrates in Figure 3. The general idea of filming exploration of the human body, whether by X-ray or CT or other is a pass-ray window from the source and received on the other side which leads to a type of diffraction or absorption of part of the transmitted ray at the receiver, as a kind of change in the properties of radiation transmitted. Using mathematical analysis draw colourful 2D pictures or graded lighting for human body parts that had passed through these rays will be possible.

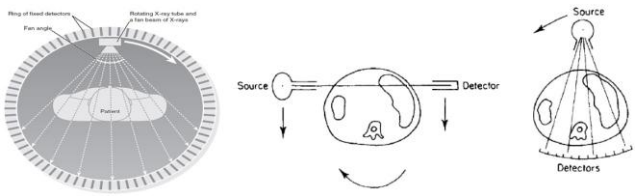

Figure 3: Schematics for CT imaging idea [8]

Changes in colour or lighting gradient unit's gives information about the type of material from which passed rays which can be specialists to determine the internal organs of the human body and its status (intact or a tumour or fragility ...etc.) as presented in Figure 4.

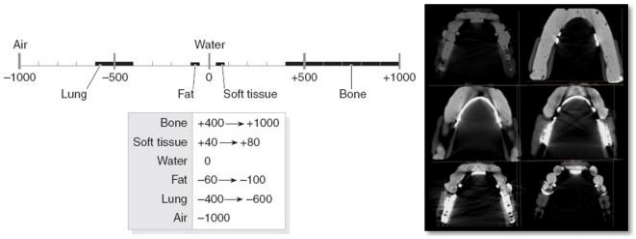

Figure 4: Brightness scale, and sample CT scan image [9]

Like any digital image, each point represents coordinates and information about the colour and intensity of light including resemble, with the presence of the tripartite scanners feature is the availability of these data for each holographic imaging under not only the outer surface. This result enables the user of some modern software(s) as Mimics, Amera, ...etc. To convert the CT scanner files (Dicom format) to form set of equally spaced 2D geometric forms, whether represented surface or volume in 3D. These programs select portions of the scanned body and using the “threshold” to select range of brightness values marking only one type of tissue (ex. bone), to create organ geometry. However, the further threshold on bone images removes also much of the cancellous bone tissue, since it is fewer dense than the cortical bone thus, leaving holes and openings on the required body mask. These opening were subsequently filled by software user, which results in a solid mask with all the bony tissues in the cloud. The mask was then added to the real image, yielding a segmented bone image only. Thus there is a high potential in dental researches to use CT-based image processing technique [8]. The stress shielding produced by numerous jawbone types might also be evaluated through the use of CT-scanned images at a set of intervals during the healing stage. This led to noticeably improved knowledge, for both the implant designer and implant surgeon, of how the jawbone will remodel. An alternative method to evaluate stress shielding within the jawbone experimentally through photoelastic stress analysis technique [9].

Advances in dental materials

The advances in four major categories of dental materials will be discussed, that covers most of the dental branches applications.

Dental composites in restorative dentistry

Amalgam restorations had been used for a long time, without enough attention to its mercury content toxicity [10]. Another major issue is the colour of amalgam for aesthetic considerations, and alternative materials are being sought to replace [11].

Restorations using composite materials have promising aesthetics however these materials are very technique sensitive, and its mechanical properties are not as good as of amalgams [12].

Bowen developed the Bisphenol A-Glycidyl Dimethacrylate (Bis-GMA) resins and used silane couplers, which composite fillings became an essential component of the restorative armamentarium. The last decade has witnessed rapid advances in dental restorative materials including the resin-based composites [13]. As dental composites are based on resin polymer enforced by other materials. Distribution of nano-filler particles as "homogenous", or "non-homogenous" and presence of nanoclusters results in composites with different bulk and surface properties that can be tailor-made according to the site of application [14].

Non-Metallic Dental Implants PEEK

Recent studies reported that Poly-Ether-Ether-Ketone (PEEK) as an alternative material for Titanium dental implants. PEEK is a biocompatible material with Young's modulus of 3.6GPa, which can be modified by reinforcing it with carbon fibres "CFR-PEEK", to reach 18GPa, similar to that of cortical bone [15].

Nowadays, orthopaedic applications PEEK implants are manufactured with a variety of physical, mechanical, and surface properties, and in different shapes to fit a wide range of dental clinical cases [16]. That, the reinforced PEEK by suitable fillers are prepared in simple sequence (known as compression moulding) [17].

Sarot et al., [18] compared the stress distribution in the peri-implant support bone of 30% CFR-PEEK or titanium, using FEA. Assuming perfect osseointegration, the results of PEEK showed high-stress concentration in the implant neck and the adjacent bone. However, PEEK implants is widely used nowadays to support complete overdentures as its performance is considerably improved when distributed adequately along the jawbone [17][19].

Nano-materials

Enamel, dentin and cementum as dental hard tissues are naturally composed of nanoscale structural units [20], and their mechanical properties may vary from one point to the other [21][22]. Nanomaterials have numerous advantages like superior properties as hardness, flexural strength, modulus of elasticity, translucency, durability in comparison to traditional materials. Currently, nano-technology embedded in many applications including bone/tissue regeneration, implantology, restorative materials, and biomarkers to detect diseases as demonstrated in Figure 5.

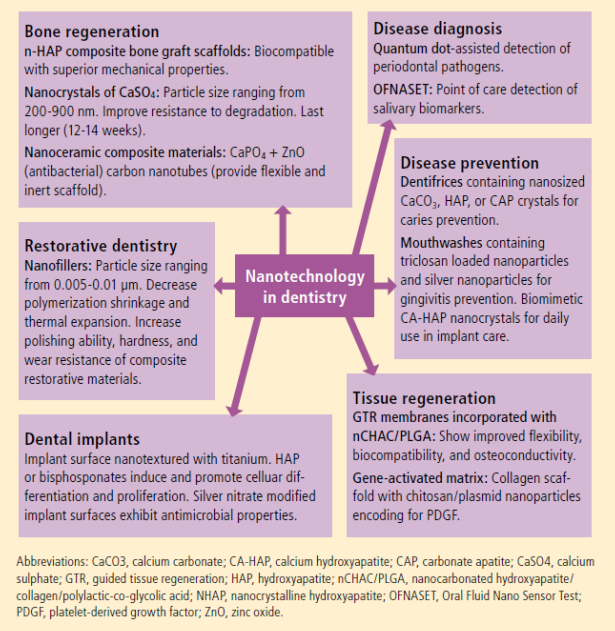

Figure 5: Current applications of nanotechnology in dentistry [25]

Dental ceramics

Classification of ceramics in dentistry is an impossible task due to vast improvements made in the compositions. Thus many classifications can be found for dental ceramics. One example classification according to the chemical composition as Alumina based, Leucite reinforced based, …etc. While another classification is based on processing technique (sintered, castable, slip cast, machinable and pressable). Most of dental ceramics are used in the prosthesis, while recent researches on immediate loading tend to use ceramics as part of implant-abutment or replacing the tooth. In previous studies tended too, bioceramic implants generate fewer stresses to surrounding bones than Titanium one, while its osseointegration behaviour is much better [6].

Recent researches formulated its failure stress by FEA as linearly related to Young's modulus differences (ceramic-substrate) and the square of the ceramic thickness. Great differences in Young's modulus between dentine, cement, and the ceramic crown is the responsible for the prosthesis expected failure [24]. This relation improved the understand of single crown failures, as a mechanism, which does not involve damage from wear facets but stresses on the cementation surface due to occlusal loading.

Metallic Alloys in Endodontics

Metals and alloys have many uses in dentistry. Steel alloys are commonly used for the construction of instruments for orthodontics. Gold alloys and alloys containing chromium are used for making crowns, inlays and denture bases while dental amalgam is the most widely used dental filling material.

Nickel-titanium (NiTi) has unique properties of shape memory and super-elasticity[25], that its endodontic instruments are three times more flexible than stainless steel instruments and have the ability to revert to their original shape after flexure, and more resistant to fracture [26]. NiTi (56% Nickel and 44% Titanium) is the favorable material for manufacturing continuous rotating and reciprocating endodontic instruments, that have three microstructural phases; austenite phase (ductile) exists at higher temperatures and lower stresses; the martensite phase (hard) exists at lower temperatures and higher stresses, whereas, R-phase is an intermediate phase that forms during the forward and reverses transformation between austenite and martensite [27][28].

In 2007, a new NiTi alloy termed "M-Wire" (Dentsply Tulsa-Dental Specialties, Tulsa, OK, USA) was developed through proprietary thermomechanical processing procedure. Manufacturer's data for M-Wire indicated significantly improved fatigue resistance in comparison with conventional NiTi alloys. Currently, M-wire is used for the manufacture of GT series X instruments and Protaper Next (Dentsply Tulsa-Dental Specialties), Wave One (Dentsply Maillefer, Ballaigues, Switzerland), and Reciprocal (VDW, Munich, Germany). Many finite element studies [4][29][30][31] compared Stainless Steel, NiTi, and M-Wire endodontic instruments behaviours.

New trends in geometrical modelling in dentistry

Geometric modelling is essential to develop a database supporting engineering design. Figure 6 demonstrate the role of geometric modeling in CAD system(s). As it links the user interface and the mathematical representation (database). Additionally, any complete part representation should include both topological and geometrical data. That the geometric data offers shape and dimensions, and topology represent the connectivity and associativity of the object entities; it determines the relational information

between object entities. Geometric modeling techniques for structures and assemblies contain many difficulties usually appeared with model(s) transfer between different packages.

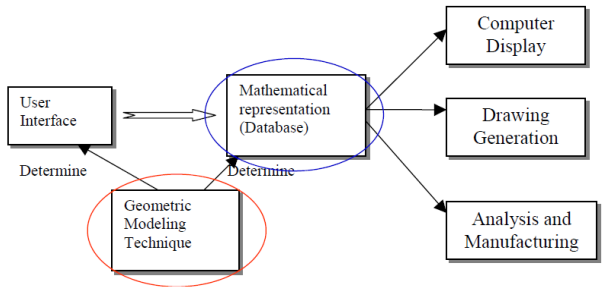

Figure 6: Role of geometric modelling in CAD system(s) [34]

Wireframe models (and 2D Projection) contain points, lines, and curves, that topological data are not included. On the other hand, surface models store topological information of the containing objects. However, surface models can't support a full range of engineering activities [32] such as stress analysis on the internal body. Solid models have complete, valid and unambiguous spatial addressability. Additionally, wireframe and surface models can be extracted from solid one(s), and reverse process is not valid [33]. Most of engineering CAD/CAM packages involve Constructive Solid Geometry (CSG) technique in representing solids using Boolean (like a union, intersection, ...etc.) combination of primitive solids (like cubes, cylinders, ...etc.).

On the other side, extracting 3D model(s) from digitisers (scanners, CT, MRI, micro-CT, CBCT, 3D Ultrasound, Confocal Microscopy) needs great effort to be recognised. Software(s) like Mimics (Materialise, Leuven, Belgium) can manipulate medical format pictures as brightness threshold and mask to separate the image parts (organs). That can generate models for a variety of engineering applications and directly export the 3D models and anatomical landmark points to 3rd party software, like CAD, or FEA packages [34].

Examples

Term "immediate loading", indicate that implants and temporary teeth are placed within two days of implant surgery and are left in position for the healing period. Then the temporary teeth are replaced with permanent crowns. Singh et al., [35] aimed to place eight implants in fresh extraction socket in two successive sessions, for early loading. CT was made for the patient mandible after teeth extraction. The CT was used as a 3D geometric model and scale the mandibular teeth to fit on it (in place). Finally placed suitable implants with correct sizes and directions to replace teeth roots. Then CT image(s) was used to illustrate the gingiva and build thin surgical template containing thicker zones with angulated holes to help the dentist to place the implants correctly. The geometric model of the template was directly sent to the milling machine to start the surgery within 48 hours. Another immediate loading technique for a single tooth [36] that place implant(s) immediately without template while zirconia crown was milled simultaneously during the implant placement in the same session.

Dental CAD/CAM system(s)

The need for a fast 3D geometric model(s) for teeth forced producers of dental CAM machine to include scanners with their systems. Recently digital impressions taken directly from the alveolar ridges and natural teeth using the intra-oral camera are used. Upper/lower scanned impressions are used for accurate estimation of missing teeth dimensions. The integrated software builds up the prosthesis by using the embedded set of thirty-two teeth (as standard shapes) as wireframe models. The CAD/CAM specialist can make scaling and points movements on the wireframe models to fit the available space before sending the model to the milling machine. Recently CAD/CAM systems are involved in the production of artificial bone, denture base, crown, bridge, veneer shades, ... etc.

Several types of research [35][36][37][38] discussed the immediate loading or immediate replacing tooth. Implant immediate loading needs fast imaging, viewing, bone thickness estimation, to determine the suitable implant(s) direction(s). Two examples will be shortly presented here:

1. Delcam (Birmingham, UK), produced special dental CAD/CAM solutions system including five-axis milling machine. While the geometric modelling to be acquired from external scanner or CT image(s), the system is capable to interface with wide range of scanners. The geometry of the scanned part was transferred as STereoLithography (STL) file [39], which is a type of Wireframe technique of geometric modelling, before extract 3D model with dimensions from Dicom files.

2. EnvisionTec (Gladbeck, Germany) offered a family of rapid prototyping printers "Perfactory PixCera". That is used to produce casts and prostheses from polymeric resins and metallic base one(s) [40]. The metallic one(s) will be covered by porcelain and veneer to match the other teeth shape and colour.

The rapid prototyping can't improve its quality at finish line thus the special care could be done manually. Contrarily the CAD/CAM system is capable of defining the finish line and matching a tool pass with a finishing tool. Finally, CAD/CAM can produce complete zirconia tooth to replace extracted one for immediate loading, which is not possible with rapid prototyping printers.

New dental instrument design(s)

Engineering CAD/CAM software(s) are used to model new dental tools before implementing stress analysis and producing prototypes for testing. New versions of CAD/CAM software(s) include stress analysis module(s), while modelling modules in stress analysis packages have limited capabilities. Designers prefer to use CAD/CAM software for geometric modelling and transfer their model(s) to finite element packages. That Initial-Graphics-Exchange-Specification (IGES) file format showed the best performance for surfaces transfer to FEA software(s). While Standard-ACIS-Text (SAT) file format is perfectly used for transferring volumes (3D solid geometric model).

Conclusions

The dental material market is flooding by several materials. Unfortunately, there are no available dental materials with ideal properties for any dental applications. With the start of nanotechnology era, improvements in existing materials' properties and development of new materials appeared in new products. In reality, nanomaterials will never completely replace the other dental materials like ceramics and composites for several reasons like economics, short-term materials usage, preparation time, ...etc. but for sure it will strongly compete with them.

Accurate geometric modelling is essential for CAM processes and stress analysis checks. Engineering CAD/CAM packages may be not suitable for many dental applications, but will never be replaced in designing new tools/instruments for dentistry. On the other hand, imaging teeth and transfer it into wireframe model before CAM is currently available. New dental solutions systems offered a lot of features for dentist and laboratory operators about implants types and size databases, locations of high dens bone, ...etc. One drawback to obtaining a suitable solid model from CT images to start stress analysis is still problematic.

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Public Health

Persuading Iranian Women toward Normal Vaginal Delivery: Using Pictorial Perception of the Labour Process

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Keywords: Parturition; Pictorial Education; Pregnant Women

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Abstract

BACKGROUND: Pictorial education could provide an innovative approach for health educators which help to increase health-related information, the attention of individuals, comprehension, and recall.

AIM: The purpose of this study was to determine the effect of pictorial perception of labour process by persuading Iranian women toward normal vaginal delivery.

MATERIALS AND METHODS: The pre and post non-randomized trial with control group carried out on non-probability sample consisted of 76 pregnant women during the third trimester of pregnancy in the four urban health care centres in Pars-Abad city, Iran, during 2014. Demographic, knowledge, attitude, subjective norms, outcome expectations, self-efficacy, and intention to do normal vaginal delivery variables were measured by using self-administered questionnaire and via the self-report method. Data analysis was performed using SPSS-21 software by Independent t-test, repeated measure, paired T-test, ANOVA, chi-square, Cochran's Q, and McNemar test. Manipulation included a pictorial education program to persuade pregnant women toward selecting normal vaginal delivery.

RESULTS: The results showed significant improvement in mean scores of knowledge, attitudes, self-efficacy and behavioural intention of labour after manipulation in the intervention group ($P < 0.001$). It was found about 60% changes for intending to choose normal vaginal delivery, and 27/06% of women in the intervention group reported normal vaginal delivery versus the control group. And 10/81% of women did a cesarean section because of medical reasons during of delivery. Reduction of cesarean section was evident. Additionally, the annual rate of cesarean section decreased about 7% in comparison to the previous year.

CONCLUSION: Pictorial education could be effective on the intention of women to choose natural vaginal delivery among pregnant women, and it can be used as an effective training technique for developing health literacy, enhancing self-efficacy and decision-making power of women in the delivery.

Introduction

Cesarean Section (CS) has become increasingly safer in the last century, but it cannot and more importantly should not replace natural vaginal delivery (NVD), because the maternal and infantile mortality associated with CS is 4-10 and 4 times higher; respectively [1] [2] [3].

While the highest reported rate of CS was for Brazil with about 50%, however, it is also high in Iran, for instance, 48% in 2009; and 58.6% based on the findings of another study in the Ardebil province, North West of Iran, which is a high figure compared to global statistics [4] [5] [6].

In comparison to NVD, CS has more complications, including uterine infection, fever, bleeding, anesthesia complications, urinary system damage during surgery, venous thrombosis in legs, higher expenditure, thromboembolism, postpartum depression, increased need for blood transfusion, placenta prevail, and fetal distress syndrome [2] [3] [7].

Tendency cause of CS is different including socio-economic factors, ageing women at first pregnancy, the preference of physicians, utilisation of technology, fear of physical harms, concern about the fetus, and determine the specific date for the birth of baby [3] [8] [9]. However, according to the World Health Organization has identified indications of CS,

but it is the preferred method of delivery [6].

According to studies, pictorial education provides an innovative approach and appropriate learning opportunity by using powerful and interesting graphics tool to create, manage, and exchange information and knowledge, and it helps to develop a mental model that for problem-solving [10] [11]. Also, pictures facilitate and accelerate the learning process, were easily learned and subsequently recalled over a prolonged time [10].

Moreover, humans have a cognitive preference for picture-based information and learning process as well rather than text-based which called "picture superiority effect" [11]. However, pictorial educations in combination with written or spoken text could have more effectiveness. In fact, it markedly increases attention to and recall, comprehension, adherence to health information, and the effectiveness of health communications in health education programs [12].

Since the training has a key role in choosing the type of delivery, so the education of pregnant women can lead to their preparation to deal with labour and thus reducing unnecessary CS, and this is important to the health education authorities [8] [13].

Hence, the audio-visual signs have greater objectivity and are considered as effective tools for learning process and health communication; the aim of current study was to develop and conduct how integrating pictorial education about labour process into routine health education programs will work and act to persuade pregnant women to make informed choices and voluntarily NVD method where there are numerous quest and forces to choose CS.

Material and Methods

The study was a pre-post randomised clinical trial with a control group which took place from April to September 2014. The non-probability sample consisted of all pregnant women including 37 women as intervention and 39 control groups who were recruited from four urban health care centres from among seven centres in Pars Abad city, Iran. Estimates for the number of women to be selected at each health services centre were calculated in proportion to the size of the population of women in child bring ages in each health care delivery centre covered. Eligibility criteria included: (1) being graduate in high school, (2) primiparous and multiparous women (grade 2) with gestational age of 26-30 weeks, (3) multiparous women (grade 2) with previous NVD, (4) pregnant women without miscarriage, (5) confirmed the ultrasound single fetus pregnancy, (6) confirmed the fetal ultrasound health, (7) no history of depression or chronic illness in the past, (8) not having an abnormal child, (9) have a normal pregnancy without any medical indication to make CS inevitable to termination of pregnancy during the study, (10) not seen CS and NVD films in the past, (11) not having recent history of prenatal education about CS and NVD, and (12) agreement to participate in the study. Also, exclusion criteria were premature delivery, multiple pregnancy diagnoses, small pelvis, diabetes, high blood pressure, CS indications, and the continued absence of women in the training sessions, and the unavailability of pregnant women at the time of completing the questionnaire and during conduct the study. The participants ranged in age from 18 to 35 years (Mean = 25.19, SD = 4.48). Of all 80 participants, 76 women completed the written questionnaire completely and participated in all training sessions. A self-report and researcher-designed questionnaire was used to collect relevant data.

The study was recorded in IRCT, and the Tabriz University of Medical Sciences' Ethics Committee approved the study and 92233 as ethical code received from the Department of Research and Technology University. The women received a demonstration on how to fill in the questionnaire, and they signed an informed consent before completing the questionnaire.

Measures included the demographic data and knowledge of NVD benefits and CS complication using 11-item (e.g., the risk of postoperative infection is lower in NVD) and 17-item (e.g., the risk of blooding is higher in CS); respectively. The options were yes, no, or I don't know. Other scales were positive attitude toward NVD (e.g. NVD is easier than CS), subjective norms of NVD (e.g., my mother and sister are recommending me to choose the natural vaginal delivery method), outcome expectations of NVD (e.g., I will have less pain after the natural vaginal delivery), and self-efficacy for NVD (e.g., the natural vaginal delivery is hard; but, I will do) using 15, 8, 12 , and 5-item scales; respectively, which were measured based on a 5-point Likert-type scaling (1 = strongly disagree to 5 = strongly agree).

Table 1: Instrument construction

Scales	# of Items	CVI*	CVR#	Reliability coefficient
Knowledge of NVD Benefits	11	0.89	0.91	0.86
Knowledge of CS Complication	17	0.85	0.94	0.92
Attitude toward NVD	15	0.95	0. 97	0.69
Subjective Norms of NVD	8	1.00	1.00	0.83
Outcome Expectations of NVD	12	0.94	0.97	0.93
Self-Efficacy for NVD	5	1.00	1.00	0.85
Intention to Choose NVD	2	1.00	1.00	0.90

*-Content Validity Index; #-Content Validity Ratio.

Furthermore, intention to choose NVD was another measure which measured via two-item scale. 1) "Which one of delivery method do you intend to choose?" and 2) "If your physician make you free to choose the delivery method which one of NVD or CS you will prefer?" Two options were available for each item, namely, CS or NVD.

Tests were used after confirmed the normality by Kolmogorov-Smirnov test using the SPSS-21 software. Descriptive statistics were used to summarise and organise the demographics data. Chi-square was performed to examine the relationship between demographic variables with the choice of delivery method. To compare the mean score of all scales except intention to choose NVD were used the Paired Samples T-test and ANOVA with repeated measures based on Greenhouse-Geiser test in the control and intervention groups in the pre and post stages of the intervention, respectively; and between the two groups were used the Independent T-test before the intervention and Covariance Analysis by adjusting the basic variables after the intervention. Mc-Nemar test and Cochran's test were used to compare the intention to choose NVD before and after the intervention in the control and intervention groups, respectively; and Chi-square test was used between the two groups in both of pre and post stages of the intervention. To analysis choice of the delivery method before the intervention and after the delivery between the two groups was used Chi-square test. To express the favourable and unfavourable changes in the intention to choose NVD and the choice of delivery method (Behavior) was performed Odds Ratio Paired. Also, P-value <0.05 was considered significant in all analyses.

From four health centres that were simple randomly assigned to two intervention and control groups, eligible women for the study were selected by convenience sampling method. Eligible pregnant women in the intervention group were invited via a phone call to participate in this study and then they were informed about all the aims and procedures of manipulation.

Manipulation included a pictorial and audio-visual education program to persuade pregnant women toward selecting NVD and was performed during four 60-minute training session, using pictures and videos, interactive communication, and question and answer after training. Most of the educational content of intervention was done according to diagnostic assessment. In addition to the intervention group participated in training sessions, benefited from routine prenatal care also, but the control group received only routine prenatal care.

The content of the training sessions included: a) the first session related to the anatomy and physiology of pregnancy, b) the second session was about the harms and benefits of NVD and CS, effective factors on the type of delivery and indications of CS, c) the third session related to movies and stages of NVD and CS, and d) the fourth session was about the pictures of delivery room, available obstetric facilities in the hospital and interviews with postpartum, and were answered to their questions after the session.

Training sessions took place while receiving regular prenatal care. Each training session was a continuation of the previous session, and if a pregnant woman had received a training session, next training session was formed. Also, the phone number was placed at their disposal if they need to call and ask their questions.

All of the training sessions ended to 36-37 weeks of pregnancy, and the post-test was conducted in three stages; 1) after the second interventional session, 2) immediately after completing all of the manipulation programs, and 3) the next referring to pregnant women to the health centre after completing all of the manipulation programs. Then the groups completed the initial questionnaire. In the end, their delivery method and their reason for it**s** choice were recorded.

Results

The mean age of pregnant women in the intervention and control groups, were, 24/43 ± 3/50 and 24/56 ± 3/46 and meant age of husbands with 29/49 ± 4/31 and 30/13 ± 4/47; respectively.

Table 2: Characteristics of participants and their relationship with the choice of delivery method (n = 76)

Variables	Intervention Group (n = 37)		Control Group (n = 39)	
	n (%)	P-value*	n (%)	P-value*
Educational level				
High school	25 (67.6%)	0.011#	26 (66.7%)	0.107
University	12 (32.4%)		13 (33.3%)	
Spouse Educational level				
Elementary	1 (2.7%)		6 (15.4%)	
Middle school	3 (8.1%)	0.541	7 (17.9%)	0.351
High school	15 (40.5%)		12 (30.8%)	
University	18 (48.6%)		14 (35.9%)	
Employment status				
Housewife	34 (91.9%)	0.554#	39 (100%)	NS
Employed	3 (8.1%)		0	
Spouse Employment status				
Staffer	8 (21.6%)		2 (5.1%)	
Worker	3 (8.1%)	0.431	2 (5.1%)	0.471
Self-employment	26 (70.3%)		35 (89.7%)	
Housing finance Status				
Landlord	18 (48.6%)	0.124	20 (51.3%)	0.151
Leased	19 (51.4%)		19 (48.7%)	
Having an independent life				
Yes	28 (75.5%)	0.711#	24 (61.5%)	0.571
No	9 (24.3%)		15 (38.5%)	
History of smoking				
Yes	0	NS	0	NS
No	37 (100%)		39 (100%)	
Spouse History of smoking				
Yes	4 (10.8%)	0.283#	1 (2.6%)	0.410#
No	33 (89.2%)		38 (97.4%)	
Household income				
Low	15 (40.5%)	0.531	10 (25.6%)	0. 264#
Average	22 (59.5%)		29 (74.4%)	
High	0		0	
Having health insurance				
Yes	20 (54.1)	0.457	27 (69.2%)	0. 726#
No	17 (45.9%)		12 (30.8%)	
Having supplemental insurance				
Yes	7 (18.9%)	0.677#	6 (15.4%)	0.370#
No	30 (81.1%)		33 (84.6%)	
Pregnancy				
Wanted	30 (81.1%)	0.408#	36 (92.3%)	0.557#
Unwanted	7 (18.9%)		3 (7.7%)	
Type of prenatal care				
Private physicians	0		2 (5.1%)	
Health care centre	17 (45.9%)	0.204	17 (43.6%)	0.099
Both	20 (54.1%)		20 (51.3%)	
Medical doctor's recommendation to choosing the CS				
Yes	1 (2.7%)	0.405#	1 (2.6%)	1.000#
No	36 (97.3%)		38 (97.4%)	
Place of delivery				
Private hospital	1 (2.7%)	1.000	0	NS
Public hospital	36 (97.3%)		39 (100%)	

*P-value based on chi-square test; #P-value based on Fisher's Exact test; No statistics are computed because this is a constant.

Choice of delivery and maternal education in the intervention group were statistically correlated.

67/6٪ (25/37) of mothers in the intervention group who were high school graduates chose NVD. No other significant associations were found.

The results of Greenhouse-Geisser test and paired t-test showed significant difference with the maternal knowledge about complications of CS and advantages of NVD in the intervention and control group; respectively, but the increased awareness in the intervention was more than the control group before and after the intervention. Women's attitude about NVD changed in a positive direction, but very little change was observed in the control group.

Table 3: Comparing the means scores Knowledge, Attitude, Subjective Norms related to NVD, Outcome Expectations of NVD, and Self-Efficacy for NVD among Participants; before and after the intervention

Variables	Measurement	Intervention Group (n = 37)	Control Group (n = 39)	P-value[a,b]
		Mean ± SD	Mean ± SD	
Knowledge of NVD Benefits				
	B	17.32 ± 3.33	17.59 ± 3.66	0.371 [a†]
	A_1	20.73 ± 1.83[*]		
	A_2	21.03 ± 1.34[*#]		
	A_3	21.35 ± 0.94[*#]	19.61 ± 2.94	<0.001[b]
P-value [c,d]		<0.001[c]	<0.001[d]	
Knowledge of CS Complication				
	B	20.5 ± 4.58	20.13 ± 5.32	0.474[a†]
	A_1	30.08 ± 5.38[*]		
	A_2	32.16 ± 4.91[*#]		
	A_3	32.57 ± 2.15 [*#]	23.62 ± 5.19	<0.001[b]
P-value [c,d]		<0.001[c]	<0.001[d]	
Positive Attitude toward NVD				
	B	53.30 ± 9.45	52.46 ± 10.43	0.356[a†]
	A_1	55.49 ± 8.14		
	A_2	60.46 ± 8.69[*#]		
	A_3	61.05 ± 10.96[*#]	52.79 ± 11.93	<0.001[b]
P-value [c,d]		<0.001[c]	0.301[d]	
Subjective Norms of NVD				
	B	19.92 ± 9.36	19.79 ± 8.12	0.475[a†]
	A_1	19.62 ± 5.28		
	A_2	19.65 ± 6.30		
	A_3	18.84 ± 6.76	17.95 ± 8.42	0.559[b]
P-value [c,d]		0.686[c]	<0.001[d]	
Outcome Expectations of NVD				
	B	52.03 ± 10.56	50.69 ± 9.00	0.277 [a†]
	A_1	54.04 ± 6.73		
	A_2	55.59 ± 5.41		
	A_3	55.23 ± 8.21	52.36 ± 9.05	<0.135[b]
P-value [c,d]		<0.059[c]	<0.007[d]	
Self-Efficacy for NVD	B	15.00 ± 8.03	12.85 ± 6.93	0.107[a†]
	A_1	13.78 ± 6.25		
	A_2	15.57 ± 6.52[#]		
	A_3	16.16 ± 6.26[#]	12.23 ± 7.44	0.028[b]
P-value [c,d]		0.057[c]	<0.107[d]	

B-Before the intervention; A_1-The first stage after the intervention (after completion the second training session); A_2-The second stage after the intervention (immediately after the completion of the training sessions); A_3-The third stage after the intervention (First visit after the completion of the training sessions); *-Significant intra-group differences with pre-intervention stage values; #-Significant intra-group differences with the values of the first stage after the intervention; a-P-value based on Independent T-test samples and a single tide is reported; †-Variables are reported on the basis of equal variances of Independent T-test; b-The P-value based on ANKOVA (Covariance); c-P-value based on Greenhouse-Geisser; d-P-value based on Paired-samples T-test and a single tide is reported; - The range of questions is knowledge of CS complication (0-34); - The range of questions is knowledge of NVD benefits (0-22); - The range of questions is positive attitude toward NVD (15-75); - The range of questions is subjective norms of NVD (8-40); - The range of questions is outcome expectations of NVD (12-60); - The range of questions is self-efficacy of NVD (5-25).

Also, using analysis of Covariance was observed a significant relationship in the mean scores of awareness, positive attitude toward NVD, self-efficacy for NVD, and intention of doing NVD between two groups after intervention, but this change did not occur in the mean scores for positive subjective norms of NVD and expectations outcome of NVD, and this is while t-test did not showed significant difference before intervention.

Table 4: Intention to Choose NVD among Participants; before and after the intervention

Variables	Measurement		Intervention Group (n = 37)	Control Group (n = 39)	P-value[*]
			n(٪)	n(٪)	
Intention to Choose NVD					
	B	CS	22 (٪59.55)	24 (٪61.5)	0.853 [a]
		NVD	15 (٪40.5)	15 (٪40.5)	
	A_1	CS	18 (٪48.6)		
		NVD	19 (٪51.4)		
	A_2	CS	14 (٪37.8)[*]		
		NVD	23 (٪62.2)		
	A_3	CS	12 (٪32.4)[*]	22 (٪56.4)	< 0.036 [a]
		NVD	25 (٪67.6)	17 (٪43.6)	
P-value [c,d]			<0.001[b]	<0.343[d]	

B-Before the intervention; A_1-The first stage after the intervention (after completing the second training session); A_2-The second stage after the intervention (immediately after the completion of the training sessions); A_3-The third stage after the intervention (immediately after the completion of the training sessions); a-P-value based on Chi-square test; b-The P-value based on Cochran's test; c-P-value based on Mc-Nemar test and a single tide are reported; * Significant intra-group differences with the pre-intervention stage.

Moreover, Cochran's Q test showed a significant relationship in the intention of doing NVD at pre and post-intervention periods in the intervention group, and it was increased. Also, despite the increase of NVD frequency and reduce CS frequency was observed no significant correlation compared to the selected method of delivery between the two groups in the pre-intervention and after giving birth phase using Chi-square test

Table 5: Choice of delivery method among Participants; before and after the delivery

Variables		Intervention Group (n = 37)	Control Group (n = 39)	P-value[*]
		N(٪)	N(٪)	
Choice of delivery method Before the delivery	CS	22 (٪59.5)	23 (٪59.0)	0.966
	NVD	15 (٪40.5)	16 (٪41.0)	
After the delivery	CS	19 (٪51.4)	24 (٪61.5)	0.370
	NVD	18 (٪48.6)	15 (٪38.5)	

* P-value based on Chi-square test.

Generally in the intervention group 27/02% (n = 10) from 37 samples had the intention changes from the CS to NVD that 16/21% (n = 6) of this number were performed NVD, and 10/81% (n = 4) were performed CS at the stage of labor because of medical reasons and emergency situations such as sudden increase in blood pressure and dystocia or lack of progress of labor. In other words, almost 60% of the intended changes from the CS to NVD was led to behaviour (NVD) in the intervention group. Also in the control group, 10/25% (n = 4) from 39 samples had the intention changes from the CS to NVD, that same percentage was maintained via performance of NVD.

It should be noted that 3 and 5 samples in the intervention and control group that had the intention of doing NVD since the beginning of the intervention respectively; were forced to have CS because of dystocia, breech presentation and preeclampsia in labour stage.

It is important to note that according to the time off provided statistics of CS rate by Pars Abad

city that was applying this intervention, 7% reduction was observed in all rate of urban CS.

Discussion

This interventional study was evaluated an innovative approach using fixed and mobile pictures to facilitate a better understanding of individuals that was different from other studies in this field.

Generally the results showed favorable changes in the intention to choose NVD (27/06%) in the intervention group that 60% of the desired changes were evident in the post-partum in the behavior, in addition to the annual assessment of the city was reported 7% reduction in the rate of CS which was included the implementation of the intervention.

Unlike other interventional studies that are focused on investigation of pre and post intervention; this study of the effect of intervention sessions sequences showed a significant intergroup relationship with the pre-intervention stage and the relationship of the stages with each other at the factors of knowledge of NVD benefits, knowledge of CS complication, positive attitude toward NVD, self-efficacy for NVD, and the intention to choose NVD.

In recent researches in 2013 in Iran, the knowledge of the majority of pregnant women (over 50%) was poor about delivery methods, and their advantages and disadvantages which it's the reason were described inadequacy of the training in prenatal in health centres [14]. But despite the knowledge and attitude of pregnant women of this study was moderate in this field, so was felt a need to upgrade the information. Therefore, these findings revealed the importance of parental education about different delivery methods, and it showed the effect of visual education that could through increased transfer of information to participants cause creation and promotion their positive attitude toward NVD.

Also, after training intervention, the knowledge about CS and NVD and the positive attitude toward NVD was improved in the intervention group, and significant correlation was reported between pre- and post-intervention stages between two the groups, which other studies confirmed these findings [7] [8] [11] [15].

Versus, the studies by Shahraky et al. and Toghyani reported lack of effect of the training program on knowledge and attitude toward childbirth and declared that this might be due to the inadequate educational content in the realm of emotional [9] [16]. But the cause of significant attitude construct in this study can be expressed properly designed interventional training based on attitude and behavioural beliefs of pregnant women in this field, because images, slide and video have more objective and are of most interest to change the attitude [17]. Moreover, despite an increase in the mean score of knowledge of delivery method in the control group, attitude construct did not change, that this was also evident in the study by Rahimikian et al., [15]. This increased knowledge in the control group could be possible because of receiving knowledge about childbirth from other information sources such as mass media, educational books, personnel of health centres, etc. which this trend of gaining unsystematic of information caused that slight promotion of knowledge and lack of changing attitude among them.

In the current study, subjective norms aimed to determine the effects of different people including doctors, midwives, wife, family, and friends and acquaintances about the pregnant women decision for choice of NVD. Despite other studies showed an increase in the mean scores of subjective norms associated with NVD and the significant relationship between pre and post intervention in the intervention group and between two the intervention and control groups [8] [9], in this intervention a smaller decrease was revealed in this scale in the intervention group compared with the control group and this can be revealed that the positive impact of different individuals views in relation to NVD in the pregnant women of the intervention group was more than the control group, and therefore, the primary effect of these comments has been maintained to some extent in the intervention group.

Also, there was no increase in this score in the intervention group, probably it's reason be to obtain a training program, observation of images objectively and videos of delivery methods by this group and thus promotion of a positive attitude toward NVD that in their decision to birth are focused more on personal beliefs rather than social or norm beliefs and prefer NVD regardless of social demand; totally Ajzen & Fishbein stated that are expected to vary the relative importance of attitudes and subjective norms to predict the intentions and needs of people from society to society [18].

Moreover, researchers have shown that NVD selection in pregnant women is associated with high self-efficacy [14]. In fact, the self-efficacy is individuals' assessment of own ability to cope with stressful situations and necessary reactions, especially during labour and pair of childbirth [19]. Women who have low self-efficacy, consider NVD as impossible issue and experience much fear during pregnancy [20]. Therefore, these women may prefer CS to avoid or escape from the pain of childbirth associated with NVD [21]. Therefore self-efficacy was explained as a mediator factor in childbirth, and a key element in the choice of delivery method and control of childbirth fear that can enable the person to cope with childbirth [20] [21]. Thus self-efficacy plays an important role in behavioural intention [19] [22] and act as a bridge between think, intention and a specific

behaviour [14]. The results also showed a positive effect of training program on this important factor to choose the delivery method, so that after intervention between two groups there was statistically significant in mean scores of self-efficacy that this result was consistent with Myoghwa 's study findings [10].

Also, since the acquisition of skills through past successes and positive experiences reinforces the self-efficacy, so multiparous pregnant women have greater self-efficacy than nulliparous [20] [23]. Hence cause of the lack of significance in mean scores of self-efficacy in the intervention group pre and post-intervention phase is probably related to this fact that the majority of mothers under current study were nulliparous.

In fact, people with high self-efficacy expect to gain success in their efforts, these people have favourable outcome expectation from certain behaviour, which this focuses on the relationship between self-efficacy and outcome expectations that had been revealed this relation in childbirth also [20] [23] [24] [25]. But in this study in spite of significance in the mean scores of self-efficacy between the two groups, was not a significant difference in the mean score of the outcome expectation of NVD. Although the studies have shown a relationship between self-efficacy and outcome expectation, these two variables are different from each other [20]. As Tudoran & Williams and et al. explained that outcome expectation is important in forming an intention and can be used as a tool in changing individuals' behaviour and alone be sufficient to explain the behaviour [25] [26] [27].

A person may be aware from the beneficial outcomes of behaviour, but had no confidence in her ability to do it; the person may have more confidence, but doubt in reaching to the outcome expectation [21]. In fact, an interaction between the outcome expectation and self-efficacy has been found in the investigations, so that the people believe that they can do a certain healthy behaviour, but do not do it, because they ask from the results of such behaviour [26] [28] [29]. So if self-efficacy is allocated as an interface between the outcome expectation and intention, the direct relation of the outcome expectation with intention will disintegrate; so the outcome expectation may be associated with intention through two different methods: 1) directly, 2) indirectly through its effect on self-efficacy and the subsequent impact on intention [26].

Therefore in the current study, the lack of significant increase in the mean scores of outcome expectation is probably its indirect effect on intention; in other words, perhaps increase in the mean score of outcome expectation has been revealed with its impact on self-efficacy, and it's significant. Moreover researchers have been showed that a person's beliefs about a certain behavior as outcome expectation and values associated with attitudes, this means that person's attitudes toward behavioral intention may be formed from a linear combination of the outcome expectation and the expected value [25] [30] which this can be another probability of the lack of significant apparent increase in the mean score of the favorable outcome expectation of NVD and its indirect impact on intention with the increase in the mean score of positive attitude toward NVD. In general, it can be said that self-efficacy and outcome expectation is effective in the formation of intention and are important as tools in the development of behaviour [27] [31].

Despite the significant relationship in labor behavioral intention between pre-post training in the intervention group and among two groups after training that were similar with many researchers results [7] [8] [9], but was not observed this significance in choice of delivery method between two groups that were not consistent with the findings of other studies [7] [8] [9] [13] [15].

Generally, behavioural intention as a person's mental willingness to do behaviour is the most determinant and predictor of behaviour which is a sign of person's readiness to carry out the desired behaviour [18] [32]. Although in Poss et al. study is confirmed strong relationship between intention and behavior and it is common quite that people behave in accordance with their intentions, but for carry out the behavior is not enough only behavioral intention [18] [32] [33], because there are factors that participate in convert the intention to behavior and filling out the gap between intention and behavior; among these factors, the factors are that facilitate the conversion of the intention to behavior like facilities, resources, needfull skills to change the behavior which John Hubley express it as enabling factors and or unpredictable obstacles or factors are that lead to the surrender of the person against temptations, such as a person's ability to cope and strength in dealing with barriers after intention as self-efficacy survival and planning to do behavior in dealing with obstacles after decision-making which have been raised by Schwarzer et al as meditors after intention to overcome intention-behavior gap and become intention to behavior [18] [22] [31].

While in the conducted surveys, the main reasons for high tendency and preference for CS among Iranian pregnant women were described as non-medical reasons, social beliefs, cultural and economic factors [34] [35], but according to Besharati et al. findings [8], in this study also, the cause of lack of convert a favorable behavioral intention (ie modified intention from CS to NVD) to the desired behavior (NVD) in the intervention group was medical reasons and mother's emergency conditions in the process of giving birth such as a sudden increase in blood pressure or lack of progress of labor or dystocia; so by the necessary arrangements except for the medical cases that diagnosed originally, other casess should be cotrolled in order to reduce the emergency CS rate resulting in such cases; because the more

understanding of the determinants of a behavioral will be caued the more success of interventions [36].

The findings of present study reflected the impact of visual training on reducing CS among the pregnant women, regardless of CS that was necessary because of emergency reasons, the rest of the modified intention was observed as desirable in the behaviour of pregnant women with NVD.

Studies showed that pictures stimulate memory, elevates attention, understanding, help to recall information, and are important to learning speed and strengthen communication [11] [12] [37]. Visual training is an innovative approach to learn and appropriate use of health-related issues because it enables people with different educational levels by strengthening self-efficacy, knowledge and increase understanding [10].

While most studies about visual training were focused among individuals with low education or lack of sufficient skills in reading and on issues other than childbirth [11] [12], but in this study were evident the effectiveness of this intervention training among individuals with high education level and familiarity with the delivery methods.

Moreover, as researchers have shown in education and psychology that people have a priority and cognitive preference for visual information rather than contextual information [10] [11]. However, if the pictures are provided with verbal explanations, in addition, to increase recall and effective help to patient understanding, causes to avoid wrong interpretation of the pictures [10] [11]. Hence, the findings of this study also showed the way impact of combining of visual training with audio training among participants.

As a limitations of this study are: 1) way of self-report for completing questionnaires that is likely bring recall bias, 2) how sampling depending on its non-random nature that causes lack of generalizing the results to other populations, 3) collecting information using self-structure instruments compared to standard tools, there are the carelessness likelihood and lack of understanding by participants in answering, 4) non large studied area, which bring out the possibility of communication and transfer of information between two groups or in other words the possibility of communication bias, and 5) The lack of audio-visual tools in some health centers under study. Therefore, it is suggested that in addition to taking into consideration the cited limitations by further researchers, in order to better achievement to research objectives, this training should be provided for pregnant women with different educational levels and their spouses and mothers in companion with routine prenatal care. Also, it is recommended to check out the entire process after intervention in control groups simultaneously with intervention group to confirm the effects of each of the training sessions.

In conclusion**,** the current study showed the effectiveness conducted visual training in selecting NVD and prevention of the unnecessary CS rate, and as a new approach could provide pregnant women with the advantage of increasing awareness of both delivery methods, increasing positive attitudes toward NVD, increasing self-efficacy of NVD, and favorable behavioral intentions through presentation of pictures and related. So this method can be used to developing health literacy, enhancing self-efficacy, decision-making power in women's skills about NVD, and the future decisions of health authorities to more encouraging mothers to do NVD, reducing unnecessary CS and providing maternal and their child health as a suitable educational tool. Thus the health communication efficiency can be increased significantly by this training in designing new cases of health education and be effective.

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Trend and Causes of Maternal Mortality in a Nigerian Tertiary Hospital: A 5-year Retrospective Study (2010-2014) at the University of Calabar Teaching Hospital, Calabar, Nigeria

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Abstract

BACKGROUND: Maternal mortality ratios (MMR) are still unacceptably high in many low-income countries especially in sub-Saharan Africa. MMR had been reported to have improved from an initial 3,026 per 100,000 live births in 1999 to 941 in 2009, at the University of Calabar Teaching Hospital (UCTH), Calabar, a tertiary health facility in Nigeria. Post-partum haemorrhage and hypertensive diseases of pregnancy have been the common causes of maternal deaths in the facility.

AIM: This study was aimed at determining the trend in maternal mortality in the same facility, following institution of some facility-based intervention measures.

METHODOLOGY: A retrospective study design was utilised with extraction and review of medical records of pregnancy-related deaths in UCTH, Calabar, from January 2010 to December 2014. The beginning of the review period coincided with the period the "Woman Intervention Trial" was set up to reduce maternal mortality in the facility. This trial consists of the use of Tranexamic acid for prevention of post-partum haemorrhage, as well as more proactive attendance to parturition.

RESULTS: There were 13,605 live births and sixty-one (61) pregnancy-related deaths in UCTH during the study period. This yielded a facility Maternal Mortality Ratio of 448 per 100,000 live births. In the previous 11-year period of review, there was sustained the decline in MMR by 72.9% in the initial four years (from 793 in 2010 to 215 in 2013), with the onset of resurgence to 366 in the last year (2014). Mean age at maternal death was 27 ± 6.5 years, with most subjects (45, 73.8%) being within 20-34 years age group. Forty-eight (78.7%) were married, 26 (42.6%) were unemployed, and 33 (55.7%) had at least secondary level of education. Septic abortion (13, 21.3%) and hypertensive diseases of pregnancy (10, 16.4%) were the leading causes of death. Over three quarters (47, 77.0%) had not received care from any health facility. Most deaths (46, 75.5%) occurred between 24 and 97 hours of admission.

CONCLUSION: Compared with previous trends, there has been a significant improvement in maternal mortality ratio in the study setting. There is also a significant change in the leading cause of maternal deaths, with septic abortion and hypertensive disease of pregnancy now replacing post-partum haemorrhage and puerperal sepsis that was previously reported. This success may be attributable to the institution of the Woman trial intervention which is still ongoing in other parts of the world. There is, however, need to sustain effort at a further reduction in MMR towards the attainment of set sustainable development goals (SDGs), through improvement in the provision of maternal health services in low-income countries.

Introduction

Maternal deaths are still unacceptably high in many low-income countries, where pregnancy and childbirth are high-risk events that constitute major public health challenge [1]. Consequently, concerted efforts have been made, including long-term goals and programs aimed at improving maternal morbidity and mortality [2] [3]. One of such goals (the 5th Millennium Development Goal (MDG-5)), which aimed at reducing maternal deaths by three-quarters between 1990 and 2015, has been attained by only sixteen countries globally [1]. Notwithstanding, there has been an improvement in reduction of maternal mortality ratios (MMR) globally, with a report indicating 0.3% reduction in the initial five years and 2.7% reduction in the later years of the 15-year MDG span [1]. Great achievement in the MDG-5 target is particularly notable in the south, east, and Southeast

Asian countries, while West and Central African regions without exception to Nigeria have made slow progress [1].

In Nigeria, MMR had initially risen from 704 per 100,000 live births in 2000, to 800 in 2003 before gradual reduction to 545 in 2007, 400 in 2008 to 280 in 2014 [1] [4]. This recent figure is short of the MDG target of 75% reduction in MMR by the end of 2015. There is however wide regional variation with higher rates in Northern compared with Southern Nigeria [5] [6] [7] [8]. Improvement in MMR has also not been consistent in many regions, due to intermittent financing for implementation of maternal health intervention policies and programs as seen in other parts of the African continent [9]. Consequently, varying intensities of implementation of maternal health interventions, have led to varying trends and leading causes of maternal deaths in the communities [10] [11] [12].

A retrospective study of the medical records in the University of Calabar Teaching Hospital (UCTH), a tertiary hospital in Calabar, Cross River State, Nigeria, found a decreasing trend in maternal mortality, from 3,026 per 100,000 live births in 1999, to 941 in 2009, [6]. During the 11-year study period, there were 231 maternal deaths, yielding an average maternal mortality ratio of 1,513 per 100,000 live births, with most women being 20-34 years old (63.3%), married (67.1%) and unemployed (55.4 (63.3%). Obstetric haemorrhage (33.4%) was the commonest cause of death, followed by hypertensive diseases of pregnancy (21.2%), septic abortion (14.3%) and obstructed labour (9.1%) [6].

Archibong et al., in their review of existing maternal health policies including an in-depth interview with key stakeholders in Cross River state health sector, identified factors that may have significantly impacted on the state's maternal morbidity and mortality status [13]. Specific programs such as Midwife Service Scheme (MSS), National Health Insurance Scheme (NHIS) and Integrated Maternal Neonatal and Child Health (IMNCH), were found to have been adopted and made functional, especially through the state's counterpart funding in collaboration with non-governmental partners. Though the authors identified that only 40% of budgetary allocation to the health sector was usually released, they were however unable to obtain adequate information on the state's budgetary income funding especially from donor agencies [13].

Also, despite poor budgetary allocation and release to the health sector, there has been significant improvement in water and electricity supply to primary healthcare facilities, as well as the construction of several road networks by the government of Cross River State [13]. These activities with potentially significant indirect effect on health outcomes were aimed at improving retention and attraction of health personnel to rural settings, and reduction in delays of providing and receiving essential health services. Assessment and evaluation of the impact of these infrastructural interventions may be useful towards identifying areas of strengths and weaknesses in our prevention efforts.

Therefore, there is still much work to be done in containing preventable maternal deaths, especially in low-income countries. This position has been supported by relevant stakeholders leading to the current Sustainable Development Goals (SDG) target of having less than 70 maternal deaths per 100,000 live births by 2030, as a consolidation of the gains of the MDG strategic achievements [14] [15]. Consequently, it is key to institute and assess maternal death prevention programs and practices, to identify areas of strength and weakness towards re-evaluation and promotion of best practices. This evaluation is especially important in settings with dwindling economies such as Nigeria, where there is increasing need to do much more with less money amidst other competing needs [16].

The University of Calabar Teaching Hospital (UCTH) was one of the leading recruitment sites for the world Maternal Antifibrinolytic trial (Woman Trial). This is a large pragmatic randomised double-blind, placebo-controlled trial to quantify the effect of early administration of tranexamic acid for postpartum haemorrhage on maternal death, Peripartum hysterectomy and other outcomes. The multi-centre trial is coordinated by the London School of Hygiene and Tropical Medicine [17]. The Trial was commenced in 2009, and over 16000 women were randomised, and the target sample size of 20,000 eligible women was attained in August 2016. The first and the second nested study aimed to evaluate the effect of tranexamic acid on markers of coagulation and hemostatic effect of tranexamic acid in randomised women respectively. Although the trial has been concluded, there is need to evaluate its impact on MMR in the region. This study was therefore aimed at assessing a 5-year trend in maternal mortality in UCTH Calabar, Nigeria, following institution of these interventions.

Methodology

A retrospective review of clinical data was carried out for all maternal deaths that occurred in UCTH within the study period of January 2010 when the interventions were commenced, to December 2014, using hospital records. This activity was conducted with assistance from three (3) trained medical research assistants. Records that were mutilated beyond recognition of basic sociodemographic data, as well as those that lacked information on the cause of maternal death, were excluded. Available records of live births during the

study period were also obtained from the medical records unit. Ethical approval for the study was obtained from UCTH health research ethics committee. Maternal death was defined as the death of a woman while pregnant or within 42 days of termination of pregnancy, irrespective of the duration and site of the pregnancy, from any cause related to or aggravated by the pregnancy or its management but not from accidental or incidental causes [18]. Socio-demographic characteristics, as well as the number and causes of death, were determined. Place of initial care and duration of admission before death was also determined. The number of live births during the study period was also determined. Data were entered and analysed using SPSS version 21.0.

Results

Sixty-one (61) maternal deaths occurred in UCTH, Calabar, during the study period (2010-2014), with an estimated maternal mortality ratio of 448 maternal deaths per 100,000 live births (Table 1).

Table 1: Trend in Maternal Mortality Ratio in UCTH (2010-2014)

Year	No. Maternal deaths n (%)	No. Live Births	MMR per 100,000 live births
2010	21 (34.4)	2,647	793
2011	18 (29.5)	3,482	517
2012	10 (16.4)	3,051	328
2013	6 (9.8)	2,787	215
2014	6 (9.8)	1,638	366
Total	61 (100)	13,605	448

MMR=Maternal Mortality Ratio

This excludes one subject whose hospital record was mutilated beyond recognition of basic socio-demographic data and maternal cause of death. There was a significant decline in maternal mortality ratio from 793 in 2010 to 215 in 2013 and gradual resurgence in 2014.

Table 2: Socio-demographic characteristics of maternal deaths (subjects) (N=61)

Variable	Frequency	Percent
Age group		
< 15	1	1.6
15-19	5	8.2
20-24	10	16.4
25-29	22	36.1
30-34	13	21.3
35-39	8	13.1
≥40	2	3.3
Total	61	100
Marital status		
Married	48	78.7
Single	10	16.4
Cohabiting	3	4.9
Total	61	100
Occupation		
Civil servant	7	11.5
Business woman / trader	18	29.5
Student	5	8.2
House wife	5	8.2
Unemployed	26	42.6
Total	61	100.0
Educational level		
Primary	25	41.0
Secondary	31	50.8
Tertiary	3	4.9
Not stated	2	3.3
Total	61	100.0

Mean age at maternal death was 27.7 ± 6.5 years, with most deaths (45, 73.8%) occurring within 20 to 34 years of age. Over three-quarters (78.7%) were married, while 25 (41.0%) had an occupation as civil servant (7, 11.5%) or businesswoman/trader (18, 29.5%). Thirty-three (55.7%) had at least secondary level of education (Table 2).

Approximately three-quarters (45, 73.8%) of maternal deaths were attributable to direct causes. Septic abortion (13, 21.3%) was the commonest cause of maternal death, followed by hypertensive diseases of pregnancy (10, 16.4%). Relatively less common causes were antepartum haemorrhage (5, 8.2%), prolonged obstructed labour (4, 6.6%) and molar pregnancy (4, 6.6%). Malaria in pregnancy (4, 6.6%), and AIDS (4, 6.6%) were the commonest indirect cause of death. Other medical causes of maternal deaths included congestive cardiac failure (3, 4.9%), pulmonary embolism (2, 3.3%), while hematologic causes were aplastic anaemia (1, 1.6%) and sickle cell disease (1, 1.6%), (Table 3).

Table 3: Comparison of causes of maternal death in UCTH

Cause of Death	1999 – 2009*		2010 - 2014		Percentage change
	Frequency	Percentage	Frequency	Percentage	
Direct causes					
Septic abortion	15	6.5	13	21.3	+14.8
Hypertensive disease of pregnancy	49	21.2	10	16.4	-4.8
Antepartum hemorrhage	11	4.8	5	8.2	+3.4
Prolonged obstructed labor	21	9.1	4	6.6	-2.5
Molar pregnancy	0	0	4	6.6	+6.6
Puerperal sepsis	33	14.3	3	4.9	-9.4
Intrauterine fetal death septicemia	0	0	2	3.3	+3.3
Post-partum hemorrhage	66	28.6	2	3.3	-25.3
Ectopic pregnancy	6	2.6	1	1.6	-1
Amniotic fluid embolism	0	0	1	1.6	+1.6
Indirect causes					
Malaria in pregnancy	12	5.2	4	6.6	+1.4
Aids	11	4.7	4	6.6	+1.9
Congestive cardiac failure	0	0	3	4.9	+4.9
Pulmonary embolism	0	0	2	3.3	+3.3
Anesthetic complication	4	1.7	1	1.6	-0.1
Aplastic anemia	0	0	1	1.6	+1.6
Sickle cell disease	0	0	1	1.6	+1.6
Hepatitis/jaundice in pregnancy	3	1.3	0	0	-1.3
Total	231	100	61	100	-

*-Obtained from table 4 of the previous study on trend in maternal causes of death (1999-2009) in same study setting[6].

The common age groups for the occurrence of maternal death due to septic abortion were 15-19 (4, 30.8%) and 25-29 (5, 38.5%) years**).** Half of the maternal deaths due to hypertensive diseases of pregnancy, prolonged obstructed labour and molar pregnancy occurred among women who were 25-29 (5, 50%), 20-24 (2, 50%) and greater than 39 (2, 50%) years old respectively. All the four maternal deaths due to malaria occurred at 30 years of age (Table 4).

Most subjects (58, 95.0%) had initially sought none (47, 77.0%) or hospital of lower quality (11, 18.0%) before presenting at the study centre. Approximately three-quarters of maternal deaths (46, 75.5%) occurred within 25 and 96 hours of admission (Table 5).

Table 4: Age distribution of common causes of maternal deaths in UCTH (2010-2014)

Variable(years)	Frequency	Per cent	Mean age (years)
Septic Abortion			
15-19	4	30.8	24.2 ± 6.9
20-24	2	15.4	
25-29	5	38.5	
35-39	2	15.4	
Total	13	100	
Hypertensive diseases of pregnancy			
20-24	2	20	27.6 ± 4.8
25-29	5	50	
30-34	2	20	
35-39	1	10	
Total	10	100	
Prolonged obstructed labour			
<15	1	25	21.8 ± 6.3
20-24	2	50	
25-29	1	25	
Total	4	100	
Molar pregnancy			
25-29	1	25	39.3 ± 8.0
35-39	1	25	
≥40	2	50	
Total	4	100	
Malaria in pregnancy			
30-34	4	100	30.0 ± 0.0

Discussion

There has been varying degrees but significant reduction in maternal deaths globally, with many low-income countries, however lagging behind. In this facility-based study, annual maternal mortality ratio was found to have decreased quite consistently in the last five years. This is in keeping with global expectations towards the attainment of the MDG-5 target [15]. This success could be attributable to several on-going intervention programs. Previous studies have shown that a significant proportion of maternal mortality and life-threatening complications occurred in women who did not receive antenatal care but referred to the maternity unit in emergency condition [6]. In 2010, the Cross River State legislators approved the Governor's request for free maternal care services to all categories of pregnant women in the State [13].

Table 5: Pattern of the health-seeking behaviour of subjects

Variable	Frequency	Percentage
Place of initial care		
None	47	77.0
Hospital of lower status	11	18.0
Same hospital	3	5.0
Total	61	100.0
Duration of admission before death		
≤24 hours	8	13.1
25-48 hours	19	31.2
49-96 hours	27	44.3
97 hours - 10 days	6	9.8
>10 days	1	1.6
Total	61	100.0

This was to promote the number of antenatal clinic attendants and encourage women to utilised skilled birth attendants in the state government-owned health facilities for labour and deliveries. Increased utilisation of skilled birth attendants might have contributed to the decline in incidences of obstetric complications because, in the last five years, the Cross River State government has been offering free maternal and newborn care in all the primary and secondary health facilities in the state [13]. This has increased the number of antenatal attendants; hospital supervised deliveries and prompt referral. Analysis of health facility-based data by Pathfinder International in a stakeholders' forum has shown a gradual decline in maternal mortality and a remarkable uptake in obstetrics and neonatal services including HIV and contraception programs in the state [19].

Much of the success of improvement in maternal mortality seen in this study might have also been influenced by the dedicated implementation of the Woman Trial. In this trial, every woman with postpartum haemorrhage was given 1 to 2 gm of tranexamic acid or placebo. During the five year period more than 500 women with PPH were recruited in which perhaps, more than have of them might have been treated. This is reflected in the significant difference in the proportion of maternal deaths caused by postpartum haemorrhage (PPH) comparing pre-intervention and intervention periods [6]. In this study, a significant reduction in the occurrence of PPH was the main cause of the significant reduction in maternal deaths [20]. In a recent Cochrane review [21] that included 12 trials involving 3285 women undergoing caesarean section (9 trials, 2453 participants) and spontaneous labour (3trials, 832 participants), Tranexamic acid (TXA), in addition to other uterotonics, decreased postpartum blood loss and prevented PPH and blood transfusion.

Furthermore, in a double-blinded trial by Xu J and colleagues [22], TXA was shown to significantly reduce obstetric haemorrhage during caesarean section compared to the control group ($P < 0.01$). Also, the quantity of total blood loss from placental delivery to 2 hours postpartum was significantly reduced ($P = 0.02$) in TXA group than the control. Woman trial which recruited 20,000 women with PPH was concluded in March 2016, and the data were analysed in September 2016 and has been published in Lancet in December 2016. The results showed a significant reduction in primary outcomes- maternal mortality and peripartum hysterectomy in the treated group compared with the placebo group.

Also, through the Cross River State essential drugs program, iron and folic acid supplements are readily available in most primary and secondary health facilities which are dispensed free to pregnant women. This may have contributed to the low prevalence of anaemia in pregnancy. It is estimated that anaemia may be the underlying factor-responsible for as much as 20% of all maternal deaths in sub- Sahara Africa [23]. The most suitable mass intervention to prevent anaemia in pregnancy is the administration of haematinics to pregnant women aimed at raising the haemoglobin concentration. Furthermore, availability of highly subsidised contraceptive services may be responsible for the

decreased incidence of death from unsafe abortion due to a possible reduction in some unwanted pregnancies [24].

Worthy of note is the effort of various NGOs in improving maternal and newborn care during the study period. For instance, Tulsi-Chanrai foundation, an Indian NGO in partnership with the state ministry of health since 2009, has trained different categories of health workers in the state on the use of partograph, infection prevention, management of preeclampsia/ eclampsia and active management of the third stage of labour [25]. Protocols and guidelines for treatment of obstetric complications are now conspicuously displayed in the form of wall-chart in the maternity units of hospitals and health centres. Sustained training on Focused Antenatal Care has been conducted across the three major districts courtesy of Malaria Action Program for States (MAPS) in conjunction with USAID and Global Fund. Among other benefits, this emphasises on complication readiness, recognition of danger signs, birth preparedness and family-centred care during pregnancy [26]. Previous studies have shown that when maternal and newborn care services are offered free highly subsidised, there is a positive impact on health demography of the population [27].

In this study, septic abortion is now a leading cause of maternal death, replacing post-partum haemorrhage which was reported in the previous study in the same setting. In the data presented by Pathfinder International [19], 'stock out' of parenteral antibiotics was commonly reported in the monthly summary form compared to other essential drugs like misoprostol. Significant reduction in the occurrence of PPH as the cause of maternal death may be due to increased utilisation of misoprostol for management and prevention of PPH during the study period [28]. This is in keeping with similar reports in Bangladesh which reported significantly lower mortality ratios in higher compared with lower and no misoprostol coverage areas (38 vs 45 vs 51) maternal deaths per 100,000 live births, respectively [29]. These findings were reported despite similar and inadequate utilisation of skilled birth attendants in the study areas [29].

Malaria in pregnancy constituting 6.6% was the commonest indirect cause of death in this study. This proportion is slightly higher than what was reported in the previous study in the same setting by Agan et al. (6.6% vs 5.2%), suggesting the need for improvement in the implementation of malaria prevention measures [6]. Difficulty in the improvement of malaria in pregnancy prevention in the study setting could be due to the high prevalence of undetected asymptomatic malaria parasitaemia reported in previous studies, with potential progression to more severe and even fatal disease [30] [31]. This study is however limited by the inability to assess maternal deaths that may have occurred in transit to health facilities, as well as the effect of sociopolitical and other factors that may have contributed to the improvement in maternal mortality during the study period.

In conclusion, there is a significant decline in maternal mortality in the study centre in the last 5 years. This might be explained by the institution of the Woman trial intervention which reduced the occurrence and severity of PPH, as well as more proactivity of caregivers in their attendance to parturition. Given a high proportion of deliveries usually occurring in rural settings, there is need to consider shortly, institution of the Woman trial in rural settings. Such scale-up using the existing primary health care facilities may yield accelerated attainment of the SDGs, especially in resource-poor settings. Septic abortion and hypertensive diseases of pregnancy have replaced post-partum haemorrhage, which was previously noted as leading cause of maternal death. This indicates the need for implementation of community and facility-based strategies for infection control towards containment of septic abortion which is the new leading cause of maternal death.

Ethical approval and consent

Ethical approval and consent was obtained from the Research Ethical Committee of the UCTH and the Research Ethics Committee of the Cross River State Ministry of Health

Author's contribution

TUA initiated and conceived the idea and project and wrote the initial draft; EM was involved with literature search and synthesis, UBA was involved with data analysis and collation as well as literature search, OEO was the sole biostatistician in the project and JEE did literature synthesis and analysis as well. All authors were involved at different stages of the manuscript writing and review. All authors read and approved the final draft before submission to the journal.

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Effect of Exposure to Cement Dust among the Workers: An Evaluation of Health Related Complications

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Keywords: cement dust; respiratory complication; inflammation; allergy

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Abstract

BACKGROUND: Cement contains various types of chemicals in addition to lime and silica, and such chemicals cause different health complications and pathogenesis in addition to respiratory disorders. The most important occupational hazards for cement workers are allergy and complication related to respiratory system.

AIM: The current study was performed by analysing the questionnaire distributed among the workers and also by the sputum collected from them to study the general health conditions and other life activities.

METHODS: Sputum samples were assayed for cytological analysis by Hematoxylin and Eosin staining.

RESULTS: In this study, it was observed that majority of these workers suffered from different types of respiratory complications, such as a cough, asthma and lung infections. In addition to this, few subjects showed allergy and other complication like hypertension, diabetes and backache. Moreover, cytological analysis of the sputum was made, and it was observed that majority of the subjects showed severe inflammation.

CONCLUSION: Based on these finding, we concluded that long-term cement dust exposure and inhalation causes respiratory complications due to epithelial tissue damage and that can lead to secondary complications as well.

Introduction

Environmental and occupational pollution has always been a major cause of morbidity and mortality. The incidence of the occupational disease is constantly increasing throughout the world, especially in developing countries due to the lack of proper quality control documentation and the practical approach towards this mammoth problem.

The smoke and dust produced by some industries cause various types of pathogenesis. The unhygienic exposure of the smoke and dust are linked with an increased risk of chronic obstructive pulmonary diseases [1]. In this vista, cement workers may also be exposed to numerous types of occupational hazards, and these materials are the major culprits in mortality and morbidity.

Cement dust of portland contains various types of metal oxides including calcium oxide, silicon oxide, aluminium trioxide, ferric oxide, magnesium oxide, sand and other impurities [2]. Respiratory problems with high prevalence and varying degrees of airway obstruction about portland cement exposure have been reported by earlier investigators [3] [4] [5]. In a related study, it has been reported that the cement industry workers, who are directly exposed to the dust for longer durations, suffer from more shortness of breath, as compared to the workers who take precautionary measures [6] [7].

In this scenario, the study was performed, based on the distributed questionnaire and the collected sputum from the concerned cement dust

exposed workers to evaluate the chronic respiratory complications or changes in lung function. Results of the study concluded that most of the subjects suffer from inflammation of the respiratory epithelial cells and other respiratory related complications including an asthma and productive cough.

Material and Methods

The study was conducted on cement dust exposed workers, and fifty subjects were included in this study which was especially subjected to cement dust exposure. Only the workers with minimum of more than three years' exposure were included in this study. 20 samples from the normal healthy population, who did not have any smoking history and exposure of cement particle, were considered as control groups in the study. A questionnaire was used to inquire about the symptoms of respiratory such as a productive cough, asthma, lung infection, allergy and other health-related issues. A sputum sample was collected from each subject in 50 ml plastic jars with tight lids. The jars were transported to the laboratory in proper transportation boxes at earliest and stored at 4^{o}C till further investigations. The smears were prepared from each sputum sample on glass slides and were fixed with methanol. The slides were analysed after Hematoxylin and Eosin staining through light microscopy. Briefly, the slide with smear was immersed in the filtered Hematoxylin and was rinsed with the help of running tap water. The same slides were then immersed in eosin and again rinsed with running tap water. Running tap water removes the excess Hematoxylin or Eosin. Dehydration of section was made via ascending alcohol solutions. Mounting of the slide was done with DPX and slides were observed using a light microscope to evaluate the cellular alterations.

Results

From the distributed questionnaire and the collected sputum from the concerned workers, we obtained with following information detailed here.

A total of 50 Subjects were included in the study; the age distribution among the subjects showed that 20 cases (40 %) were in the age group between 20-30 years, 17 cases (34%) were in the age group 31-40 years, and 13 cases (26%) were in the age group between 41-50 years (Figure 1).

Nowadays smoking and tobacco chewing is very common among workers during working hours and is a major cause of health hazard. In this study, it was observed that cement workers are allowed to smoke and tobacco chewing during work.

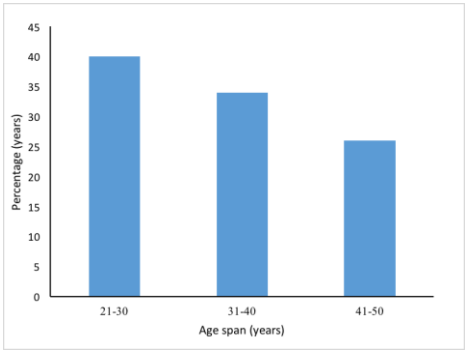

Figure 1: Analysis of the age distribution among the workers

It was found that more than 50% of workers had either smoking or tobacco chewing habits. Furthermore, it was observed that 23 subjects (46%) were smokers, 3 cases (6%) were chewing tobacco, and 2 subjects (4%) were having both smoking and chewing habits. In addition to this, 22 persons (44%) were not having either tobacco chewing or smoking habits (Figure 2).

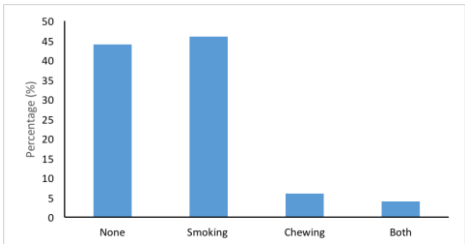

Figure 2: Distribution of subjects according to tobacco chewing and smoking habits

It was observed that most of the workers were in this sector were more than three years of work experience. It was obvious to study the general health conditions among these workers. It was studied that 5 persons (10% subjects) showed a productive cough, 7 workers (14%) were having the complication of asthma, 7 individuals (14%) were having lungs infection. Moreover, 1 person (2%) showed allergy and 11 (22%) were suffering from other complication including hypertension, diabetes and back pain (Figure 3). It was further analysed that the health conditions of these workers were not so severe before joining this cement industry.

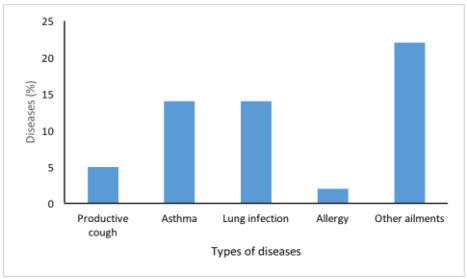

Figure 3: Study of general health conditions among the workers

All fifty sputum samples were collected in separate tight lid plastic jars with a detailed history of each subject to evaluate the alterations in cells. All the samples were performed for H&E staining. We observed 35 subjects (70%) showed severe inflammation and most of the cases were also having a long history of smoking and tobacco chewing (Figure 4).

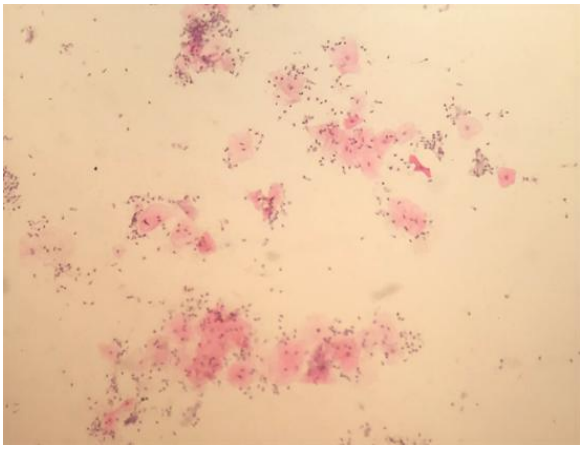

Figure 4: Severe inflammation was noted through Hematoxylin and Eosin staining (Original mag. X10)

We did not notice other types of alterations like metaplasia and dysplasia. In parallel, control samples were also run for H&E staining to compare the results with cement exposed workers. No severe inflammation was observed in the control groups (Figure 5).

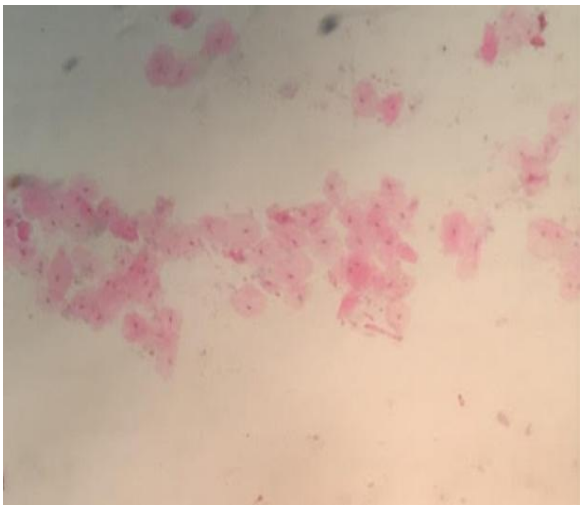

Figure 5: Less inflammatory cell was seen in control groups samples (Original mag. X10)

Discussion

Respiratory diseases associated with inhalation of airborne dust are the most vital group of occupational diseases [8]. Previous study subjects with chronic obstructive pulmonary disease advocate that workplace exposures are powerfully linked with an increased risk of chronic obstructive pulmonary disease [9]. Chronic respiratory diseases account for a public health challenge in both industrialised as well as developing countries because of their health and economic impacts [10]. Cement is one of the most important building materials in the world [11]. Moreover, cement dust constitutes numerous materials including calcium oxide, silicon oxide, aluminium trioxide, ferric oxide, magnesium oxide, sand and other impurities [2]. The cement dust or constituents of cement causes pathogenesis of various lung diseases including chronic bronchitis, asthma, lung cancer, pneumonia and tuberculosis. The study based on workers reported that condition of the oral mucosa in cement plant workers, where clinical examination established features of mechanical trauma and oral mucosal inflammation in all workers exposed to cement dust [12]. In a previous finding based on Iran of 200 workers, increased prevalence of respiratory symptoms and reduced lung function indices were noticed post-shift [13].

In the current study, we also noticed that various types of health complication as 5 (10%) a productive cough, 7 (14%) having the complication of asthma including having shortness of breath or feeling tight in the chest, 7 (14%) were having lungs infection. Moreover, 1 (2%) subjects showed skin allergy, and 11 (22%) were other complication including hypertension, diabetes and back pain. Another study finding concluded that acute respiratory health effects among the workers are most likely due to exposure to high concentrations of irritant cement dust [14]. A study based on Malaysian population reported the association of total dust exposure and respiratory symptoms such as a cough, phlegm, chest tightness and also with lung function indices [15].

The study was performed in a cement factory based in the United Arab Emirates to assess cement dust exposure and its relationship to respiratory symptoms among workers. Result confirmed that prevalence of respiratory symptoms was higher among the exposed workers, but the difference from that of unexposed workers was statistically significant only for a cough and phlegm [16]. In the current study, it was observed that 35 subjects (70%) showed severe inflammation and most of the cases were also having a long history of smoking and tobacco chewing. We did not notice other types of alterations like metaplasia and dysplasia. Another study was made to investigate the risk of the respiratory epithelium in regards to occupational exposure to cement dust. The result of the study reported that atypia (dysplasia), squamous metaplasia, acute inflammatory infiltrated cells and chronic inflammatory infiltrated cells were detected [17].

Our finding concluded that cement particle exposure or inhalation causes respiratory

complications and its accumulation in the lung causes epithelial damage and causes inflammation.

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The Correlation between Islamic Lifestyle and Pregnancy-Specific Stress: A Cross-Sectional, Correlational Study

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Keywords: Life Style; Pregnancy; Stress; Psychological; Religion; Islam

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Abstract

BACKGROUND: Pregnancy is associated with great psychological, emotional and physical stress. In addition to undergoing hormonal changes, pregnant women experience a change in their attitude toward life and learn to re-assess their skills and lifestyle. Lifestyle, in general, and Islamic lifestyle, in particular, is concerned with the different psychological, social and physical aspects of the individual's life.

AIM: This study was conducted to evaluate the correlation between Islamic lifestyle and pregnancy-specific stress in pregnant women.

MATERIAL AND METHODS: This cross-sectional, correlational study was conducted on 300 pregnant women presenting to prenatal care clinics. Data were collected using a demographic, the Islamic lifestyle and the pregnancy-related stress questionnaires.

RESULTS: The results showed a statistically significant negative correlation ($r = -0.284$) between Islamic lifestyle and pregnancy-specific stress ($P < 0.01$). The stepwise regression showed that the mean score obtained in the pregnancy-related stress questionnaire decreased by 0.75 per year of marriage and by 0.14 per point in the Islamic lifestyle questionnaire.

CONCLUSION: There was a significant negative correlation between Islamic lifestyle and pregnancy-specific stress. Training mothers, raising their awareness and encouraging them to adopt an Islamic lifestyle may play a significant role in controlling pregnancy-specific stress.

Introduction

Pregnancy is a happy and enjoyable time in women's lives in which the parents expect the arrival of a child and make plans to raise and love him. This sweet period and the natural process can be disturbed by internal and external stressors, and the routine of life can be disturbed by internal and external stressors [1]. Most pregnant women experience stress and concerns when faced with the physical symptoms of pregnancy, significant biological-biochemical changes specific to pregnancy, changes in family-personal relationships, socioeconomic problems, pregnancy-related medical-obstetric factors, infant health issues and the stages of childbirth [1] [2]. Different studies have been conducted in various societies on the causes of stress in pregnant women and its consequences. A study conducted to examine the prevalence of stress in early pregnancy in Sweden revealed that 16.5% of mothers experience stress in early pregnancy [3]. Another study in Ireland, after Europe's financial crisis, showed that 74% of pregnant women experience stress [4]. The results of a study in Iran showed moderate state anxiety in 63.3% and severe anxiety in 4.40% of the pregnant women [5]. Concerns and other stressful feelings during pregnancy can harm the mother and the fetus. Epidemiological evidence suggests that mothers' psychosocial stress is a risk factor of preterm birth, low birth weight, high-risk pregnancy, increased nausea and vomiting, intrauterine growth restriction and asphyxia, and consequently, increased cesarean sections [6] [7] [8]. Also, maternal stress is associated with high levels of stress hormones (epinephrine and adrenocorticotropic) in the fetal blood circulation. These hormones directly affect the nervous system and increase blood pressure, heart rate and degree of activity. The likelihood of hyperactive, irritable and low-weight children is higher in mothers with high

levels of anxiety compared to mothers with low anxiety levels [9]. From a public health point of view, it is very important to identify women who suffer from mental stress during their pregnancy, because psychological and social factors may also be responsible for some of the complications of pregnancy and midwifery in addition to biomedical factors [10]. Pregnancy is associated with great psychological, emotional and physical stress and many physiological and psychological changes [11]. These changes are severe at the onset of pregnancy because pregnant women tend to change their attitude toward life and re-assess their skills and lifestyle in addition to undergoing hormonal changes [12].

Lifestyle is a set of ideas, programs and typical examples of behaviours, aspirations and desires and a method for explaining social or personal conditions and determining a particular type of personal interaction [13]. The Islamic lifestyle is focused on the meaning and philosophy of life from an Islamic point of view [14]. The essence of the Islamic lifestyle is in performing a set of behaviours based on Islam and Islamic teachings [15]. Living based on Islamic teachings reduces poor attitudes that are themselves the cause of depression and anxiety. The reason for this reduction is that people who adopt such lifestyle keep themselves away from negative and poor attitudes that are a major cause of many mental illnesses with faith in divine power and by adherence to Islamic precepts and rules [16]. Feeling of belonging to superior power and the faith in God's hand in stressful life conditions and spiritual support help religious suffer less from their life problems and enjoy better mental health [17].

Given the considerable body of evidence suggesting that religious activities and beliefs are central to the personal efforts made for coping with stressful events [18] [19] [20] [21] [22], and since lifestyle, in general, and Islamic lifestyle, in particular, are concerned with the different psychological, social and physical aspects of the individual's life [16], and since research into pregnancy-specific stresses and concerns is still scarce [23], and also considering the known complications of pregnancy-specific stress and the lack of studies on the link between pregnancy-specific stress and an Islamic lifestyle, the present study seeks to determine the correlation between Islamic lifestyle and pregnancy-specific stress.

Methods

This cross-sectional, correlational study was conducted in 2017 on 300 pregnant Iranian women presenting to select hospitals for prenatal care services. The sample size was determined using the following equation, where r is the correlation between Islamic lifestyle and pregnancy-specific stress and was set as 0.20 due to the novelty of the subject matter and the unavailability of similar studies, α is type-I error and equals 0.05, β is type-II error and equals 0.10, and the test power is 90%.

$$n \geq \left[\frac{\left(z_{1-\alpha/2} + z_{1-\beta}\right)}{0.5 \times \ln\left[\left(1+r\right)/\left(1-r\right)\right]} \right]^2 + 3$$

The inclusion criteria consisted of age 15-45, reading and writing literacy, Iranian nationality, wanted pregnancy, no high-risk pregnancy based on obstetric definitions, living with the husband, no known physical and psychological diseases and being a Shiite Muslim. Data were collected using a demographic form, the Asset Index Scale, the Islamic Lifestyle Questionnaire and the Pregnancy-Related Anxiety Questionnaire. The demographic questionnaire inquired about the subjects' personal and obstetric data, including age, number of children, education, and duration of marriage and place of residence. The asset index scale assesses ten economic variables (owning a vacuum cleaner, a separate kitchen, a computer, a washing machine, a bathroom, a freezer, a dishwasher, a car [not for work or to make money], a cell phone and colour TV) and is calculated in percentage. The obtained variable (asset index) is classified into five categories, including 0-20 (poorest), 21-40 (poor), 41-60 (medium), 61-80 (wealthy) and 81-100 (wealthiest) [24] [25]. The Islamic Lifestyle Questionnaire consists of 75 items answered by the participants concerning their current living status and based on a 4-point scale from "very little" to "very much". Each item is given a point from 1-4 based on its significance. The total score ranges from 141 to 570. This questionnaire consists of ten subscales, including social (11 items), beliefs (6 items), religious and worshipping (6 items), ethical (11 items), financial (12 items), familial (8 items), health (7 items), science and contemplation (5 items), security-defense (4 items) and timeliness (5 items). The reliability coefficient of the entire scale was 0.71. The results of the factor analysis also showed a favourable validity and factor structure for the scale. The concurrent validity of the scale with the Religious Orientation Test was obtained as 0.64 [26].

The pregnancy-related anxiety questionnaire combines the 10-item questionnaire developed by Huizink et al. in 2004 (the short form of van den Bergh's 55-item scale) and some personal-familial factors also addressed in van den Bergh's original tool. This questionnaire consists of 25 items within six subscales, including maternal health (6 items), infant health (5 items), childbirth and the experience of motherhood (4 items), maternal-fetal attachment (2 items), personal-familial (5 items) and personal-occupational (3 items). It is scored based on a 5-point Likert scale (Never = 0, Seldom = 1, Sometimes = 2, Often = 3, and Always = 4) from zero to 100. Navidpour et al. reported a favourable validity and

reliability for this scale. The reliability coefficient and content validity of the entire scale were reported as 0.89 and 0.94, respectively [27].

Once the title of the study was approved, arrangements were made with the authorities of Shahid Beheshti Nursing and Midwifery School and Shahid Beheshti University of Medical Sciences and reference letters were obtained. After receiving permission from the presidents of Tehran and Iran universities of medical sciences, the researcher visited the prenatal care clinics of select hospitals with prior arrangements, introduced herself, presented the reference letters to the authorities and obtained their permission, and then visited the study settings a few days during the week and selected eligible candidates. After submitting their informed written consent, the participants were briefed on the study objectives, ensured the confidentiality of their information and the voluntary nature of participation. They were then given the data collection instruments to fill out. The researcher instructed the participants on how to answer the questionnaires. Data collected through the questionnaires were then analysed.

Results

The mean age of the 300 women under study was 28.68 ± 5.21, and the mean age of their husbands was 33.07 ± 5.72 years. Most of the participants had a high school diploma (44%) and were a housewife (92%). The husbands were mostly self-employed (57.7%). A total of 28% of the participants were in their first trimester, 33.7% in their second trimester and 38.3% in their third trimester. The results showed that 42% of the women had become pregnant for the first time, 46% had no other children, 80.7% had no history of abortion, and 97.7% had no history of stillbirth. Table 1 shows the maximum and minimum scores obtained in the measurement instruments.

Table 1: The maximum and minimum scores obtained in the measurement instruments

Variable	Mean	SD	Minimum	Maximum
Age	28.68	5.21	16	45
Husband's Age	33.07	5.72	21	51
Asset Index	69.70	18.42	0	100
Total Stress	31.72	19.81	0	100
Total Lifestyle	440.30	37.57	316	542
Total Depression	16.31	11.16	0	55

According to Pearson's correlation coefficient, there was a significant negative correlation between the participant's age and pregnancy-specific stress. There was also a significant negative correlation between the husband's age and pregnancy-specific stress. A significant positive correlation was observed between the husband's age and the total asset index. The Islamic lifestyle did not correlate with the participant's and the husband's age. The Islamic lifestyle and pregnancy-specific stress also did not correlate with the asset index. Meanwhile, there was a significant negative correlation between Islamic lifestyle and pregnancy-specific stress (Table 2).

Table 2: The correlation between the demographic variables, pregnancy-specific stress and Islamic lifestyle

	Age	Husband's Age	Total Lifestyle	Total Pregnancy-Specific Stress	Total Asset Index
Age	1	0.694**	0.053	-0.127*	0.098
Husband's Age		1	0.051	-0.115*	0.115*
Total Lifestyle			1	-0.284**	0.074
Total Pregnancy-Specific Stress				1	-0.074
Total Asset Index					1

** The correlation is significant at the 0.01 level (2-tailed); * The correlation is significant at the 0.05 level (2-tailed).

The stepwise regression was used to examine the effect of the Islamic lifestyle score and the demographic variables on the score of pregnancy-specific stress; Table 3 presents the results.

Table 3: The regression analysis of pregnancy-specific stress in the participating pregnant women by demographic variable and Islamic lifestyle*

Model		Unstandardized Coefficients		Standardized Coefficients	t	Significance Level
		B	Std. Error	Beta		
1	Constant	97.692	12.944		7.547	0.000
	Islamic Lifestyle	-0.150	0.029	-0.284	-5.115	0.000
2	Constant	99.691	12.756		7.815	0.000
	Islamic Lifestyle	-0.143	0.029	-0.271	-4.951	0.000
	Duration of Marriage	-0.749	0.230	-0.178	-3.253	0.001

* Dependent variable: Pregnancy-specific stress.

Based on the results presented in Table 3, only the duration of marriage had a significant effect on pregnancy-specific stress and the other demographic variables were eliminated from the model. The results also revealed a significant correlation between Islamic lifestyle and the duration of the marriage, such that the mean score of pregnancy-specific stress decreased by 0.75 per year of marriage and by 0.14 per point in the Islamic lifestyle questionnaire.

Discussion

This study was conducted to evaluate the correlation between Islamic lifestyle and pregnancy-specific stress in pregnant women presenting to the prenatal care clinics of hospitals affiliated to universities of medical sciences in Tehran in 2017. The results showed a statistically significant negative correlation between Islamic lifestyle and pregnancy-specific stress; pregnancy-specific stress decreased as the score obtained in the Islamic lifestyle questionnaire increased. Pregnancy is considered an important stage in a woman's life, and although a natural condition, it makes women vulnerable because

they are exposed to inconsistent physical, psychological and social conditions that predispose them to psychological disorders [28]. This result is in line with the results of studies that showed that an Islamic lifestyle promotes social health, mental health, happiness and satisfaction [29] [30].

This hypothesis can be explained by arguing that religious people are healthier than others because they adopt healthy behaviours, and mental health is associated with inherent religiosity [31]. Psychological theories and research have emphasised the role of religious beliefs in preventing psychological damage. For instance, Spilka et al. studied the positive effect of religion on mental health and morality using psychological studies. Beliefs and behaviours such as the trust in God, patience, praying, pilgrimage, etc., can make people inwardly peaceful by fostering hope and positive attitudes. Having meaning and purpose in life, the feeling of belonging to supreme power, the faith in God's hand in times of difficulty and enjoying social and spiritual support are some of the means by which religious people can potentially suffer less in the face of adverse life events and pressures [32]. Living by Islamic teachings reduces inefficient attitudes that are the cause of depression and anxiety because such people have faith in the divine power and obey Islamic precepts and rules and thus keep themselves away from negative and poor attitudes that are a major cause of psychological disease [16]. The results also showed that of all the demographic variables assessed, only the duration of marriage affected pregnancy-specific stress; that is, pregnancy-specific stress decreased with an increase in the duration of the marriage.

In the Iranian society, religion is one of the most effective sources of psychological support that can give meaning to life as long as one lives and can thus save lives from being meaningless. Religion can be a particularly important source of help in difficult and critical conditions [33].

The results showed that when participants' life was more in tune with the Islamic lifestyle, they experienced less pregnancy-specific stress, because the faith in a God who controls the situation and overlooks the people reduces situation-specific anxiety to a significant degree, such that most religious people define their relationship with God as the relationship with a very close friend and believe that they can somehow control the impact of uncontrollable situations by relying on God [34]. A limitation of the present study is that there were no similar studies on Islamic lifestyle and there were no relevant references for comparison. Given the novelty of the research subject, more studies are recommended to be conducted on this issue.

In conclusion, a negative correlation was observed between Islamic lifestyle and pregnancy-specific stress, as pregnancy-specific stress decreased with an increase in the score obtained in the Islamic lifestyle questionnaire. By encouraging the pregnant women to adopt an Islamic lifestyle, pregnancy-specific stress and its complications can be controlled in pregnant women. Further studies are recommended on this subject.

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Are Resilient Factors Increasing the Risk for Childhood Psychological Victimization?

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Abstract

BACKGROUND: Understanding the resilient factors and why some children do well despite early adverse experiences is crucial, because it can inform more effective policies and programs that help more children reach their full potential.

AIM: The main objective of the study is to describe the associations between psychological abuse in childhood and resilient risk factors on individual, relational, contextual level among adolescents in the country and see the probability of resiliency to predict psychological victimisation.

MATERIAL AND METHOD: Cross-sectional study on two-stage quota sample of 622 university students was applied in the study, including adolescents at first and second year at the main public Ss Cyril and Methodius University of Skopje, from the 12 faculties in the country. *Adverse Childhood Experiences Study International Questionnaires* was used for collecting information on psychological abuse, while the individual, relational and contextual resilient factors were measured using the *Child and Youth Resilience Measure - Youth version.* The study was conducted from March to September 2017. Statistical significance was set up at $P < 0.05$.

RESULTS: The results from the study have shown statistically significant negative correlation between exposure to psychological abuse in childhood and individual (rpb = -0.159), relational (rpb = -0.263), contextual factors (rpb = -0.147), and resilience in total (rpb = -0.232). The regressive model presents that 5.2% of the variance of the variable experienced psychological abuse is explained with resilience ($F(1, 527) = 28.909$; $P < 0.001$), showing that resilience is negatively significant predictor for being psychologically abused in childhood ($\beta = -0.228$; $t = -5.377$; $P < 0.001$). The regressive model explains the individual contribution of the predictor variables for the psychological abuse, presenting that only caregiver resiliency is a significant predictor for psychological abuse ($\beta = -0.282$; $t = -4.986$; $P < 0.001$).

CONCLUSION: Supporting children through prevention means foster competence and prevent problems. Preventive programmes represent developing protective factors in childhood, increasing competence and skills for the growth of resilience and decreasing the likelihood of developing psychopathology in adolescence and adulthood. It is of common interest of society for implementation of evidence-based interventions with fostering settings and in the long run enabling positive childhood basis for future generations.

Introduction

Epidemiological evidence links adverse experiences of abuse and neglect in childhood with resilience. Understanding the resilient factors [1] [2] [3] and why some children do well despite adverse early experiences is crucial, because it can inform more effective policies and programs that help more children reach their full potential.

The most influential study of Garmezy, 1970 conducted on children living with a schizophrenic parent, established a foundation for the study of resilience [4]. For the first time, he introduced the term "protective factors" that help individuals to overcome the negative effect of adverse experiences, resulting in positive development [2]. According to the findings of his research, although children living with a schizophrenic parent increases the risk of developing illnesses, an incredible 90% of children in this study do not develop the disease [4]. Researchers began to present findings that speak of positive results that can be achieved despite the negative life events and

experiences of children to plan interventions to promote mental health among children at risk. Evidence-based interventions were evaluated that promote mental health among children at risk. Also, the resilience may be different in different life phases [5] [6]. Resource studies focused on factors or features that help individuals successfully cope with the negative experiences [5]. As research in the field of resilience has progressed, it has become increasingly evident that in addition to research aimed at understanding the characteristics of resilience, attention should also be focused on the study of interventions and prevention programs [7] [8].

Early efforts were primarily focused on personal qualities of "resilient children," such as autonomy or high self-esteem [9]. How work in the area evolved, however, researchers increasingly acknowledged that resilience might often derive from factors external to the child. Subsequent research led to the delineation of three sets of factors implicated in the development of resilience: (1) attributes of the children themselves, (2) aspects of their families, and (3) characteristics of their wider social environments [9] [10] [11].

The development of children around the world is threatened by disasters, violence, pandemics, and other adversities that can have life-altering consequences for individuals, families, and the future of all societies [12]. These adversities have raised global concerns about dangers posed to children as well as the future of societies and renewed attention to resilience across many fields of research.

During the last few decades, there has been a growing interest in the concept of resilience [13]. The starting point in understanding the term resilience refers to the phenomenon of responding to stress or adversity. The concept of resilience has been defined in many various ways depending on from the components of the construct of resiliency, its dimensions, underlying processes, conceptual models, identifying factors contributing to resiliency, and empirical findings. Resiliency, or resilience, is commonly explained and studied in the context of a two-dimensional construct concerning 1) the exposure of adversity and 2) the positive adjustment outcomes of that adversity [14]. Rutter has defined resilience including... "protective factors which modify, ameliorate or alter a person's response to some environmental hazard that predisposes to a maladaptive outcome" [13]. Masten [12] has broadly defined resilience …as the capacity of a dynamic system to adapt successfully to disturbances that threaten system function, viability, or development. Luthar and colleagues consider resilience as … ''a dynamic process encompassing positive adaptation within the context of significant adversity'' [14].

Lee and Cranford defined resilience…''as the capacity of individuals to cope successfully with significant change, adversity or risk'' [15], and also Leipold & Greve sees resilience as capacity to resist and recover despite adversity ...''as an individual's stability or quick recovery (or even growth) under significant adverse conditions'' [16].

Subsequently, ecological systems theory, articulated by Bronfenbrenner [17], and Garmezy (5), functioned as a way to examine the interplay between individuals and their environments and the resulting impact upon the individual's development.

Regarding the term adversity, Luthar et al., (2000) stated that adversity ''typically encompasses negative life circumstances that are known to be statistically associated with adjustment difficulties'' [14].

Analyzing resilience begins with an assessment of exposure to adversity and the impact risk factors have on children's experience of wellbeing. Large cohort studies have shown that adverse childhood events such as neglect and exposure to family violence exert long-term deleterious effects on mental and physical health [18]. These processes of successful adaptation in situations where there is abnormally high environmental load (the quality and quantity of the adversity that is experienced) have been attributed to a range of biological, psychological, relational, and sociocultural factors, some more likely to respond to clinical interventions than others [19]. Given the multidimensionality of the processes associated with resilience, the likelihood of individual children withstanding the impact of cumulative stressors is not a measure of their invulnerability [20]. Instead, resilience is predicted by both the capacity of individuals, and the capacity of their social and physical ecologies to facilitate their coping in culturally meaningful ways [21].

Based on the work of the Resilience Research Centre, Michael Ungar (2005) makes comprehensive explorations of resilience based on mixed methods (quantitative and qualitative) study including over 1500 youth across various cultures [21] [22]. They explained that resilience is "a multidimensional construct, the definition of which is negotiated between individuals and their communities, with tendencies to display both homogeneity and homogeneity across culturally diverse research settings" [21]. In the later work he [23] [24]. Understand resilience as a social-ecological construct. According to him [24] ..."resilience is defined as: 1) the capacity of individuals to navigate their ways to resources that sustain well-being; 2) the capacity of individuals' physical and social ecologies to provide those resources; and 3) the capacity of individuals and their families and communities to negotiate culturally meaningful ways to share resources."

The paper intends to explore the relationship between the experience of psychological abuse in childhood and resilience risk factors on individual, relational, contextual level in adolescents.

Additionally, we want to explore the likelihood of psychological victimisation related to the resilience.

Material and Methods

Study design and sample

The cross-sectional study on two-stage quota sample comprised of 622 first and second-year university students aged 17-19 years, 417 (67%) of whom were male and 215 (33%) female. According to the ethnicity, 80% of students are Macedonians, 10.1% Albanians, 1.2 Serbians, 0.3% are Roma and under others are 8,2% students. Predominantly 83% of students are from urban areas, while only 17% are coming from the rural environment. Predominantly (83%) of study participants were from urban areas as presented in Table 1.

Table 1: Descriptive statistics: gender, ethicity, urban/rural

Variables	n (%)
Gender	
Male	206 (33.12)
Female	416 (66.88)
Ethnicity	
Macedonian	498 (80.06)
Albanian	63 (10.13)
Roma	2 (0.32)
Other	56 (9)
Refuse to answer	3 (0.48)
Place of living	
Urban	516 (82.96)
Rural	106 (17.04)

The sampling procedure applied two stages. At the first stage of sampling procedure, selection has been made randomly by electing every second faculty from the website list of the 23 faculties from the biggest public university "Ss Cyril and Methodius University of Skopje" [25]. The following faculties have been included in the sample: Faculty of Architecture, Faculty of Economics, Faculty of Medicine, Faculty of Law; Faculty of Dentistry; Faculty of Veterinary Medicine, Faculty of Drama Arts, Faculty of Agricultural Sciences and Food, Faculty of Fine Arts; Faculty of Physical Education, Sport and Health; Faculty of Philosophy; and Faculty of Forestry.

In the second stage of sampling procedure, participants were selected so that the same percentage was included in the sample as per the percentage of the students enrolled in the first year in the academic 2015/2016 according to the State Statistical Office data [26].

Procedure

The participants were recruited from the largest public university in Republic of Macedonia "Ss Cyril and Methodius University of Skopje". At the beginning of the study, the selected 12th Faculties have been contacted, and accordingly, an agreement has been done for conducting the study for the first-year university students. At the beginning of the study, the researcher explained issues of confidentiality, informed consent and the right to withdraw at any time or not answer certain questions. Participants were informed in written form of the general aims of the study and were asked to read and sign the consent form before participating.

After reading the information sheet and signing the consent form, participants completed questionnaire to assess abuse and resilience, including demographic characteristics. The questionnaire was administrated in the same order for all participants. Completion time required for the questionnaire was approximately 45 minutes. The researcher indicated that participation in the study was voluntary and that participants had the right to withdraw from the study at any time. The study was conducted from February to September 2017.

Measurement

Adverse Childhood Experiences Study International Questionnaires [27] was used for collecting information on psychological abuse in childhood. The questions on psychological victimisation in childhood were with multiple choice (many times-code 5; a few times-code 4; sometimes-code 3; rare-code 2; and never code 1. The questions begin with the phrase: When you were growing up, during the first 18 years of your life . . . Did a parent, guardian or other household member yell, scream or swear at you, insult or humiliate you? ..or …Did a parent, guardian or other household member threaten to, or actually, abandon you or throw you out of the house?

Resilience (individual, relational and contextual resilient factors) was measured by using the *Child and Youth Resilience Measure 28 - Youth version* (CYRM-28), [24]. CYRM-28 has three sub-scales: individual capacities/resources, relationships with primary caregivers and contextual factors that facilitate a sense of belonging. Study participants were asked to state to what extent the sentences describe them asking to circle one answer for each statement on a five-point response scale (Not at All, A Little, Somewhat, Quite a Bit, A Lot) for some of the following statements: I have people I look up to; I know how to behave in different social situations; My parent(s)/caregiver(s) know a lot about me; am treated fairly in my community …and other similar questions [24].

Permission for usage of the instruments has been obtained by the authors/institutions prior to the study. The study has been approved by the Scientific Advisory Board of the Medical Faculty at the "University St Cyril and Methodius, Skopje" in February 2017.

Statistical analysis

The Statistical Package for Social Sciences (SPSS v17) was employed, and a significance level of 0.5 was adopted. Before commencing statistical tests, data were screened for the accuracy of entered responses, missing data, and violations and assumptions. Statistical analysis included descriptive statistics (Mean and Standard Deviation), correlations and linear logistic regression.

Results

Descriptive statistics

The data regarding the descriptive statistics such as median (M), standard deviation (SD) and obtained a range of the scores for the variables: psychological abuse, individual, caregiver and contextual resilience, and resilience total in adolescents are presented in Table 2. The mean for psychological abuse is 3.68, and standard deviation of 2.01, scores ranging from 2-10. The mean for resilience is 114.4, while the standard deviation is 15.7, with scores ranging from 29-140. The descriptive statistics for the other types of resilience are presented in Table.

Table 2: Descriptive statistics for variables: psychological abuse, resiliency total, individual, caregiver and contextual resilience

	M	SD	Obtained range of scores
Psychological abuse	3.68	2.01	2-10
Resilience total	114.4	15.7	29-140
Individual resilience	46.6	6.5	11-55
Relationship with Primary Caregiver	29.5	5.0	6-45
Contextual resilience	38.3	6.6	11-50

N = 622

Correlation and linear regression analysis

The findings showed statistically significant negative correlation between the individual resilient factors (r = -0.159), relational (r = -0.263), and contextual resilient factors (r = -0.147) as well as resilience in total (r = -0.232) with exposure to psychological abuse in childhood as presented in Table 3.

Table 3: Correlation coefficients (r) between psychological abuse and individual, caregiver, contextual resilience and resilience in total

	Individual resilience (r)	Relationship with Primary Caregiver (r)	Contextual resilience (r)	Resilience total (r)
Psychological abuse	-0.159**	-0.263**	-0.147**	-0.232**

N = 622; *P < 0.05; **P < 0.01.

The results have shown that adolescents with higher level experiences of psychological abuse have lower levels of individual, relational, contextual and resiliency in total as presented in Table 3. The findings showed a statistically significant negative correlation between the resilient (individual,) factors and exposure to psychological abuse in childhood that is in line with the actual literature evidence. The higher level experiences of adverse experience such as psychological abuse were associated with lower levels in resilient (individual, relational, contextual) factors.

In Table 4, the data were presented from the linear regressive analysis predicting psychological abuse by resilience, were total score of resilience has been calculated.

Table 4: Simple linear regression analysis results: predicting psychological abuse by the resiliency

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	3.644	0.333		10.934	0.000	2.989	4.298
	Resilience	-0.429	0.080	-0.228	-5.377	0.000	-0.585	-0.272

r = 0.228; R^2 = 0.052.

The regressive model revealed that 5.2% of the variance of the criteria variable psychological abuse is explained by resilience (F (1.527) = 28.909; P < 0.001). The findings show that resilience is an important predictor for experiencing psychological victimization in childhood (β = -0.228; t = -5.377; P < 0.001). Lower resiliency in adolescents increase the odds for the experience of psychological abuse. The linear regression analysis also confirmed that lower resilience factor is a statistically significant predictor for experiencing psychological victimisation.

Table 5: a Hierarchical linear regression analysis: predicting psychological violence by individual, caregiver and contextual resiliency

Model		Unstandardized Coefficients		Standardized Coefficients	T	Sig.	95% CI for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	3.050	0.314		9.711	0.000	2.433	3.667
	Individual resiliency	-0.275	0.072	-0.163	-3.804	0.000	-0.417	-0.133
2	(Constant)	3.661	0.326		11.219	0.000	3.020	4.302
	Individual resiliency	-0.003	0.087	-0.002	-0.037	0.970	-0.173	0.167
	Caregiver resiliency	-0.418	0.077	-0.278	-5.395	0.000	-0.570	-0.266
3	(Constant)	3.648	0.333		10.938	0.000	2.992	4.303
	Individual resiliency	-0.008	0.090	-0.005	-0.091	0.928	-0.186	0.169
	Caregiver resiliency	-0.425	0.085	-0.282	-4.986	0.000	-0.592	-0.257
	Contextual resiliency	0.016	0.084	0.011	0.194	0.846	-0.149	0.182

r = 0.279; R^2 = 0.078.

The hierarchical linear regression analysis suggests that three types of individual, caregiver and contextual resiliency explain 7.8 of the variance for the psychological abuse. Significant 2.7% is explained by individual resiliency (F (1.527) = 14.471; P < 0.001). Major significant part of 5.1% is explained by caregiver resiliency (F (1.526) = 29.101; P < 0.01), while contextual resiliency does not have significant contribution.

The third regressive model explains the individual contribution of the predictor variables for the psychological abuse, presenting that only caregiver resiliency is a significant predictor for psychological abuse (β = -0.282; t = -4.986; P < 0.001). The caregiver resiliency significantly contributes as a predictor for decreasing the risk for psychological victimisation.

Discussion

At the beginning of the investigation of the construct of resilience in the 1970s, researchers investigating children at risk for psychopathology noted that some children had good outcomes despite being exposed to risk [3] [28]. This discovery stimulated a search for specific differences in children who thrive in the face of adversity and generated a field of research on resilience [29]. The concept of resilience was significant as it signified a change in focus from mental illness to mental health [30]. This changing focus created an increase of research on protective factors that promote mental health and positive development in the face of risk and adversity [28].

The results of the current research showed that higher levels of psychological abuse experiences in childhood are associated with lower levels of resilient (individual, relational and contextual) factors, which is in line with other studies [13] [30]. As other study suggested, not all maltreated children develop maladaptively. Many abused and neglected children show positive, resilient functioning despite the pernicious experiences they have encountered and the ignominious treatment they have received [28] [31]. Adolescents who have higher levels of experience of psychological abuse in childhood, a lower level of resistance appears, not only for the overall resilience but also for individual, relational and contextual factors of resilience in the current study. Similar data were also obtained when studying the resilience in psychosocial studies, in which it was noted that children could adapt and deal despite the negative experiences that they had [5] [32].

The findings show that resilience is an important predictor of the being psychologically victimised among adolescents involved in the study. Adolescents with higher resilience are less likely to experience psychological abuse than those with lower levels of resilience. Longitudinal studies have produced similar empirical evidence of understanding of resilience [11] [33]. An influential study conducted by Hawaii-born children provided key information that resilience is a result of the impact of multiple risks [11] [33], giving prospective ecological studying the factors as suggested by Ungar [24]. According to this study, about one-third of children were resilient despite the risks they experienced. These children continued to be resilient as adults [11]. The growth and development of children in environments dominated by protective factors are of paramount importance in reducing the likelihoods of experiencing psychological victimisation [22] [35].

There are available interventions based on evidence of reducing child abuse and neglect with an emphasis on protective factors that encourage the growth and development of children [27]. These protective factors can have a positive long-term impact on the development of the overall potential of future generations [36] [37]. Research demonstrates that resilient are important factors that can either increase or reduce the risk of victimisation, although the conclusions were drawn from relatively privileged population group of adolescents enrolled at the university.

The study findings highlight the need for investing in protective (individual, relational, contextual) factors in childhood as critical features in the development of resilience in young adolescents [36] [37] as well as increasing the self-esteem, competence and decreasing the likelihood of developing psychopathology. More evidence is needed analysing influence on various factors of adversity such as physical, sexual abuse, community violence, and family dysfunction to come up with more general conclusions and recommendations. To advance the understanding of resilience, it is essential that more longitudinal research is conducted that investigates the pathways to the resilient functioning and that simultaneously examines biological and psychological systems. Still, evidence-based interventions to reduce adverse childhood experiences are available with design and implementation of resilience-promoting interventions and in the long run setting up positive childhood surroundings for future generations.

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Review Article

How to Improve Clinical Outcome of Epileptic Seizure Control Based on Medication Adherence? A Literature Review

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Abstract

Anti-Epileptic Drugs (AEDs) are the main therapy for epilepsy to prevent seizures. Non-adherence situation plays an important factor in the failure of seizure control. Such a condition may generate several impacts on clinical, social, and economic aspect. Several methods are used to measure adherence in epilepsy patients, including direct and indirect measurement. The direct measure involves measurement of drug levels in hair or body fluids such as blood and saliva. Whereas, indirect measure involves the non-biological tools, for example, a self-report measure, pill counts, appointment attendance, medication refills, and seizure frequency. Numerous factors may affect adherence in epilepsy patients, such as age, sex, and seizure aetiology, seizure sites, which are categorised as irreversible factors and hardly to be improved. However, there are factors that can be influenced to improve adherence such as patient knowledge, medication, cultural, health care professionals, and national health policies, which are related to treatment and education factor which is associated with behaviour to be likely adherence.

Introduction

Epilepsy is an unprovoked seizure condition, while epileptic status is a seizure activity that does not improve spontaneously or seizures repeatedly without any improvement between seizures [1] [2]. Based on the duration, seizure, when the duration is less than 5 minutes, if the duration between 5 to 30 minutes is categorised as prolonged seizure, while status epilepticus is defined as seizure, occurs with duration greater than 30 minutes, or repeated seizure activity (2 x or more) with or without any improvement in awareness among repeated seizures [3]. Epilepsy is a chronic brain disease characterised by recurrent seizures (2 or more times), involving partial body, involuntary movements, and often accompanied by loss of consciousness and control of gastrointestinal or urinary tract function. Approximately, 4 to 10 in 1000 people are predicted suffering from active epilepsy and persistent seizures. Additionally, a study of epilepsy in developing countries population indicated that the prevalence of epilepsy is higher, with a ratio of 7 to 14 in 1000 people [4].

Anti-epileptic drugs (AEDs) are the main therapy for epilepsy to prevent seizures thoroughly and an easy to follow a treatment schedule. Approximately 67% of patients with epilepsy could reduce or even combat their seizure frequency effectively using AEDs [5]. As a result, it is indicated that the substance could control epilepsy symptom, which is shown as seizure free at least for 18 months [6]. On the other hand, uncontrolled epilepsy is illustrated with considerably uncontrolled seizures with a high frequency of its symptoms. A study implied that this condition might cause disruption and even degradation of the quality of life of the patients [7].

Non-adherence is considered as one of the most important factors for controlling epileptic

seizures. It is acknowledged as a worldwide health problem and has impacts on clinical, social, and economic outcomes [8]. Non-adherence includes prescription or delay inappropriate failure dose, and missed dose of treatment [9]. Many retrospective studies have demonstrated the most crucial impact of non-adherence in AEDs. It was identified that non-adherence would increase costs due to hospitalisation as well as a decrease in productivity [10] [11] [12]. Therefore, identifying obstacles to AEDs adherence is important to allow practitioners are developing appropriate strategies to improve adherence [10]. Patients need support and specific information to achieve adherence.

This literature review is developed to address the importance of AEDs adherence factors for improving clinical outcomes for patients with epilepsy.

Discussion

Adherence is the extent to which a person's behaviour-taking medication, following a diet, and/or executing lifestyle changes, corresponds with agreed recommendations from a healthcare provider [13]. Adherence implies that health professionals have a responsibility to form a therapeutic relationship with patients, to encourage them to agree to a recommended treatment regimen. This means that patients should be better informed about their medicines, and in theory, have greater power to decline treatment [14].

Adherence in AEDs, defined as the extent to which patients follow the agreed instructions given by the prescribers and persistence in a regimen of treatment epilepsy such as the duration of initiation for discontinuation of therapy [15]. The World Health Organization (WHO) has concluded that increasing the effectiveness of adherence may have a greater impact on the health of the population than any improvement in specific medical treatments [16].

Drug adherence can be used to predict the remission condition in epilepsy. Non-adherence term may cause breakthrough attacks at several months or years after the previous episode of seizure [17]. It is indicated that patients who adhere a routine use AEDs, have 6, 12 and 24 months continuous remissions compared to those non-adherence patients. An adherence to epilepsy is a milestone of the success and the effectiveness of pharmacological therapies. It has impacts in helping to prevent seizure events and reducing the cumulative negative impact of seizures in daily life [18].

Non-adherence of AEDs in epilepsy patients may cause an increased risk of convulsive status epilepticus [19]. And the most serious consequence of non-adherence would be the increased risk of sudden unexplained death in epilepsy patients. Due to those consequences, identifying the barriers to adherence of AEDs is observed as imperative to allow practitioners are developing some appropriate strategies to improve adherence rates [20].

Table: 1 Method to Measure Adherence in Epilepsy Patients [21]

Measures adherence	Method	Disadvantages	Advantages
Direct Measures			
Plasma or serum Antiepileptic drug levels	Plasma or serum levels of medication are measured	Patient factors (albumin, hepatic & kidney condition) and drug types can Cause measurement variability	Commonly used, well understood by Clinicians, effective in extreme low adherence situations, to determine the range of therapy and the possibility of overdose-related side effects
Detection in human hair	Hair medication levels analysed using gas or liquid chromatography	Researchers disagree on the effectiveness	Less invasive than blood testing
Saliva antiepileptic drug concentration	Levels of aeds excreted in saliva are measured	Measurement must be calibrated to individual saliva production and serum/saliva medication ratios	Painless, does not require venous access (good for pediatric and geriatric patients); as accurate as blood plasma or serum monitoring
Indirect Measures			
Self-report measures	Patient reports medication adherence through methods such as survey or interviews	No standardised and validated Measures exist for epilepsy treatment adherence; can be inaccurate due to patient misperceptions or tendency to give a desirable response	Low cost; not physically invasive; adaptable to the target population
Pill counts	Doses monitored through counts of remaining pills or use of an event recorder	Does not guarantee that medication is taken outside of a controlled environment	Not physically invasive; event recorders measure regularity of dosing
Appointment attendance	The regularity of attendance at appointments is documented	Tends to be the limited term, while epilepsy is lifelong; not proof of proper medication use	Easy to collect information can be related to others adherence behaviours
Medication refills	Medical/pharmacy records reviewed to see if medication is refilled at appropriate intervals	Not proof of proper medication use	Can be correlated to blood serum levels
Seizure Frequency	The frequency of seizures is logged over time	Non-adherence patients may not have frequent seizures	An essential indication of the degree to which epilepsy is managed

From the literature, there are two types of poor adherence, which are primary non-adherence and secondary non-adherence. Primary non-adherence is implied in a condition where the patient does not have a prescription for medicine dispensed. Meanwhile, the secondary non-adherence is defined as the patient has medicine dispensed but does not take medicine according to the prescribed regimen [14]. Another study implied there are three types of non-adherence: (i) in medication; (ii) in dietary/exercise; and (iii) in an appointment. First, non-adherence in medication defined as a non-adherence which includes failure to have the prescription dispensed or renewed, the omission of doses, errors of dosage, incorrect administration, errors in the time or frequency of administration, and premature discontinuation of the drug regimen. Second, a non-adherence in dietary/exercise occurs if the patient fails to follow the diet and exercise recommendations. Last, a non-adherence in an appointment occurs if the patient fails to come at clinics for the scheduled check-up [15].

Two different methods to measure adherence are direct and indirect measurements. Direct measurement includes a measurement of drug levels in hair or body fluids such as blood or saliva. Indirect measurement involves non-biological tools, such as self-report measures, pill counts, appointment attendance, medication refills, and seizure frequency

(Table 1) [21].

A study on adherence of epilepsy patients with an indirect measure to treatment includes adherence to outpatient epilepsy treatment in Saudi Arabia. There were 38.3% of patients who did not adhere to the prescribed drug based on self-reported measurement of Morisky Medication Adherence Scale (MMAS) score [9]. Another study on adherence in some hospitals in Ethiopia for treating epilepsy indicated that prevalence of AEDs non-adherence is about 37.8% [22].

WHO has identified five dimensions of influencing factors on patient adherence, which are: (i) social/economic factors; (ii) health system/healthcare team factors; (iii) condition-related factors; (iv) therapy-related factors; and (v) patient-related factors [13]. A systematic review regarding adherence in epilepsy therapy implied that the factors that influence patient adherence are including factors in patients, confidence in treatment, depression and anxiety, treatment management, disease factors, factors related to medication, health care factors, and socio-economic factors (Table 2) [20].

Patient age is associated with different levels of adherence. For example, adolescents tend to deny that a medication regimen can impose on their lives. On the other hand, a patient with older ages might be not adhered to medication due to the complexity of medication regimens and presence of multiple comorbidities [14].

The seizures that characterise primary epilepsy have been considered to be signs of demonic possession. It also can be affected by depression and anxiety patients, thus influencing the adherence. A study from Shallcross et al., [23] about non-adherence to a regimen with AEDs reported that there is a significant correlation between being depressed and non-adherence with a score of -0.379 on Pearson correlation coefficient and rates of probability values being $P < 0.05$. Another study conducted in Southern Ethiopia indicated that the most common of non-adherence were forgetfulness (75.4%) and ran out of pills (10.8%). Furthermore, the study illustrated that duration of epilepsy treatment, marriage status, level of education, and the absence of comorbidity may affect adherence to medication.

Medication factor is another important factor that might affect adherence. For example, complex dose regimens are associated with poor adherence due to the difficulties of drug administration. Medication regimens can be complex because they contain multiple different medicines (polypharmacy), or because they need to be taken frequent times a day. A study indicated that adherence is much lower with regimens that require medication to be taken three or four times daily, by 65% and 51% respectively compared to once daily dose [24]. Moreover, two types of research about adherence in patients with epilepsy to take AEDs observed that forgetting the dose and administration time were given contributions for non-adherence [25] [26].

Another patient's related factor that influence of adherence is patient's belief in medication and health professionals. The types of patient's beliefs about medicines are perceived efficacy of medicine, the danger of becoming immune over time, unnaturalness of manufactured medicines, the danger of addiction and dependence, anti-drug attitude and balancing risks and benefits that all types of patient's belief can influence of adherence [14]. To overcome the problem, a good relationship and communication between patients and health professionals are likely in helping patients to adhere to their medication. Maintaining contact between patients and health professionals is required to achieve patient adherence [27]. Conversely, healthcare systems with poorly trained or overworked health professionals, short consultation times, inadequate patient education and follow-up, and lack of help for patients to pay for treatment can contribute to poor adherence [14]. Hovinga et al., [28] investigated that adherence percentage in patients who trust their doctor is higher by 34% compared to 17% in patients who have lower trust. This indicates that the impact of the patient and physician relationship can affect adherence. Furthermore, adherence participants reported being comfortable discussing missed medications with their doctor can be decreased non-adherence medication.

Other potential factors that may cause non-adherence in patients with epilepsy from an area with the low-quality health care system are level of healthcare development, cultural belief, economy, distance from health care facilities, supply antiepileptic drug, lack priority in national health policies, and belief in traditional medicine [29]. In some area, there were various misunderstandings and stigma about epilepsy that can affect adherence to epilepsy. A study in Vientiane, the capital city of Lao Peoples' Democratic Republic (Lao PDR), observed that epilepsy was acknowledged as a disease caused by supernatural powers or ancestral factors [30]. This negative stigma may diminish appropriate treatment for patients with epilepsy [31]. Moreover, the stigma may affect the level of knowledge about the proper treatment of patients, their family, and the society. The low level of knowledge about the treatment would trigger the trust in traditional medicine utilisation [29].

Table 2: Factors Affecting Medication Adherence in Epilepsy Patients

Aspect	Factors affecting medication adherence in epilepsy patients, ethnic origin (the cultural belief that epilepsy is a disease that has supernatural manifestations (associated with ancestors or evil spirits)
Patients factors	Age, sex, confidence in treatment, depression & anxiety
Disease factors	The onset of epileptic, aetiology of the seizures, the location of the epileptogenic zone
Medication factors	Treatment management (monotherapy or polytherapy), adverse effect of AED, beliefs in medication
Health care factors	Belief in health care providers (clinician, pharmacist)
Socioeconomic factors	Cost of treatment and income patients
Distance from healthcare facilities	The measure of distance from the health service can affect the level of adherence and the type of treatment the epilepsy patient receives
National health policies	The lack of prioritisation in national health policies can affect the treatment of epilepsy

Essential strategies to improve medication adherence with long-term therapy involve multiple interventions are: more convenient care, comprehensive information, a reminder to take medication, self-monitoring of treatment effectiveness, psychological therapy, and telephone follow-up [14]. Pharmacological intervention in people with epilepsy is only one aspect of their management in medication. Patients with epilepsy also require support from the society as having epilepsy has a widespread effect and impact psychologically on the social life [18].

Another approach to improve adherence is the involvement of other health workers such as pharmacist in the provision of education and changes in the behaviour patterns of epilepsy patients to treatment. A study in China described that providing educational interventions through counselling could alter the patient behaviour to be more concern about the administration schedules. This may provide a better effect on the level of medication adherence with a reduction in the percentage of the forgetful taking of drugs from more than 70% to 45% [32].

According to Cochrane database of systematic reviews, the strategies for improving adherence to AEDs consist of three interventions, which are educational, behavioural, and mixed interventions [33]. Additionally, several supporting approaches, such as reminder systems, cognitive educational, behavioural counselling, social support (family member support), and multifaceted interventions (reminder systems combined with cognitive, educational interventions) were observed as efficient methods to improve adherence [34] [35] [36].

Communication and information about pharmacological treatment and diseases are important. Providing comprehensive information as an education intervention must be focused not only on patients but also on others like family. To achieve better adherence, it is required to provide information to the patient about their disease, how to manage missing dose, the importance of adherence, in need of adopting patient-centered care, and sharing decision. Decreasing number of patients who missed the AEDs along with an increased percentage of patients taking the AEDs were the results of a positive effect of the educational intervention. Education has become the most common strategy used by many researchers to improve adherence [32].

Health education is a powerful tool, which can give a positive impact on both drug adherence and self-management ability in patients with epilepsy. From a study from Dash et al. [18], it is observed that the epilepsy health education group, can affect different MMAS score from 6.58 to 7.53 (P = 0.001), and indicated that health education is significant to improve adherence.

Another research about the impact of pharmacist educational interview on medication of Saudi Arabia showed a significantly different result in post-intervention for epilepsy patients with education intervention (P = 0.024) [37]. Medication education provided by the pharmacist helped to improve adherence, seizure control, and the knowledge of AEDs.

Provision of an educational intervention on epilepsy treatment, especially in a region that has a belief that epilepsy is a disease with ancestral cultural-influence should be conducted together with cultural approaches to the family and carried out continuously with long-term education [29]. Family support is a major strategy in the treatment of epilepsy, but it is challenging when families feel ashamed of having a family with epilepsy [38] [39]. Hence, communication and information about the disease and therapy for patients with epilepsy must be provided as an educational intervention to the family as well. In other words, an intervention with a single component or intervention (information or education) seems to be ineffective to improve adherence or other clinical outcomes, but there is some evidence that education intervention may improve knowledge.

Behavioural interventions are characterised by the cognitive behavioural techniques and therapies focused on dysfunctional emotions, behaviours and cognitions with the aim to promote healthy lifestyles and positive changes toward symptoms or treatment [40]. Additionally, behavioural interventions are aimed to change individual behaviour in those aspects related to their daily life, which can modify patient's behaviour toward treatment. Intensive treatment reminders provide favourable effects on adherence (moderate-quality evidence) and can be conducted by suggesting the patient consume the drug with the place and other routine activities, thus minimising the forgetting to take the drug. This tool for improving adherence in patients who are forgetting to take their medications, much less research has shown that forgetfulness is found to be the main reason for non-adherence in patients with epilepsy [41].

Mixed intervention is a combination of one or more interventions, both educational and behavioural, which can be performed by health professionals. The mixed intervention was intended to help patients incorporate drug administration into their daily lives. The European Declaration on Epilepsy recommends interdisciplinary action to help people with epilepsy understand their condition and do the search for a proper treatment possible in order to improve their quality of life such as pharmaceutical care and give significantly improves quality of life (QOLIE-31 score for the intervention group was 12.45 points (P-value < 0.001) compared to the control group it was 2.61 (P-value = 0.072) [17]. All interventions to improve the quality of treatment of epilepsy patients should be based on local perceptions, social conditions and cultural backgrounds as well as the spirits possession of epilepsy patients [31]. And understanding why patients do not take their medicines can help to avoid some of this frustration and, in some cases, enable

health professionals to improve patient adherence.

In conclusion, adherence to AEDs for patients with epilepsy is an important factor and affects the outcome of seizure control. Several irreversible factors may affect treatment adherence in epilepsy patients, including such as age, sex, seizure aetiology, seizure sites. However, there are reversible factors that can be altered by conducting the approaches to improve patient adherence including patient knowledge and treatment regimens, cultural factor, health care factors, and national health policies. Various interventions to improve clinical outcome of epileptic seizure control are behaviour interventions, educational or combination of some of this intervention.

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