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Basic Science

Induction of Immune Responses by DNA Vaccines Formulated with Dendrimer and Poly (Methyl Methacrylate) (PMMA) Nano-Adjuvants in BALB/c Mice Infected with *Leishmania major*

Fatemeh Tabatabaie[1], Nasim Samarghandi[2], Somayeh Zarrati[3], Fatemeh Maleki[4], Mehdi Shafiee Ardestani[5], Taher Elmi[1], Sayed Hussain Mosawi[6*]

[1]Department of Parasitology and Mycology, Faculty of Medicine, Iran University of Medical Sciences, Tehran, Iran; [2]Department of Immunology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran; [3]Microbiology Department, Science & Research Branch, Islamic Azad University, Tehran, Iran; [4]Department of Parasitology, Faculty of Para Medical Sciences, Iran University of Medical Sciences, Tehran, Iran; [5]Department of Radiopharmacy, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran; [6]Department of Biology and Microbiology, Faculty of Medical Technology, Khatam Al Nabieen University, Kabul, Afghanistan

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***Correspondence:** Sayed Hussain Mosawi. Sayed Hussain Mosawi, Department of Biology and Microbiology, Faculty of Medical Technology. Khatam Al Nabieen University, Kabul, Afghanistan. E-mail: hossein.nostalgia@yahoo.com

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Abstract

BACKGROUND: Leishmaniasis is a parasitic disease induced by a protozoan from the genus *Leishmania*. No effective vaccine has yet been developed against the disease.

AIM: In this work, two nano-vaccines, TSA recombinant plasmid and dendrimer and poly (methyl methacrylate) (PMMA) nanoparticles (as adjuvants), were designed and tested for their immunogenicity in BALB/c mice.

METHODS: After the plasmid construction and preparation of adjuvants, three intramuscular injections of the nano-vaccines (100 µg) and the recombinant TSA protein (20 µg) were subcutaneously performed. Eventually, the challenged animals were infected with the parasites ($1*10^6$ promastigotes). After the last injections of the nano-vaccines, the responses of their antibody subclasses and cytokines were assessed via ELISA method before and after the challenge.

RESULTS: This study revealed that the new nano-vaccines were strong and effective in inducing specific antibody and cellular responses and reducing the parasite burden in the spleen compared to the control groups of *Leishmania major*-infected BALB/c mice.

CONCLUSION: Based on the results, we can suggest that the formulated vaccines are suitable candidates for further studies in the field of leishmaniasis control.

Introduction

Leishmaniasis is a widespread parasitic disease in many tropical and subtropical regions of the world, which is transmitted via the bites of infected sand flies. Re-infection is prevented by the immunity achieved via cutaneous infection with *Leishmania* spp. This suggests that prophylactic immunisation is achievable. No vaccines have been approved to be effective against leishmaniasis. DNA vaccination is a recent immunisation plan with many potential benefits over any other vaccine strategies. DNA vaccines can elicit broader immune responses than formal vaccines. Thus, to elevate immunity, DNA vaccine is complemented with adjuvants. Thiol-Specific Antioxidant (TSA) protein is one of the dominant antigens of *L. major* promastigote and amastigote and is considered as a primary DNA vaccine candidate against leishmaniasis. Many attempts to improve an efficient anti-*Leishmania* vaccine have failed due to lacking a suitable adjuvant [1][2].

Nanoparticles represent a group of macromolecular materials that exhibit promising therapeutic or prophylactic properties to be used as adjuvants for delivering antigens via mucosal surfaces and intradermal routes. However, the size of a particle affects both antigen delivery and the type of immune

responses it produces. As antigen carriers, these particles may act as a depot for the regulated release of antigens to enhance immune cell responses [3]. Dendrimers represent another group of repetitively branched molecules with the ability of gene and drug delivery. They can also be used in the synthesis of monodisperse metallic nanoparticles [4][5].

Given the recent developments of nanotechnology in the field of drug delivery and the unique features of carriers, such as dendrimers, which alleviate the problems of low solubility and bioavailability of drugs, we applied biocompatible and biodegradable dendrimers with polyethylene glycol (PEG) core and citric acid branches in this study. Today, thanks to nanotechnology, researchers in the pharmaceutical industry have developed drug carriers, which resolve such problems as low solubility and poor absorption of drugs by cells. They can not only increase drug bioavailability and help targeted delivery to a specific tissue, but also control the amount of drug release. The polyvalent natures of peptide dendrimers enhance their peptide-specific affinities to interact with peptides, proteins, and carbohydrates [6].

Despite its approval by the US Food and Drug Administration (FDA) for certain clinical human uses, poly (methyl methacrylate) (PMMA) as a phagocytised particle may trigger strong immune responses by inducing the production of inflammatory cytokines [7].

Therefore, we appraised the effectiveness of dendrimer and PMMA as nano-adjuvants with the DNA-encoding TSA antigen of *L. major* in BALB/c mice in a bid to obtain a vaccine of improved efficacy against leishmaniasis.

Materials and Methods

L. major promastigotes

L. major MHRO/IR/75/ER, which is an Iranian strain separated by Nadim et al. in 1964, was obtained from Iranian Pasteur Institute. Promastigotes were cultured in RPMI 1640 medium (Sigma®) and supplemented with 10% heat-inactivated Fetal Calf Serum (FCS) (Gibco®, BRL) and 100 lg/ml of gentamicin (Sigma®) at 26°C. The stationary phase was catched by centrifugation and used at $1*10^6$ promastigotes/ml. The procedures of this study were also approved by the Ethical Committee of the Faculty of Medicine (Iran University of Medical Sciences) with code number: IR.IUMS.REC1390.15896.

Plasmid construction

After preparation, TSA recombinant plasmid DNA was transmuted into *E. coli* DH5-α, purified by plasmid extraction Kit (Bioneer, Germany), dispersed in sterile deionised distilled water, and kept at -20°C until used. Then, a purification step was followed by using Endo-Free plasmid purification Giga Kit (Qiagen, CA, USA) according to the manufacturer's instructions. DNA concentration was concluded by taking the dimensions at the Optical Density (OD) of 260 nm. To ensure that the purified DNA was protein-free, the OD260/OD280 ratio was obtained to be 1.80-1.95 [8].

Preparation of adjuvants

Here, we introduced a new method for the synthesis of G2 dendrimer with PEG core and citric acid branches. The method was characterised by simplicity and the use of non-toxic materials. Also, in this approach, consecutive steps of purification were taken, and impurity removal was done in one run using Sephadex column without a previous G1 purification. The method was thus highly fast, cheap, and efficient. In this approach, 2 ml of PEG 600 equivalent to 3.7 mmol and a dry dimethyl sulfoxide (DMSO) solvent were utilised in a test tube. An amount of 3.7 x 2 mmol of dicyclohexylcarbodiimide (DCC) was then added to the test tube to activate the reaction, and the lid was immediately closed. The reaction tube was stirred for 15 min before the addition of an amount of 3.7x2 mM of citric acid followed by one h of stirring. Upon skipping a reaction stop for G1 purification, we added 3.7x6 mM of DCC and the reacting components were further stirred for 15 min. The stirring was continued again for one h after the addition of 3.7 x 6 mM of citric acid and 10 ml of DMSO. The reaction was ended by the addition of 30 ml of double-distilled water. For G2 dendrimer purification, we utilised Sephadex column G-75 (Merck, Germany). To this end, an amount of 6.0 g of Sephadex powder was dissolved in 20 ml of double-distilled water and maintained at ambient temperature for 24 h. The Sephadex was then transferred to a column and eluted once with double-distilled water. Afterwards, G2 dendrimer solution was separated from the sedimented DCC using a filter paper and transferred to the Sephadex column. The eluted and purified solution was thus collected. This step was repeated to remove all the impurities and obtain the purified water-soluble G2 dendrimer, which was lyophilised.

The required amount of the contributing substance was determined with the aid of its corresponding stoichiometric relationship, density, and molecular weight. Since PEG has two functional carboxyl groups capable of binding to citric acid, two moles of citric acid was applied per one PEG besides DCC as its activator. To ensure the synthesis of the required dendrimer, Thin-Layer Chromatography (TLC) was performed using a solvent system of gradient methanol-chloroform. The size and surface

charge of G2 dendrimer was determined by Dynamic Light Scattering (DLS) using double-distilled water as a solvent. Gamma irradiation polymerisation method was applied to produce PMMA nanoparticles in the absence of antigens [4]. A nano-vaccine candidate was then prepared by loading pcDNA3/TSA recombinant plasmid into PMMA nanoparticles. In short, 1 Mm of PMMA nanoparticle solution was used to cross-link to 10 Mm of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) reagent under incubation with soft mixing at room temperature for 10 min. Then, 1 ml of plasmid DNA (100 lg/ml of the solution) was added to an equal volume of the former and placed in a cold room overnight. The solution was finally purified by comprehensive dialysis, and the resulting PMMA-plasmid DNA nanoparticles were suspended in double-purified water. The nanoparticle size was determined by using a Zeta Sizer (Malvern, UK) (data not shown) [1]. TSA recombinant peptide booster (22 KD) was a gift from Miss Nargestehrani, a faculty member of the Islamic Azad University of Tehran [4][5][6].

Immunization and experimental infection of the mice

Inbred female BALB/c mice matured 6-8 weeks were obtained from the Animal Center of Pasteur Institute of Iran (Karaj) and treated by the National Animal Care and Use protocol adopted by the Iranian University of Medical Sciences. The mice were divided into 3 test (T) and four control (C) groups (20 mice/group). The test group received DNA vaccine (pcDNA3/TSA), nano-vaccine (pcDNA3/TSA+dendrimer), and nano-vaccine (pcDNA3/TSA+PMMA), while the control group received pcDNA3, dendrimer, PMMA, and PBS at the doses of 100 µg. For experimentation, the mice were anesthetized by an intraperitoneal injection of 25 µl g^{-1} of a combination of 10% ketamine and 2% xylazine. All the treatments were intramuscularly administrated, and the injection sites were immediately subjected to 8 electric 60-V pulses for 20 ms at a 200-ms interval by using a BTX ECM 830 generator (Harvard Apparatus, USA) equipped with tweezer-type electrodes (CUY 650, Sonidel Limited, Ireland). Immunization of the mice was done by injecting 50 µl of PBS into each anterior tibialis muscle. The immunisation schedule was performed with three inoculations of equal doses of DNA, dendrimer, and PMMA at 3-week intervals. The booster peptide (20 µg) and incomplete Freund's adjuvant was subcutaneously injected two weeks after the injection of the last nano-vaccines. The immunised mice were intradermally challenged with $1*10^6$ promastigotes of *L. major* at the base of their tails three weeks later. The animals were then sacrificed after five post-challenge weeks, and their serum samples and spleens were immunologically analyzed [7][8][9].

Lymphocyte proliferation assay

The spleen of each sole mouse was dismembered and suspended in sterile, cold Phosphate-Buffered Saline (PBS) containing 2% Fetal Bovine Serum (FBS). The Red Blood Cells (RBCs) were lysed, and a single-cell suspension was prepared in RPMI 1640 (Gibco, Germany) at $3*10^6$ cells/ml, which was complemented with 10% of FBS, 4 mM of L-glutamine, 1 mM of sodium pyruvate, 50 µm of 2-ME, 100 µg/ml of streptomycin, and 100 IU/ml of penicillin. Flat-bottom 96-well culture plates were used to dispense 100 µl of the cell suspension motivated with ten µg/ml of the recombinant TSA protein expressed in *E. coli* cells for antigen recall. Phytohemagglutinin-A (5 µg/ml, Gibco) and unmotivated wells were utilized as the positive and negative controls, respectively. The whole culture medium was similarly applied as blank. All the tests were done in triplicate. The plates were incubated for 72 h before supplementing 100 µl of 5-Bromo-2-deoxy-uridine (BrdU) labelling solution into each well and incubating them for 18 further hours. The plates were then subjected to centrifugation to remove the culture medium before drying and fixing the wells with 100 µl of fixation/ permeabilisation buffer. Subsequently, each well received 100 µl of anti-BrdU antibody before washing the plates four times and supplementing them with tetramethylbenzidine (TMB) substrate. The test was halted by concluding 100 µl of 2 NH_2SO_4. The OD of each well was assessed at 450 nm. The stimulation indices were estimated in accord with the following formula: OD of the stimulated well/OD of the unstimulated well.

Cytokine evaluation before and after the challenge with L. major

The single-cell suspension ($3*10^6$ cells/ml) derived from each mouse spleen was dispensed into the 24-well plates, aroused *in vitro* with ten µg/ml of recombinant TSA protein, and incubated in 5% CO_2 at 37°C. After 72 hrs of antigen recall, the supernatants were obtained by centrifugation at 300*g for 10 min and supplied at -70°C for cytokine analysis. Then, using commercial ELISA Kits (Mabtech, Sweden) according to the manufacturer's instructions, IFN-γ and IL-4 cytokines were quantified. Each cytokine was quantified as pg/ml based on the plotted standard bend.

ELISA of the total antibodies and the subclasses of IgG1, IgG2a

Assay of the sera of the empirical groups was done using an optimised indirect ELISA approach to assess humoral immune responses based on the specific antibodies before and after *L. major* challenge. In short, 100 µl of antigen (10 µg/ml) in PBS buffer was supplemented into 96-well ELISA MaxiSorp plates (Nunc, Naperville, IL) and incubated

at 37°C for 24 h. After being washed with PBS containing 0.05% Tween 20 (washing buffer), the plates were blocked with 5% skimmed milk in PBS (blocking buffer) at 37°C for one h. The plates were again rinsed with a rinsing buffer before the addition of 100 µl of the diluted sera (1/100) to each well and then incubated at 37°C for two h. The wells were rinsed five times and incubated with 100 µl of the diluted (1/7,000) anti-mouse sera conjugated to HRP (Sigma, USA) for two h. The wells were again rinsed five times before further incubation with 100 µl of TMB substrate in the dark for 30 min. The reaction was then stopped by the addition of 2 N H2SO4. The ODs were estimated with an ELISA plate reader at λ 450 nm. Using the secondary antibodies of goat anti-mouse IgG1 and IgG2a (Sigma, USA) based on the manufacture's instruction, the specific subclasses of IgG1 and IgG2a were detected [10][11].

Parasite load distinction

The parasite burden was determined by sacrificing three mice per each group, which had been challenged seven weeks earlier. Then, their spleens were drained using the limiting dilution method. Briefly, a slice of a spleen was removed and weighed to be homogenised with a tissue grinder in 2 ml of RPMI 1640 medium (Gibco, Germany) complemented with 20% heat-inactivated FCS and Gentamicin (0.1%). Serial dilutions were prepared in 96-well micro titration plates under sterile conditions. After seven days of incubation at 26°C, the plates were examined using an inverted microscope at a magnification of 40*. The presence or absence of mobile promastigotes was recorded in each well. The last titer was the last dilution, in which the number of parasites per gram was estimated in the following way: _log10 (parasite dilution/tissue weight) [2][10].

Statistical analysis

The significance of the differences among various groups was tested using the one-way ANOVA test. Besides, Post-hoc LSD test was utilised to compare the means of the different groups under treatment. The statistics were regarded significant at $P < 0.05$.

Results

Lymphocyte proliferation assay

Lymphocyte proliferation analysis before the challenge through BrdU method revealed no significant differences between the vaccinated groups after three injections of DNA and the nano-vaccines (pcTSA+dendrimer and pcTSA+PMMA and pcTSA) at $P = 0.337$ although 3 test groups demonstrated significantly different proliferative activities from those of the control groups at $P < 0.001$. After challenging with *L. major*, the immunised groups displayed no significant differences among themselves ($P > 0.549$), but all produced higher degrees of proliferation responses than those of the control groups ($P < 0.001$) (Fig. 1).

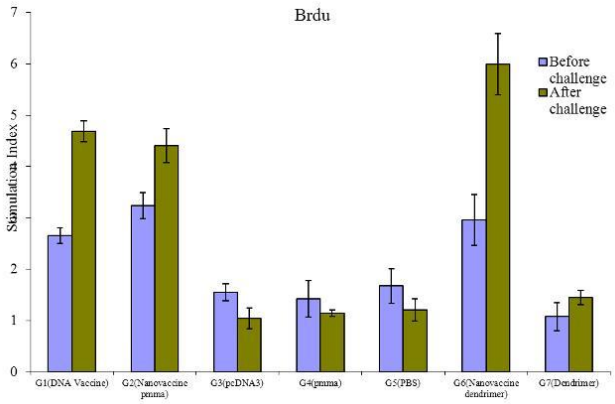

*Figure 1: Lymphocyte proliferation responses before and after the challenge: The mice were immunised with DNA vaccine with or without a dendrimer and PMMA (n = 5 mice per group) in the DNA prime/peptide raising plan. The four groups of mice were injected with pcDNA3 vector, dendrimer, PMMA, and PBS as the negative controls (n=4 mice per group). Proliferative reactions were followed for the unique mice in triplicate and evaluated using BrdU method as represented in the section of "Materials and Method". The data depict mean ± SD (95% C.I.). *P < 0.001 considered for the vaccinated groups before and after the challenge showed a significantly higher rank for rapid growth compared to those of the control groups*

The models of IL-4 and IFN-γ cytokines

In an attempt to appraise the pattern of cytokine secretion caused by vaccination, the special mouse splenocyte culture was re-stimulated *in vitro* with recombinant TSA protein and cleansed in *E. coli* cells (data not shown). The collected supernatants were tested for IFN-γ and IL-4 quantities causing the types of the induced immune responses (T helper one vs T helper 2). The results represented that before the challenge, IFN-γ secretion level was significantly higher in the vaccinated than in the control groups ($P<0.001$) meaning that immunisation increases IFN-γ production by lymphocytes. Behind the challenge, IFN-γ level remarkably augmented in the mice immunized with the nano-vaccines (pcTSA+dendrimer, pcTSA+PMMA) compared to the control groups and those immunized with DNA vaccine (PcTSA) ($P<0.001$) (Fig. 2a). Before the challenge, the mice immunized with the DNA vaccine and formulated with the nanoparticles revealed a significant enhancement of IL-4 level produced by lymphocytes compared to the control and DNA-vaccinated mice groups ($P < 0.001$). However, after the challenge with *L. major*, no statistically significant differences between the test and control groups were

noticed (P > 0.733) regarding IL-4 production (Fig. 2b).

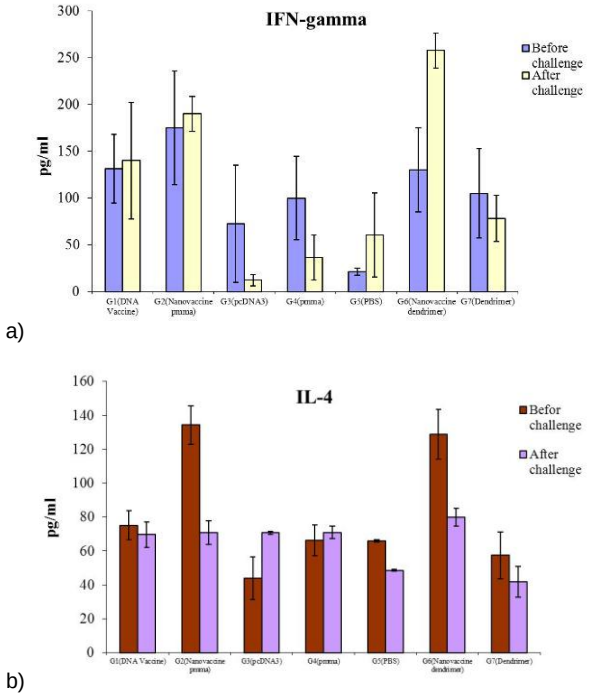

*Figure 2: Cytokine yields (IFN-γ (a) and IL-4 (b)) by the spleen cells of BALB/c mice after the immunization periods and the challenge: The mice were immunized with DNA vaccine with or without the dendrimer and PMMA (n = 5 mice per group) in the DNA prime/peptide life plan. The four groups of mice were injected with pcDNA3 vector, dendrimer, PMMA, or PBS as the controls (n=4 mice per group). Cytokine analyses were observed during the study by using ELISA approach as mentioned in the section of "Materials and Methods". The tests were carried out in duplicate for the special mice. The merits represent mean ± SD (95% C.I.). *P < 0.005 was considered for the nano-vaccine groups compared to all the other groups. *P < 0.001 was regarded for the immunised groups compared to the control groups. *P < 0.001 for the groups of pcTSA+dendrimer and pcTSA+PMMA was considered to be similar to all the other groups after the challenge with L. major (Fig. 2a). *P < 0.025 was taken for the nano-vaccine groups compared to all the other groups before the challenge. *P < 0.011 for the vaccinated groups was regarded to be similar to the PBS group after the challenge (Fig. 2b)*

Antibody responses

In general, the immunised groups showed a significant rise in their total antibody productions before the challenge compared to the control groups (P < 0.003). After the challenge, the mice produced elevated levels of antibodies and had been thus immunised with the nano-vaccines, but they did not indicate statistically significant differences among themselves (P > 0.059). The nano-vaccines exhibited a significant rise in the total antibody production after the challenge in comparison to the control and vaccinated groups (P < 0.003) (Fig. 3a,b). The effects of IgG isotyping demonstrated that the test and control groups had similar IgG1 levels before the challenge with no significant differences between them (P > 0.059). However, after the challenge, all the immunised groups produced significantly increased IgG1 isotypes as compared to the control groups (P<0.030). Before and after the challenge, IgG2a titer was more significant in the immunised than in the control groups (Fig. 3c,d).

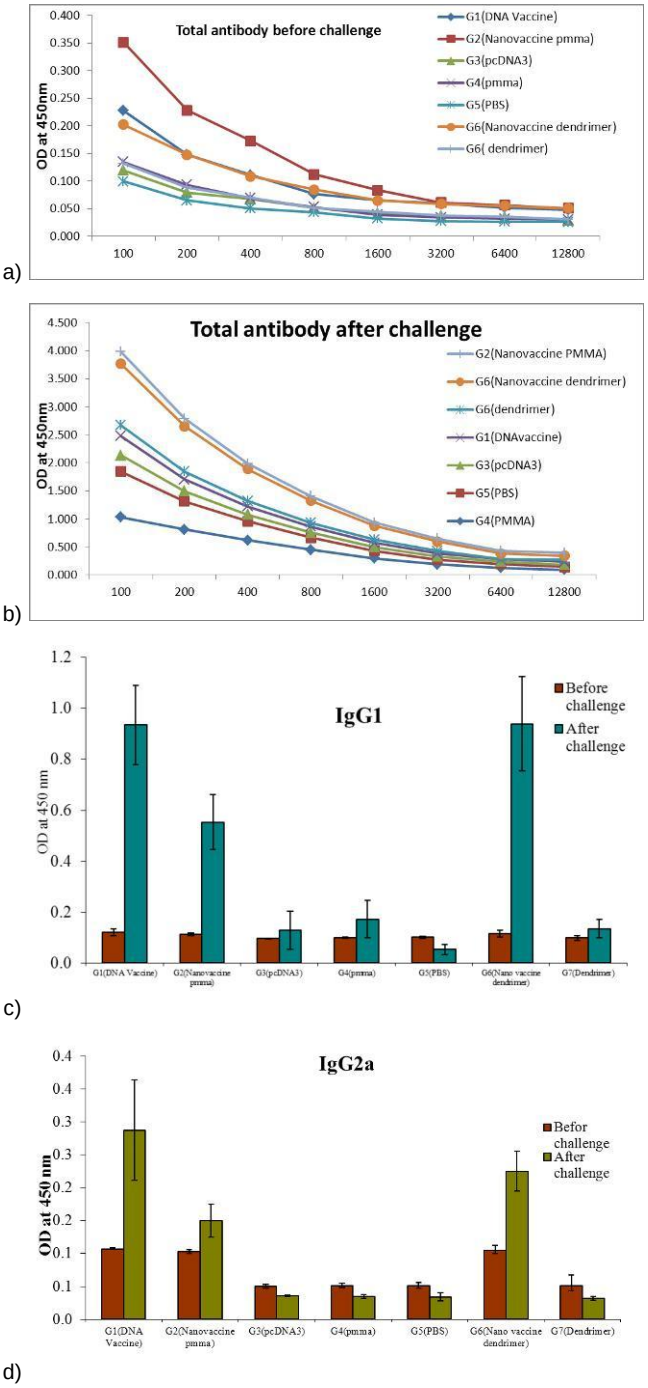

Figure 3: Specific antibody production against TSA recombinant protein in BALB/c mice immunized with DNA vaccine and nano-vaccines before the challenge (a) and after the challenge (b): The specific total IgG, IgG1, and IgG2a were measured through ELISA approach as mentioned in the section of "Materials and Methods". The sera obtained from each group were diluted 1:200 and assessed for the presence of IgG1 and IgG2a. Specific changes in IgG1 (c) and IgG2a (d) levels were detected throughout the study. TMB substrate was employed for the detection and OD was determined at 450 nm. Mean ± SD (95% C.I.) is represented throughout the data

Splenic parasite burden

The splenic parasite burden assays of all the empirical groups revealed that the numbers of viable splenic parasites were different among the vaccinated and unvaccinated groups following the immunisation and seven weeks after the challenge with *L. major*. The immunised mice displayed a significantly lower number of alive parasites compared to the control groups ($P < 0.001$) (Fig. 4).

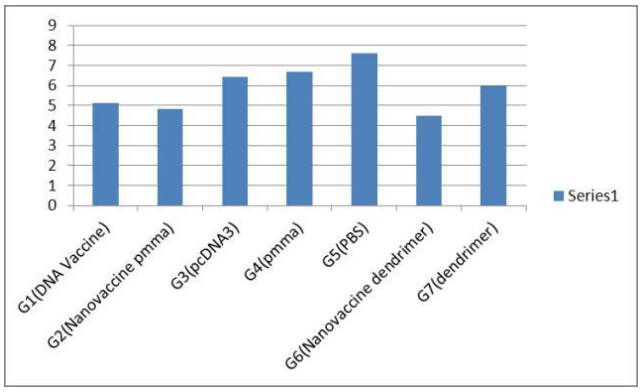

*Figure 4: Parasite burden associated with DNA vaccine and nano-vaccines in the prime/boost strategy and control groups seven weeks after the challenge: Calculations of the total numbers of the viable parasites within the spleens of the infected animals were done based on parasite dilution per tissue weight. Mean ± SD was used to represent the values obtained for the individual mice (n = 3). A significantly less parasite burden was found in the spleens of the vaccinated mice compared to those of the control groups (*P < 0.001)*

Discussion

In the present study, we attempted to design novel nano-vaccines containing TSA plasmid DNA and estimate their immunogenicities in BALB/c mice. Currently, there are no effective vaccines available to produce a protective response against leishmaniasis. This is despite the fact that many vaccination strategies ranging from involving the killed parasites to recombinant antigens or DNA vaccines have been tested. Antigens, such as recombinant proteins induce only antibody responses while DNA vaccines involve both MHC-I and MHC-II pathways. Therefore, DNA vaccines can cause strong, long-lasting, and effective humeral and cellular immunities. The prime-boost immunisation method can influence the quality and quantity of immune responses. However, certain approaches are needed to increase the qualities and efficacy of DNA vaccines, such as *in vivo* electroporation. TSA as the immune-dominant antigen of *L. major* is antigenic in murine and human systems and induces CTL activity and safety against the parasite. Excellent protection against leishmaniasis has been reported for the recombinant leishmanial antigens LmSTI1 and TSA [12][13][14]. Nevertheless, using an adjuvant seemed necessary to boost any modern vaccines against leishmaniasis [15]. The results of this research revealed that the mice immunization with the nano-vaccines and DNA vaccine increased humoral and cellular responses as compared with the control groups.

In this investigation, we used dendrimer nanoparticles as adjuvants to elicit stronger immune responses to the candidate vaccines. This is because dendrimers had nowadays several practical applications in medicine and attracted the attentions of many researchers to obtain novel synthetic designs with reduced toxicities.

An anionic PEG-citrate G2 dendrimer was chosen because of its biocompatibility and biodegradability. Dendrimers are nano-carriers with a high potential to carry hydrophobic drugs and increase their solubilities in water as well as their cellular uptakes. The unique properties of dendrimers, including monodispersity and surface modification capability along with their sizes and structure sets, have made them ideal candidates for drug delivery. Scientific research during the last two decades has shown that dendrimers are appropriate and effective carriers for drug delivery and enhancement of the mobilities of hydrophobic drugs. Another feature of a dendrimer is its possession of suitable spaces, within which various hydrophobic and hydrophilic drug molecules can be accommodated. The presence of multiple well-known functional groups on the surface of these spherical particles make them as suitable carriers to fit various drug molecules or ligands and consequently enable them to help a targeted drug delivery. The results of the studies performed on these dendrimers have shown that these chemicals have great potentials for use in drug delivery systems. Shafiee Alavijeh et al. examined cell death mechanisms (apoptosis and necrosis) caused by a dendrimer in HT1080 cell lines. Based on their results, dendrimers had no significant detrimental effects at a concentration of 0.5 mg/ml. They stated that these hybrid structures would be a very large potentiality for application in the various fields of nano-medicine. In another study carried out by Haririan et al., two conjugates were prepared from cisplatin in aqueous solutions with the two generations (G1 and G2) of biodegradable anionic citric acid dendrimers. Based on the *in vitro* results obtained from their research, a conjugate of G2+platinum had higher toxicity to cancer cells than that of G1+platinum and cisplatin and hence showed a better therapeutic effect. They stated that these conjugates with such a high potentiality and minimum hemolysis are good candidates as new and effective anti-tumour agents. It should be noted that some types of dendrimers, such as viologen with a chemical structure containing bipyridinium salts or other structures like Caminade, PAMAM, polyanionic phenyl dicarboxylic acid (BRI6195), or carbosilane have shown antiviral properties, particularly against HIV-1 with EC [50] = 0.26 ± 0.08 μM (e.g., viologen). The mechanism

behind these observations was reported to be caused by an interference with viral replication or gp-120 protein function, especially in the case of PAMAM. This would strengthen the hypothesis that the simultaneous use of dendrimeric structures and curcumin can produce synergistic or additive effects. In this research, PEG was chosen due to its environmental compatibility and biodegradability, as well as its easy reaction with citric acid and constitution of the core of a dendrimer. The presence of citric acid in the structure of this dendrimer as a macromolecule, which can be subjected to metabolism by the cellular citric acid cycle upon entering the cell, has made it environmentally compatible and biodegradable. The dendrimer is not cytotoxic in therapeutic doses depending on the study type because of its size (about 80 nm) and negative charge. Cells have negative charges similar to a subjected dendrimer and thus cell surface absorption as the main cause of cell toxicity may not occur. In this study, DCC was applied instead of chlorinated compounds, such as dichloromethane, which is usually used to activate synthetic reactions. Chlorinated compounds are highly toxic and cause a serious damage to human respiratory system, but DCC has a much lower toxicity and can be thus considered advantageous in our method. The obtained product was a two-generation dendrimer (G2), which was completely soluble in water and could, therefore, be a good candidate to increase the solubility of water-insoluble drugs. Studies have shown that this dendrimer has no unfavourable effects on cells at a concentration of 5.0 mg/ml. In this research, we employed a new method to synthesize nanoparticles, which not only shortened the reaction and production times but also enabled the use of less toxic materials [6][16].

The nanoparticle conjugates of the dendrimer produced valid antibody responses and protections against some antigens. Among numerous nanoparticles, those which are biodegradable, safe, and simple and easy to be produced can be selected for the drug delivery under study [17][18][19][20]. The findings of the previous studies indicated that dendrimers have antibacterial effects. In this work, PMMA nanoparticle was utilised as an adjuvant to enhance specific humoral and cellular immune responses to our candidate vaccines due to its good antibody responses in addition to its conferring higher stability to the vaccines. The utilisation of PMMA adjuvant in split influenza vaccines demonstrated a safety record and excellent and powerful protection [21][22]. This nanoparticle may also enhance humoral responses against Hiv-2. Some authors suggested that PMMA adjuvant can increase antibody production and hence the efficacy of candidate vaccines [23][24]. Our findings revealed that both the specific IgG1 and IgG2a were augmented upon immunisation with PMMA nano-vaccine and dendrimer. Considering that IgG1 and IgG2a are Th2 and Th1 markers, respectively, this funding was of prime importance. Campos-Neto et al. reported that immunisation of BALB/c mice with a TSA plasmid DNA influenced elevated titers of particular IgG1 and IgG2a antibodies vs. *Leishmania* [25][26][27]. Other studies have shown that the use of nanoparticles and a major raising plan increases protective immunity against *Leishmania* infection in animal models [26][28]. In this research, we showed that a dendrimer and PMMA can boost the efficacy of DNA vaccines encoding TSA against *L. major* disease and bring out immune responses to the delivered antigen. Our nano-vaccines were productive for lowering parasite load in the spleens of BALB/c mice infected with *Leishmania major* as compared to the control groups.

In conclusion, the vaccine formulation suggested in this investigation may provide a way to be paved for obtaining excellent candidates against *Leishmania* through further research.

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Ethics approval

The procedures of this study were also approved by the Ethical Committee of the Faculty of Medicine (Iran University of Medical Sciences) with code number: IR.IUMS.REC1390.15896.

Authors' contributions

Somayeh Zarrati searched the literature and performed experiments. Mehdi Shafiee Ardestani designed the study and analyzed the data. Sayed Hussain Mosawi and Fatemeh Tabatabaie have participated in drafting the manuscript and supervised the research. Fatemeh Tabatabaie and Nasim Samarghandi wrote the final manuscript. All authors read and approved the final manuscript.

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Histopathological Pattern and Age Distribution, of Malignant Ovarian Tumor among Sudanese Ladies

Sumeya A. Kheiri[1], Abdelillah Kunna[1], Ali Yousif Babiker[2], Sultan A. Alsuhaibani[2], Rami Yousif Ahmed[1], Mohamed Alkhatim Alsammani[1,3*]

[1]Department of Obstetrics and Gynecology, College of Medicine, University of Bahri, Khartoum, Sudan; [2]Department of Medical Laboratories, College of Applied Medical Sciences, Qassim University, Buraidah, Saudi Arabia; [3]Department of Obstetrics and Gynecology, College of Medicine, Qassim University, Buraidah, Saudi Arabia

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***Correspondence:** Mohamed Alkhatim Alsammani. Department of Obstetrics and Gynecology, College of Medicine, University of Bahri, Khartoum, Sudan. E-mail: m_sammani@yahoo.com

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Abstract

INTRODUCTION: Ovarian cancer is the cause of a high case-fatality ratio, and most of the cases are diagnosed in late stages.

OBJECTIVES: To determine the histopathological types, age distribution, and ovarian tumour stages among diagnosed with ovarian cancer at Al - Amal Tower a multi-referral polyclinic of Radiology & Isotope Center Khartoum (RICK), Sudan.

METHODS: All histopathology reports patients' case from January to June 2015 were reviewed. The cancers classified according to federation international of Obstetrics and Gynecology (FIGO).

RESULTS: There were 127 cases of ovarian cancers. Surface epithelial cancers were the most common 77.7% (n = 98), followed by sex cord-stromal cancers 11.23% (n = 14), Germ cell tumor 1.6% (n = 2). Metastatic cancers were seen from colon and breast in 6.3% and 3.9 % of cases respectively. Few cases (14%) of ovarian cancers were reported before 40 years of age, after the age of 50 is a sharp increase in the incidence of a tumour. The mean age at presentation was 52.36 ± 14.210 years, there is mean age of menarche 13.59 ± 2.706 years. Very few patients used HRT (1.6%) or had been on ovulation induction treatment (8.7%). Most of patients 39 (30.7%) presented in stage IIIC, and stage 1V 32 (25.2%) indicating a poor prognosis.

CONCLUSION: The incidence of different types of ovarian cancers in the present study is similar to worldwide incidence. The surface epithelial tumour is the commonest ovarian cancer, of which serous adenocarcinoma is the commonest and most of our patients present in late stages.

Introduction

Ovarian cancer is the second most common cause of death from gynecologic tumours in the United States [1][2]. Initially, symptoms may be vague or not apparent, but they become more noticeable as the disease progresses. Early symptoms of ovarian cancer include bloating, pelvic nausea pain, and abdominal swelling [3]. Peritoneal cavity, lymph nodes, lungs, and liver are the most common site for metastasis [4]. The risk of ovarian cancer increases with ovulation induction treatment, nulliparity, women on hormonal replacement therapy, and those begin ovulation at a younger age or reach menopause at an older age at a higher risk of ovarian cancer [5][6]. Factors that decrease the risk of ovarian cancer include the use of OCP, tubal ligation, and breastfeeding [6]. Genetic inheritances are responsible for 10% of cases; the estimated risk for women with BRCA1 or BRCA2 is 50% [6]. The most common type of ovarian malignancies is epithelial cell carcinoma which accounts for 95% of cases. There are five main subtypes of epithelial cell carcinoma, of which high - grade serous is most common. These tumours originate from inclusion cysts in the cells overlying the ovaries [5] though some may form at the Fallopian tubes [7] [8]. Infrequent types of ovarian

cancer include germ cell tumours and sex cord stromal tumours [5]. The diagnosis of ovarian cancer is confirmed by histopathology examination.

This study aimed to determine the histopathological pattern of ovarian cancer stages, and the age distribution in the patients diagnosed at Al - Amal Gynecologic Oncology Hospital, Khartoum, Sudan.

Material and Methods

This is a prospective cross-sectional hospital-based study conducted at Al-Amal Tower a multi-referral polyclinic of Radiology & Isotope Center Khartoum (RICK) which is the most leading Oncology Center in Sudan since founded in 1967. Where over 6,800 new cancer cases were diagnosed & managed in 2014. The study was carried out from January to June 2015. The study population composed of patients diagnosed as having ovarian cancer. Eligibility is limited to the Alamal Oncology Tower.

Data collection tools

Demographic data were gathered including age; residence, menarche and family history of breast, ovarian and colonic cancer. History of ovulation induction treatment and uses hormonal replacement therapy were recorded. Histopathology report of the examined specimen was obtained from the histology laboratory.

Ethical consideration

The study was approved by the Ethics Review Committee of the Sudan Medical Specialization Board, Council of Obstetrics and Gynaecology and AL-amal Ethics Committee. Formal consent was taken from each participant written consent was taken from each participant.

Data collection

To ensure completion of questionnaire data was collected by a senior registrar in Obstetrics and Gynecology.

Statistical analysis

The statistical package for the social sciences (SPSS version 20 for Windows) was used for data analyses. The descriptive statistical analyses used included the mean, standard deviation, and frequency distribution.

Results

The mean age of the study group was 52.36 ± 14.210 years (ranged from14 to 95 years). Their mean age at menarche was 13.59 ± 2.706 years and a mean parity of 4.41 ± 3.396 deliveries. The mean menopausal age was 33.85 ± 21.991 years, and the parity was 4.41 ± 3.99 deliveries. The majority of patients were from the central states of Sudan. Few of them had been using combined oral contraceptives, ovulation induction treatment and hormonal replacement therapy, 7.1%, 8.7%, and 1.6% respectively.

Table 1: Basic characteristics of study population

Mean maternal age	52.36 ± 14.210 years
Age of menarche	13.59 ± 2.706 years
mean parity	2.2756 ±.76301 years
Oral contraceptive pills users	9 (7.1%)
Ovulation induction medications	11 (8.7)
HRT use	2 (1.6)
Nulliparous	22 (17.3)
Multiparous	47 (37)
Grand multiparous	58(45.7)

Data present as number (%).

The family history of ovarian, breast or colonic cancer was positive in 11.8% (n = 15) of cases. The mean age for endometrioid tumours was 64 ± 4 years, while that for mucinous, serous transitional and adenosarcoma was similar. The mean age of occurrence of Sertoli cell tumours and Clear cell tumours was at late reproductive life as shown in Table 1 and 2.

Table 2: The mean age of occurrence of different types of ovarian cancer

Cancer	Age mean ± SD years
Serous adenocarcinoma	54.2 ± 14
Mucinous adenocarcinoma	50 ± 16
Endometrioid tumours	64 ± 4
Clear cell tumours	39 ± 3
Transitional cell tumours	52.4 ± 14.2
Adenosarcoma	52.3 ± 3
Granulosa tumours	53.2 ± 6
Sertoli cell tumours	45.7 ± 3

Bilateral involvement of both ovaries was reported in more than half of cases (52%, n = 66), followed by the right ovary 27.6 (n = 35) and the left ovary 20.5% (n = 2). of all surface epithelial tumours 77.1% (n = 98), 13.3% (n = 13) were borderline tumour. Of all ovarian tumours, serous adenocarcinoma was the most common type (44.1%), followed by mucinous adenocarcinoma 12.6%, detailed of other types are shown in Table 3. The second reported an ovarian tumour was sex cord-stromal tumours which comprised 11.23% of all cases detailed are shown in Table 3. Germ cell tumour was reported in only 1.6% (n = 2) of cases, while metastatic cancer is most commonly seen from the colon (6.3%) and only 3.9 % from breasts (Table 3).

Most of the patients 39 (30.7%) presented in stage IIIC, and 32 (25.2%) presented at stage IV, while 14 (11.0%) of patients presented in stage IC,

and 9 (7.1%) patients presented in stage IIA, and only 8 (6.3%) patients in stage IB, 7 (5.5%) patients at stage IA (Table 4).

Table 3: Rate of occurrence of different ovarian malignancies among the study population

Frequency of surface epithelial-stromal 77.2% (98)			
Pathology pattern		Frequency	%
Serous tumours	Borderline serous	4	3.1
	serous adenocarcinoma	56	44.1
Musinous tumours	Borderline mucinous tumor	1	0.8
	mucinous adenocarcinoma	16	12.6
Endometrioid	endometrioid borderline tumour	4	3.1
tumours	endometrioid adenocarcinoma	3	2.4
Clear cell tumours	Borderline tumours	3	2.4
	clear cell adenocarcinoma	3	2.4
Transitional cell tumours	Borderline Transitional cell	1	0.8
Epithelial stromal	Adenosarcoma	4	3.1
	Carcinosarcoma (mixed muellerian tumor)	3	2.4
Frequency of sex cord-stromal tumours 14 (11.2%)			
Granulosa tumours		12	9.4
Sertoli cell tumours		2	1.6
Frequency of germ cell tumours 2 (1.6%)			
Immature Teratoma		1	0.8
Mixed germ cell tumours		1	0.8
Frequency of metastatic cancer 13 (10.4%)			
Colonic appendiceal		8	6.3
Breast		5	3.9

Discussion

In the present study, we reported an incidence of ovarian epithelial carcinoma of 77.2%. It is approximating the 85% incidence rate quoted from European countries [8] while the even higher rate of incidence (90%) has been reported from United States [9]. The lower incidence of ovarian cancer in our study can be explained by the fact that black women are less likely to develop ovarian cancer compared to white women.

Table 4: Tumors stages at the time of presentation

Stage	Frequency	%
IA	7	5.5
IB	8	6.3
IC	14	11.0
IIA	9	7.1
IIB	6	4.7
IIC	6	4.7
IIIA	3	2.4
IIIB	3	2.4
IIIC	39	30.7
IV	32	25.2

Studies demonstrated that white women had the highest risk of developing ovarian cancer, followed by Hispanic, Asian, black, and American Indian women [10]. The variation in the incidence of ovarian cancer between nations may be due to other factors such as sample size in each study, biosocial differences of the population, and genetic and other environmental factors.

In the current study, 11.8% of studied cases had a positive family history of ovarian cancer. It was reported that positive family history is considered most important risk, probably mediated through inherited genetic mutation which was found to increase the risk by 5 – 10% compared 1.4% risk in the general population [11]. In the current study, the main age of patients at presentation was 52.36 ± 14.210 years ranged from 14 to 95 years, and almost two-thirds of patients were above 50 years of age, and only 14% of cases were < 40 years of age. Similar results have been obtained by other researchers. The American cancer society reported that ovarian cancer is rare in women less than 40 years of age. Typically the diseases develop after menopause, and almost 50%of all ovarian cancers are found in women 63 years of age or older [12]. Similarly, the US Surveillance, Epidemiology, and End Results (SEER) database reported that ovarian neoplasm is a function of age after 50 years [13]. The mean age reported in the current study for endometrioid adenocarcinoma of the ovary (64 ± 4yrears) is consistent with 60 years of age reported by other authors [14].

The average age of menarche is the study group was 13.59 ± 2.706 years. Epidemiologic studies have inconsistent reports on associations between menarcheal age and ovarian cancer risk. One meta-analysis concluded that there was inversely associated between menarcheal age and the risk of ovarian cancer [15]. It is suggested that later menarcheal age will result in a decreased incidence of ovarian cancer by decreasing a woman's lifetime number of ovulation.

There is consistent literature that infertility and low parity increase the risk of ovarian cancer and multiparity and the use of oral contraceptives decreases the risk [16]. In the present study showed that 16.5% (n = 21) were nulliparous women, 37% (n = 47) were multiparous, while the majority 45.7 % (n = -58) are grand multiparous women. The use ovulation induction medications and hormonal replacement therapy were linked to increased risk of ovarian cancer and the use of HRT for a shorter duration are associated with 20% of ovarian cancer [17]. We reported that few of our patients used ovulation induction treatment (8.7%) and HRT (1.6%).

The incidence of endometrioid adenocarcinoma of the ovary in the current study was 5.5% which is less than 10 - 25% reported in the literature [11] but consistent with a report from Africa countries (4.5%) [18]. The present study, sex - cord stroma cell comprises 11.2% of all ovarian neoplasm, and the majority were granulosa cell tumors which comprise 9.4% of cases; a higher incidence (34.4%) was reported by Akakpo from Ghana opposed to a comparatively similar (7%) incidence rate from USA [19].

We reported an overall 1.6% incidence of germ cell tumours which are mainly immature teratoma and mixed germ cell tumour. Previous studies reported an incidence of 1.1% and 2.6% from Africa [18] and USA [20]. Although the disease is rarely reported in older age, the mean age for immature teratoma in this study was 52.1 years.

In the present study, the incidence of secondary metastatic cancer to ovary was 10.4% this was mainly from colon (6.3%) and ovary (3.9%). The figure is relatively lower than the incidence reported by Stewart et al. [21] who analysed 116 patients diagnosed with metastatic ovarian cancer at the Radboud University Nijmegen Medical Centre; they reported a 15% incidence rate. The latter authors found that 39% were from the gastrointestinal followed by breast in 28% and endometrium in 20% of cases [18].

In the present study, bilateral involvement of both ovaries was 52%, while has been reported in only 25% of cases [22][23]. What the frequency of bilaterality of ovarian neoplasm depend primarily on tumour type is involved. Pejovic et al. [24] was the first to raise the question whether bilateral ovarian carcinoma is a due metastasis from another ovary or it occurs as a result of two independent primary tumours. Analysis by karyotyping and genomic hybridisation concluded that bilaterality occurs by a metastatic process [22]. The high occurrence of bilateral (52%) ovarian neoplasm in the present study could be explained by advanced tumour stages at presentation which is an indication poor 5 - year's survival rate.

In conclusion, the incidence of different types of ovarian cancers in the present study is similar to worldwide incidence. The surface epithelial tumour is the commonest ovarian cancer, of which serous adenocarcinoma is the commonest and most of our patients present in late stages. The limitations of this study are the limited number of cases included and being a single centre rather than a multicenter study which is more informative. Further study with a large number of cases is warranted to investigate the predictors of ovarian malignancies among Sudanese women.

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Evaluation of Combined Use of Temocillin Disk and Mastdisks Inhibitor Combination Set Against Polymerase Chain Reaction for Detection of Carbapenem-Resistant *Enterobacteriaceae*

Omnia A. Shaker[1], Howayda E. Gomaa[2*], Shereen A. ElMasry[1], Rania M. Abdel Halim[1], Amany H. Abdelrahman[2], Jackline S. Kamal[2]

[1]Clinical Pathology Department - Faculty of Medicine - Ain Shams University, Cairo, Egypt; [2]Clinical Pathology Department - National Research Centre - Egypt, Cairo, Egypt

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***Correspondence:** Howayda E Gomaa. Clinical Pathology Department -National Research Centre - Egypt, Cairo, Egypt. E-mail: howaydagomaa61@gmail.com

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Abstract

AIM: To evaluate the diagnostic performance of MDI and temocillin disk (30 µg) for detection of carbapenem-resistant *Enterobacteriaceae* in comparison to real-time PCR.

MATERIAL AND METHODS: Fifty specimens submitted to the Microbiology Laboratory of Ain Shams University Hospitals and showed resistance to carbapenem drugs through routine culture and susceptibility testing, were assessed by both temocillin disk (30 µg) and MDI set to detect carbapenem-resistant *Enterobacteriaceae*. Results were compared to real-time PCR for detection of carbapenemase genes *blaKPC, blaNDM, blaOXA*–48-*like*, *blaVIM*, and *blaIMP*.

RESULTS: Our work revealed that most of the CPE isolates were Klebsiella species (62%) followed by *E. coli* (24%), *Serratia* (10%) and *Citrobacter* (4%). Phenotypic detection of carbapenem-resistant classes revealed OXA - 48 in 96% of isolates, followed by MBLs (82%), and KPC (34%). All isolates were negative for *AmpC*. Detection of the genes by real-time PCR showed that the predominance was for the *blaOXA-48* gene (96%) then *blaVIM* (94%) followed by *blaNDM* (54%), *blaKPC* (46%) and finally *blaIMP* (40%). Evaluation of the MDI set against PCR showed sensitivity (82.1%) and specificity (70%). The temocillin disk had 97.9% sensitivity and 50% specificity. The evaluation of Temocillin disk and MDI in combination for detection of carbapenem-resistant Enterobacteriaceae showed 99.7% sensitivity and 35% specificity.

CONCLUSION: Adding Temocillin disk to Mastdisks ID inhibitor combination set provides a simple, easy, rapid and highly sensitive test that can be used for screening and classification of carbapenem-resistant *Enterobacteriaceae*. However, it still needs confirmation by molecular techniques.

Introduction

Multidrug-resistant organisms are markedly increasing among bacterial species, substantially, Carbapenem-resistant *Enterobacteriaceae* which have been reported worldwide [1].

Carbapenems are considered the drugs of the last choice for treating multidrug-resistant pathogens due to their high clinical efficacy and safety [2]. There is a major problem concerning resistance to carbapenem groups of drugs as it limits treatment options and thereby obligates for the use of antibiotics with high toxicity like tigecycline and colistin [3]. The most common mechanism of carbapenem resistance is the production of carbapenemase enzymes [2].

At first, carbapenemases were chromosomal - mediated in a few specific species, but they are now plasmid - mediated, or both chromosomally - and plasmid-mediated. This leads to the much more aggressive spread of resistance due to horizontal transmission among various bacterial species and genera [4].

Carbapenemases are classified according to their functional and molecular properties. Molecular classes A and D are the β - lactamases having serine at their active site, whereas molecular class B β - lactamases are all metalloenzymes with zinc at their active site [5].

The most clinically significant enzymes among the class A carbapenemases are *K. pneumoniae* carbapenemase (KPC) enzymes. KPC-

producing *K. pneumoniae* are widely disseminated among several countries [6]. The class B beta-lactamases or Metallo – beta-lactamases (MBLs) have also been identified in various enterobacterial species, including *K. pneumonia* [7]. They are mainly NewDelhi Metallo – beta-lactamase (NDM - 1), Verona integrin - encoded Metallo – beta-lactamase (VIM), and Imipenemase (IMP) type enzymes. NDM - 1 is the most commonly identified worldwide. Carbapenem - hydrolysing oxacillinase - 48 (OXA - 48) is the most frequently reported class D beta-lactamase [8].

OXA - 48-producing isolates are frequently multidrug - resistant, as they combine multiple resistance mechanisms. This enzyme shows different hydrolysing activities against β - lactam antibiotics, with high activity against penicillins but only low activity against carbapenems. OXA - 48 carbapenemase has very weak activity against third - generation and fourth - generation cephalosporins; however, these are seldom a therapeutic option because other β - lactamases, such as extended - spectrum β - lactamases (ESBL), are frequently associated [9]. The problem of OXA - 48 is that it may go undetected and classified as susceptible with imipenem and meropenem MIC of (≤ 1 μg/ml) according to Clinical and Laboratory Standards Institute (CLSI, 2015) and (≤ 2 μg/ml) according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines respectively [11]. MIC determination alone is not accurate for the screening of OXA - 48.

Rapid detection of carbapenemases is crucial to ensure early detection and implementation of control measures in hospitals. Preliminary screening by Disk Diffusion should be confirmed by Modified Hodge test (MHT) according to the recommendation of CLSI [12]. However, MHT requires at least 24 - 48 h and thereby it is time-consuming. Also, it lacks specificity and may give false positive results or fail to detect MBLs [13].

Molecular techniques remain the reference standard for the identification and differentiation of carbapenemases. The most common of which is PCR. Sequencing is sometimes done following PCR for precise identification of a carbapenemase, rather than just its group (e.g. VIM - type, KPC - type, NDM - type, and OXA - 48 - type) [13].

Materials and Methods

Patient population

This study was conducted on 50 specimens randomly selected from different clinical specimens submitted to the Microbiology Laboratory of Ain Shams University Hospitals for routine culture and susceptibility testing that showed resistance to carbapenem drugs by both Kirby Bauer disk diffusion method (They were nonsusceptible, i.e., intermediate or resistant to the carbapenems if zone diameter was < 23 for meropenem (10 μg) and imipenem (10 μg) and Broth microdilution method if MICs were > 1 μg/ml for meropenem and imipenem (10). The isolates included 31 (62%) Klebsiella species, 12 (24%) E.coli, 5 (10%) Serratia and 2 (4%) Citrobacter.

Most disks ID inhibitor combination disks (MDI)

Most disks ID inhibitor combination disks (MDI) (Mast Diagnostics) method was performed according to the manufacturer's instructions. Four disks were included: disk A, containing a carbapenem (meropenem, 10 μg); disk B, consisting of meropenem (10 μg) and an MBL inhibitor; disk C, consisting of meropenem (10 μg) with a KPC inhibitor; and disk D, containing meropenem (10 μg) with an AmpC inhibitor. The interpretation of the test is as follows. The zone of inhibition of disk A is compared to the zones of inhibition of each of disks B, C, and D. If disk B shows a zone difference of ≥ 5 mm from disk A, the organism is considered as producing MBL activity as in (Figure 1). If disk C shows a zone difference of ≥ 4 mm from disk A, the organism considered as producing KPC activity. If disk C and disk D both show a zone difference of ≥ 5 mm from disk A, the organism is considered as producing AmpC activity coupled with porin loss (impermeability).

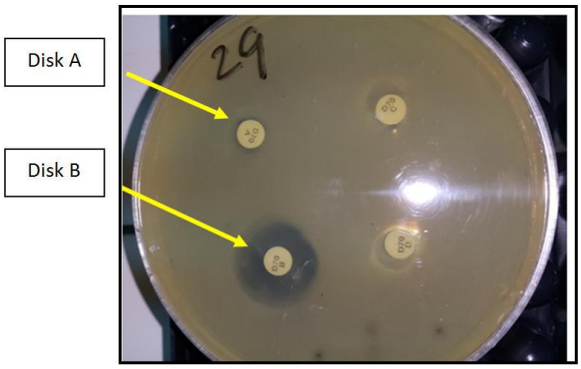

Figure 1: Mastdisks inhibitor combination set showing MBL producing strain (Disk B > disk A by 5 mm)

Detection of OXA 48

Temocillin testing using a modified zone diameter cut - off of < 12 mm (Figure 2) was used as a tool for discriminating Ambler class D carbapenemase producers [14].

E. coli ATCC 25922 and Klebsiella ATCC 700603 were included as negative control.

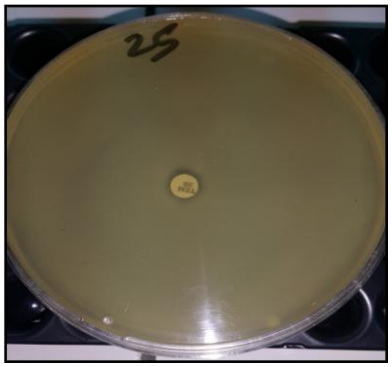

Figure 2: Disc diffusion susceptibility test showing Temocillin resistant strain (OXA48 producer)

Genotypic Identification

All test isolates were subcultured on blood agar overnight at 36°C, and five selected colonies were suspended in 1.5 ml microtubes containing 180 µL sterile distilled water, then whole genomic DNA was extracted by QIAamp DNA Mini kit (QIAGEN Sample and Assay Technologies, Germany) according to manufacturer's instruction. Suitable primers each targeting selected regions of the *blaKPC, blaNDM, blaOXA – 48 - like blaVIM* and *blaIMP* genes were used (Table 1). Amplification reactions were performed in a final volume of 25 µL containing 12.5 µL 2 x HRM PCR Master Mix (Thermo Scientific, Lithuania, EU), and two primer mix, 9.5 µL RNase - free H2O, and one µL of the template DNA. The optimized cycling protocol for High Resolution Melting (HRM) Analysis was as follow: initial denaturation at 95°C for 5 minutes followed by 40 cycles at 95°C for 10 seconds, 55°C for 30 seconds, and 72°C for 10 seconds, followed by HRM analysis of 65 - 95°C for 2 seconds (Rotor-Gene, QIAGEN) [15].

Table 1: Primers targeting selected regions of the *blaKPC, blaNDM, blaOXA-48-like, blaVIM,* and *blaIMP* genes (Monteiro et al. 2012)

Template	Primer	Sequence (5'-3')
blaOXA-48	Forward primer	TGTTTTTGGTGGCATCGAT
	Reverse primer	GTAAMRATGCTTGGTTCGC
blaNDM-1	Forward primer	TTGGCCTTGCTGTCCTTG
	Reverse primer	ACACCAGTGACAATATCACCG
blaKPC	Forward primer	TCGCTAAACTCGAACAGG
	Reverse primer	TTACTGCCCGTTGACGCCCAATCC
blaIMP	Forward primer	GAGTGGCTTAATTCTCRATC
	Reverse primer	AACTAYCCAATAYRTAAC
blaVIM	Forward primer	GTTTGGTCGCATATCGCAAC
	Reverse primer	AATGCGCAGCACCAGGATAG

Results

Phenotypic detection of carbapenem-resistant classes using carbapenemase detection set by (MastDiagnostics) and temocillin disk revealed that the highest positive results were for OXA48 (detected in 96% of samples), followed by MBLs (82%), then for KPC (34%), while all samples were negative for *AmpC*.

Some specimens showed two or more positive carbapenemase producing enzymes, 46% of specimens were positive for both OXA 48 and MBL, whereas 32% specimens were positive for OXA 48, MBL and KPC in combination. Only 2% showed positive results for both OXA 48 and KPC.

By applying real-time PCR (singleplex) to detect *blaKPC, blaNDM, blaOXA – 48 - like, blaVIM and blaIMP* genes in our samples, *blaOXA – 48 - like* gene showed the highest percentage (96%) followed by *blaVIM, blaNDM, blaKPC,* and *blaIMP* with (94%) (54%), (46%), and (40%), respectively (Figure 3).

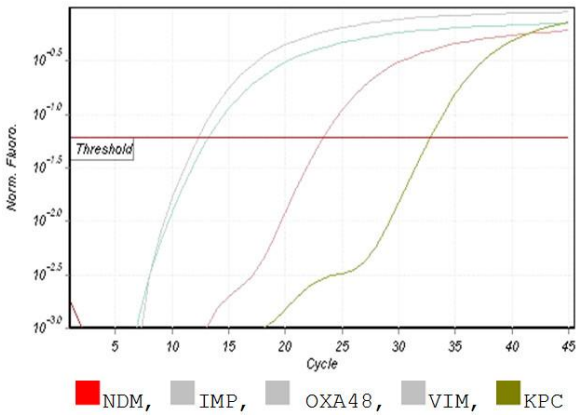

Figure 3: Melt data for HRM; The isolate was positive for OXA48, IMP, KPC, VIM and negative for NDM

Statistical evaluation of the diagnostic performance of the carbapenemase detection set and temocillin disk against PCR showed, a significant difference between both methods regarding KPC and MBLs (P-value < 0.001). However, no significant difference was detected between temocillin disk and PCR regarding OXA - 48 (P value was 2.239).

By comparing the results of temocillin disk specifically used for detection of OXA 48 and PCR, 47 out of 50 specimens were true positive (97.9%) and one specimen was a false negative (2.1%). One out of two specimens was true negative (50%), and one specimen was false positive (50%). Temocillin disk had 97.9% sensitivity, 50% specificity, 97.95% PPV, 50% NPV and efficacy was 73.95%.

Evaluation of class A (KPC) detection by using disk C compared to PCR detection of the *blaKPC* gene showed that 15 out of 23 specimens were true positive (65.2%) and eight were a false negative (34.8%). Whereas 25 out of 27 were true negative (92.6%) and two were false positive (7.4%). Sensitivity, specificity, PPV, NPV was 65.2%, 92.6%, 88.24%, 75.78% respectively and efficacy was 78.9%.

We compared results of class B (MBL) detection by using disk B with PCR to detect blaVIM, blaNDM, and blaIMP genes. It was found that 40 out of 46 (87%) specimens were true positive and six

(13%) was a false negative. Whereas three out of 4 (75%) specimens were true negative and one (25%) was a false positive. Sensitivity, specificity for PPV, NPV was 87%, 75%, 97.5%, 33% respectively and efficacy was 90.2%.

Finally, the whole detection set was evaluated according to PCR. It was shown that 33 out of 40 specimens were true positive (82.5%) and seven were a false negative (17.5%). Whereas seven out of ten specimens were true negative (70%) and three were false positive (30%). Overall sensitivity, specificity, PPV, NPV were 82.1%, 70%, 91.6%, 50% respectively and efficacy was 85.7%.

Evaluation of the combined use of Temocillin disk and Mastdisks ID inhibitor combination set (MDI) in parallel for detection of carbapenem-resistant *Enterobacteriaceae* showed 99.7% sensitivity and 35% specificity.

A high percentage of positive isolates for OXA 48 (96%) and the low percentage of negative OXA 48 isolates (4%) resulted in Low specificity for Temocillin disk as well as specificity of Temocillin disk and MDI in combination. One isolate was a false negative (2.1%), one out of two isolates (that were negative by PCR) was true negative (50%), and one was false positive (50%).

Discussion

Antibiotic resistance is a major cause for concern in *Enterobacteriaceae* family. The rate of resistance is increasing especially carbapenem-resistant *Enterobacteriaceae.* Therefore, the need for a simple and accurate method is crucial for detection of these bacteria.

Some phenotypic confirmation tests have been performed for the detection of carbapenemase-producing *Enterobacteriaceae*. These include; modified Hodge test (MHT), inhibitor - based methods using metal chelators for MBLs (e.g., MBL Etest) and boronic acid for KPCs [13].

In this study, Mastdisks ID inhibitor combination disks kit (MDI) (Mast Diagnostics) was evaluated for the detection of Enterobacteriaceae producing carbapenemases. Sanjeev and Mehra (2015), stated that simultaneous using of both inhibitors (PBA for KPC detection and DPA for MBL detection) as in our study, seems to restrict the activity of both carbapenemases against meropenem, allowing the detection of isolates that co-produce these enzymes in almost all cases [16]. Their study has shown the combined use of two inhibitors can detect and differentiate carbapenemase production. However, Mastdisks combination inhibitor set was not designed to detect OXA – 48 - like B -lactamase in *Enterobacteriaceae*. That is why we performed disk diffusion assay with temocillin disk to detect OXA – 48 - like producing *Enterobacteriaceae*. The high - level resistance to the B - lactam temocillin might be presumptive evidence for the presence of OXA - 48 [17].

We found that 96% of our isolates were sensitive to temocillin disk used for OXA - 48 detection. In areas where OXA - 48 producers are predominant, temocillin testing using a modified zone diameter cut - off of < 12 mm [18], could be an easy and useful tool for discriminating Ambler class D carbapenemase producers from extended spectrum b - lactamases and/or *AmpC* - producing isolates among carbapenem – non - susceptible *Enterobacteriaceae* isolates. About temocillin, 98.1% (317/323) of the OXA – 48 - producing *Enterobacteriaceae* isolates displayed disk inhibition zones of < 12 mm compared with only 10.0%(92/919) of the carbapenemase - negative isolates [14].

Also, 82% and 34% were positive for MBLs using disk B and KPC using disk C respectively. These results were concordant with a study conducted by Doyal and co-workers (2012) [19], who stated that 100%, 93% and 49% of their isolates were positivity for OXA - 48, MBLs and KPC detection respectively. Another study conducted by Andrea *et al.* (2014) [20], they found that 92% of their CPE isolates were OXA 48 positive, 49.5% were harbouring double positive KPC and MBL when used the Mastdisks ID inhibitor combination. Although their study was in Italy, their results were similar to our results, so we can conclude that class D is prevalent there like in Egypt while other classes are not.

A high prevalence of CPE can be found in southern Europe and Asia, Greece, Italy, Turkey, and Israel than in other parts of the world. Historical/cultural relationship and exchange of populations with other countries of high prevalence can influence the type of CPE. Cross-border transfer of patients, travel, medical tourism and refugees might also play an important role. This is particularly true for the spread of OXA - 48 in France and Belgium from North Africa, and in Germany, probably from Turkey, or the identification in UK of NDM - 1 producers of Indian origin. The increasing frequency of CPE could be related to the increasing prevalence of OXA - 48 producers in many European countries and the exportation of KPC producers from Greece and Italy to other European countries [21].

Some of our isolates showed double or triple positive for carbapenemase enzymes. Both OXA 48 and MBL were positive in 23 isolates (46%), whereas 32% showed positive results for OXA48, MBL and KPC altogether. Only (2%) were positive for OXA48 and KPC together (2%). This means that OXA - 48 was positive in most our isolates in combination with other classes. OXA - 48 enzyme in *Enterobacteriaceae*, can give weak level carbapenem

resistance and with cross-resistance with KPC enzymes and MBLs tend to confer broader effects on the resistance profile of the host strain [22]. OXA - 48 enzyme was positive in most of our isolates combined with other resistant classes that made OXA - 48 carbapenemase production more evident and which otherwise couldn't be detected if present alone.

In this study, we applied real-time PCR to detect *blaKPC, blaNDM, blaOXA – 48 - like blaVIM,* and *blaIMP* genes. OXA 48 gene showed the highest percentage (96%) then VIM (94%) followed by NDM (54%), KPC (46%) and finally IMP (40%). This could be explained by the fact that Amber class D is weak and needs the coexistence of other classes to be expressed and so had the highest percentage. Also, in a study conducted by Samar (2016); *blaOXA – 48 - like* gene's percentage was the highest (28.6%), followed *byblaKPC* (19%), *blaVIM* (9.5%) and finally *blaNDM* (2.4%) [23].

Most disks ID inhibitor combination disks had a sensitivity and specificity of 87% and 75% respectively for detecting MBL producers, 65.2% and 92.6% respectively, for detecting KPC producers. In our study, evaluation of carbapenemase detection set for detection of all classes of CPE in comparison to PCR showed sensitivity 82.1%, specificity 70%, PPV 91.6% and NPV 50%. Samar [25]; in her study stated that the combined disk test showed sensitivity 100% and specificity 88.9%. Doyal and co-workers *(*2012), stated that overall, the sensitivity and specificity for MDI were 78% and 93% respectively, MDI performed well for the detection of KPCs and NDMs but poorly for VIMs, IMPs, and OXA – 48 - like enzymes [19].

On the other hand, temocillin disk had 97.9% sensitivity, 50% specificity, 97.95% PPV and 50% NPV and efficacy was 73.95%. Another study conducted by Woodford *et al.* (2014) in England, the sensitivity and specificity of temocillin were 90.7% and 88.2% respectively with PPV 27.6 % and NPV 99.5% [24].

Huang and co-workers (2014), support previous data and confirm that high - level resistance to temocillin is a highly sensitive and specific phenotypic surrogate marker of OXA - 48 productions in strains with decreased susceptibility to a carbapenem, although in itself it could not differentiate OXA - 48 from VIM - type carbapenemases [18], [25].

However, the overall sensitivity and specificity of both Temocillin disk and carbapenemase detection set tests in our study for detection of carbapenem-resistant *Enterobacteriaceae* was 99.7% and 35% respectively.

Low specificity for Temocillin disk as well as specificity of Temocillin disk and MDI in combination could be explained by the high percentage of positive isolates for OXA 48 (96%) and the low percentage of negative isolates (4%). Only two isolates were negative by PCR, one was true negative (50%), and the other was false positive (50%) thus reducing Temocillin disk test specificity.

So, adding Temocillin disk to carbapenemase detection set provides an easy, inexpensive, rapid and highly sensitive test for screening of a large number of isolates for CPE. However, it still needs confirmation by molecular techniques.

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Basic Science

Value of SSTR2A and Claudin - 1 in Differentiating Meningioma from Schwannoma and Hemangiopericytoma

Shady E. Anis[1*], Mira Lotfalla[1], Muhammad Zain[2], Nora N. Kamel[3], Ahmed A. Soliman[1]

[1]Cairo University School of Medicine - Pathology, Cairo, Egypt; [2]Al - Azhar University School of Medicine - Pathology, Cairo, Egypt; [3]National Research Center - Pathology, Giza, Egypt

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***Correspondence:** Shady E. Anis. Cairo University School of medicine - Pathology, Cairo, Egypt. E-mail: shadyeliaanis76@yahoo.com

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Abstract

BACKGROUND: The distinction between meningioma, schwannoma and solitary fibrous tumour/ hemangiopericytoma can be challenging in some cases. This study evaluates the expression of Somatostatin receptor 2A (SSTR2A) and Claudin-1 in these different tumours.

MATERIAL AND METHODS: Thirty-five cases of meningioma, 10 cases of intracranial schwannoma and 10 cases of hemangiopericytoma were assessed. The immunohistochemical expression of SSTR2A and Claudin-1 was evaluated and scored according to the percentage of immunostained tumour cells (0: 1+, 2+ and 3). The intensity of staining was classified as weak, moderate and strong.

RESULTS: Positivity for SSTR2A and Claudin-1 was encountered in 89% and 49% of meningiomas respectively. None of the schwannomas or hemangiopericytomas was positive for any of both markers. All grade I and II meningiomas were positive for SSTR2A, and only 20% of grade III showed positive staining ($p < 0.05$). Claudin-1 positivity was detected in 50%, 43% and 60% of grade I, II and III meningioma respectively, with significantly higher intensity in grade III ($p < 0.05$).

CONCLUSION: SSTR2A is highly sensitive and specific for meningioma. Claudin-1 is highly specific for meningioma, with low sensitivity. The adjunctive use of both markers can be very helpful in the diagnosis of meningioma and its distinction from schwannoma and hemangiopericytoma.

Introduction

Meningiomas are the most common primary central nervous system (CNS) tumours accounting for 36% of all primary CNS tumours [1].

Since the first classification of meningioma introduced by Cushing in 1920 [2]; according to anatomical location; different classification schemes, adopted histology as the main factor in grading meningioma [3]. The recent WHO 2016 classification system, has grouped meningioma according to biological behavior into two groups, (1) Meningiomas with low risk of recurrence and aggressive behavior, including variants of WHO grade I meningiomas, and (2) Meningiomas with greater likelihood of recurrence and aggressive behavior, including variants of WHO grade II and grade III meningiomas and any subtype with high proliferation index, defined in one study as > 20 mitosis /10 HPF [4].

Both schwannomas of the cranio/spinal axis and meningeal Solitary fibrous tumour/ Hemangiopericytoma occur at a much lower frequency than meningioma. However, the distinction between these entities and meningioma can be challenging in some cases. Additional immunohistochemical studies are needed to resolve such cases [3]. The traditionally used immunohistochemical markers show some overlap in the expression between these entities [5][6][7].

Somatostatin receptors (SSTR) belong to a family of seven alpha-helical transmembrane spanning domains G protein-coupled receptors. They mediate the action of Somatostatin [8]. Somatostatin (SST) exerts inhibitory actions on some physiologic processes including pituitary and pancreatic hormone secretion, gastrointestinal peptide secretion and

motility. In the CNS, it plays a role as a neurotransmitter and neuromodulator affecting behaviour and cognition [9]. Finally, SST has a potent antiproliferative and antiangiogenic activity. Thus it can be used as an anti-neoplastic agent in tumours that express Somatostatin receptors [10][11].

The expression of somatostatin receptors is known to be frequent in meningioma [12]. There are five subtypes of somatostatin receptors (SSTR1-5). Among the five subtypes, SSTR2A was the most frequently detected in meningioma [8][13].

Claudin - 1 is one of the main components of tight junction, normally expressed in epithelial, endothelial and arachnoid cap cells, functioning as a regulator for paracellular space; controlling the barrier function of the cells and preserving cellular polarity and integrity [14].

Claudin - 1 has been recently identified as a tumor marker expressed in many tumors; e.g.: renal cell carcinoma, colonic adenocarcinoma and melanoma, where its increased expression and mislocalization were correlated with the bad behavior encountered in such tumors, e.g. metastatic potential [15]; through its inhibitory effect on E - cadherin and Beta-catenin inducing epithelial-mesenchymal transmission; a major step in metastatic process [16].

This study evaluates SSTR2A and Claudin-1 immunohistochemical staining in meningiomas versus cranio - spinal schwannomas and meningeal solitary fibrous tumours/hemangiopericytomas, to determine if these two markers can help in this differential diagnosis and to add specific markers for meningioma that can be targeted therapeutically.

Materials and Methods

A total of 55 cases of CNS tumours were retrieved from the neuropathology files at Cairo University Hospital between 2012 and 2015. The ethics committee of Cairo University Hospital approved the study. The cases include 35 meningiomas (22 females, 13 males), ten schwannomas (5 females, five males) and ten solitary fibrous tumours/hemangiopericytomas (6 females, four males). The mean age of patients was 42 years in cases of meningioma, 40 years in cases of schwannoma and 44 years in cases of solitary fibrous tumours/hemangiopericytoma. All cases were previously diagnosed by examination of Hematoxylin and Eosin stained sections and by routine immunohistochemical markers.

Histological review: Five microns - thick tissue sections were cut from the archived paraffin blocks and stained by Hematoxylin and Eosin for histological re-evaluation according to the WHO criteria [3], the cases of meningioma are classified as grade I (n = 16), grade II (n = 14) and grade III (n = 5). Among grade I meningiomas, five were transitional, four fibroblastic, three meningothelial, two psammomatous, one microcystic and one angiomatous. Grade II meningiomas included ten atypical and four chordoid and grade III meningiomas included three papillary and two rhabdoid variants.

Immunohistochemical staining and evaluation: Additional cuts were prepared from the paraffin blocks, heat mediated antigen retrieval was performed (with low pH for SSTR2A and high pH for Claudin - 1) in automated water bath (Dako PT Link), and sections were stained with antibodies for SSTR2A (Abcam, UMB1, rabbit monoclonal, 1:100) and Claudin - 1 (Cell Marque, rabbit polyclonal, ready to use). Staining was performed in an autostainer (Dako autostainer link 48) using a polymer-based detection system (Dako EnVision FLEX^{TM}K8000).

Immunohistochemical staining for SSTR2A and Claudin - 1 was scored according to the percentage of immunostained tumour cells (0: less than 5%, 1+: 5% to 25%, 2+: 26% to 50%, 3+: more than 50%). The intensity of staining was classified as weak, moderate and strong. In claudin - 1 evaluation, the perineurial cells in peripheral nerves are used as a control for moderate intensity [14].

Statistical methods: Comparison of numerical variables between the study groups was done using Mann Whitney *U* test for independent samples. For comparing categorical data, Chi-square (χ^2) test was performed. The exact test was used instead when the expected frequency is less than 5. *P* values less than 0.05 was considered statistically significant. All statistical calculations were done using computer program SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) release 15 for Microsoft Windows (2006).

Results

Somatostatin receptor 2A

Positive immunohistochemical staining for SSTR2A was encountered in 31 of 35 (89%) cases of meningioma (Table 1). All positive cases showed cytoplasmic and/or membranous staining. Among the positive cases, 24 of 31 (77%) showed diffuse staining in more than 50% of tumour cells (scored as 3+). Among these 24 cases, 16 showed strong immunostaining intensity, 7 showed moderate intensity, and only 1 showed weak staining (Figure 1).

In contrast, none of the cases of

schwannoma and solitary fibrous tumour/ hemangiopericytoma showed any positive staining for SST2A (Table 1). Therefore, the expression of SSTR2A was statistically significant in meningioma versus schwannoma or solitary fibrous tumours/ hemangiopericytoma (P <0.05) (Figure 2).

Table 1: Summary of immunohistochemical staining results for meningioma, schwannoma and Solitary fibrous tumors/Hemangiopericytoma [number (percentage)]

	Meningioma (n=35)	Schwannoma (n = 10)	Hemangiopericytoma (n = 10)
SSTR2A positive	31 (89)	0 (0)	0 (0)
Claudin-1 positive	17 (49)	0 (0)	0 (0)
SSTR2A&/or Claudin - 1 positive	34 (97)	0 (0)	0 (0)

SSTR2A, somatostatin receptor 2A

Regarding the different grades of meningioma, a significant correlation was found between the positive expression of SSTR2A and the lower grades of meningioma (grades I and II) (P <0.05) (Table 2).

Table 2: Summary of immunohistochemical staining results for different grades of meningioma [number (percentage)]

	Grade I (n = 16)	Grade II (n = 14)	Grade III (n = 5)
SSTR2A positive	16 (100)	14 (100)	1 (20)
Claudin-1 positive	8 (50)	6 (43)	3 (60)

In grade I, all cases (16/16) showed positive SSTR2A expression. Among these cases, 13 showed diffuse staining in more than 50% of tumour cells (Score 3+). The majority of these cases displayed strong immunostaining intensity. In grade II, all cases (14/14) showed positive SSTR2A expression. 10 out of these 14 cases showed diffuse staining in more than 50 % of cells (Score 3+), but only 4 of them showed strong intensity. In grade III, only a single (1/5) case of papillary subtype showed positive SSTR2A expression. The staining, in this case, was diffuse in more than 50% of tumour cells (Score 3+); however, the intensity was weak.

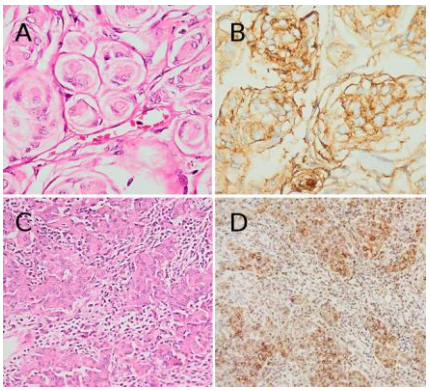

Figure 1: Transitional meningioma, WHO grade I (A: H&E, x400) showing strong diffuse SSTR2A immunostaining (B: SSTR2A, x400). Rhabdoid meningioma, WHO grade III (C: H&E, x200) showing strong diffuse Claudin - 1 immunostaining (D: Claudin-1, x200) immunostaining of claudin 1 (D: claudin 1x400)

Claudin-1

Positive immunohistochemical staining was encountered in 17 of 35 (49%) cases of meningioma. Tumor cells showed cytoplasmic and/or membranous staining. Unlike SSTR2A, Claudin - 1 showed only focal staining in less than 50% of tumour cells in all positive cases (Figure 1).

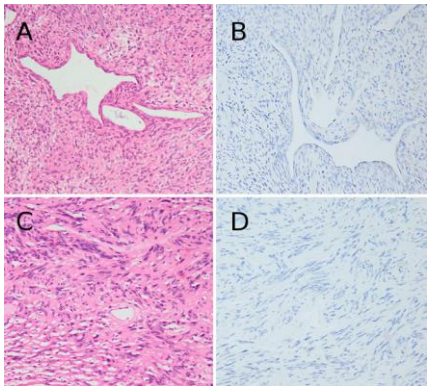

Figure 2: Hemangiopericytoma (A: H&E, x400) showing negative immunostaining for SSTR2A (B: SSTR2A, x400). Schwannoma (C: H&E, x400) showing negative

However, a significant expression of Claudin - 1 was still detected in meningioma in comparison to schwannoma and solitary fibrous tumour/ hemangiopericytoma (P< 0.05) since none of them showed any positive staining (Table 1) (Figure 2).

Table 3: Intensity of immunohistochemical staining of Claudin-1 in different grades of meningioma [number (percentage)]

	Grade I (n=16)	Grade II (n=14)	Grade III (n=5)
Negative	8 (50)	8 (57)	2 (40)
Weak	2 (13)	3 (21.5)	0 (0)
Moderate	5 (31)	3 (21.5)	0 (0)
Strong	1 (6)	0 (0)	3 (60)

As for the three grades of meningioma, positive Claudin - 1 expression was detected in 8 out of 16 grade I cases, 6 out of 14 grade II cases and 3 out of 5 grade III cases. The intensity of Claudin - 1 staining was significantly stronger in grade III than in grades I and II (P <0.05) (Table 3). Interestingly, the three positive cases of grade III were negative for SSTR2A. More details of SSTR2A and Claudin - 1 staining in different grades and subtypes of meningioma are shown in (Table 4).

Discussion

Although the diagnosis of most meningiomas can be based merely on routine examination of Hematoxylin and Eosin stained sections, the histologic mimicry between certain subtypes and other CNS tumours warrants the use

of immunohistochemical tests. A common example is a distinction between meningioma, particularly the fibroblastic subtypes, and schwannoma, especially if arising at the cerebello - pontine angle.

Table 4: Details of immunohistochemical staining of SSTR2A and Claudin - 1 in all cases of meningioma

Case	Grade	Subtype	SSTR2A		Claudin-1	
1	I	Transitional	3+	Strong	1+	Moderate
2	I	Transitional	3+	Strong	1+	Moderate
3	I	Transitional	3+	Strong	1+	Moderate
4	I	Transitional	3+	Moderate	0	0
5	I	Transitional	1+	Weak	0	0
6	I	Fibroblastic	3+	Strong	2+	Moderate
7	I	Fibroblastic	3+	Strong	0	0
8	I	Fibroblastic	3+	Strong	0	0
9	I	Fibroblastic	3+	Strong	0	0
10	I	Meningothelial	3+	Strong	1+	Weak
11	I	Meningothelial	2+	Moderate	2+	Moderate
12	I	Meningothelial	2+	Moderate	0	0
13	I	Psammomatous	3+	Strong	2+	Strong
14	I	Psammomatous	3+	Strong	0	0
15	I	Microscystic	3+	Strong	0	0
16	I	Angiomatous	3+	Strong	1+	Weak
17	II	Chordoid	3+	Moderate	1+	Weak
18	II	Chordoid	3+	Moderate	1+	Weak
19	II	Chordoid	3+	Moderate	0	0
20	II	Chordoid	2+	Moderate	0	0
21	II	Atypical	3+	Strong	1+	Moderate
22	II	Atypical	3+	Strong	1+	Weak
23	II	Atypical	3+	Strong	0	0
24	II	Atypical	3+	Strong	0	0
25	II	Atypical	3+	Moderate	1+	Moderate
26	II	Atypical	3+	Moderate	0	0
27	II	Atypical	3+	Moderate	0	0
28	II	Atypical	2+	Moderate	0	0
29	II	Atypical	2+	Weak	2+	Moderate
30	II	Atypical	1+	Weak	0	0
31	III	Papillary	3+	Weak	0	0
32	III	Papillary	0	0	1+	Strong
33	III	Papillary	0	0	0	0
34	III	Rhabdoid	0	0	2+	Strong
35	III	Rhabdoid	0	0	1+	Strong

This differential diagnosis should also be considered in patients diagnosed with Neurofibromatosis type 2 since these patients are prone to develop both tumours. Histologically, fibroblastic meningioma and schwannoma are formed of spindle cells with the variable collagenous background. Occasionally, well-formed whorls that are characteristic for meningioma are seen in schwannoma. On the other side, meningioma can show Verocay body - like structures similar to those seen in schwannoma. Although most meningiomas express epithelial membrane antigen (EMA), a small subset of cases does not. Also, S100 - which is routinely used for diagnosis of schwannoma- can stain up to 70% of fibroblastic meningiomas [5].

Another problematic case is the differential diagnosis of meningioma versus solitary fibrous tumour/hemangiopericytoma. Some meningiomas develop branching staghorn vessels like those encountered in solitary fibrous tumour/ hemangiopericytoma [5]. Previous studies had shown that occasional cases of solitary fibrous tumour/ hemangiopericytoma might focally express EMA [7]. Also, CD34, which is a marker of solitary fibrous tumour/hemangiopericytoma, can be expressed in up to 60% of fibroblastic meningiomas [6].

In the present study, we compared the immunohistochemical expression of SSTR2A and Claudin - 1 in meningioma versus their expression in schwannoma and solitary fibrous tumour/ hemangiopericytoma.

The expression of somatostatin receptors is known to be frequent in meningioma [12]. Among the five subtypes of somatostatin receptors, SSTR2A was the most frequently detected in meningioma [13]. This wide expression has made it a useful tool in tumour imaging by PET/CT using radiolabeled somatostatin analogues [17].

In our study, we detected the immunohistochemical expression of SSTR2A in meningiomas with a sensitivity of 89%. This is comparable with the findings detected by Bacchi et al. [18], Agaimy et al. [19] and Menke et al. [20] in their studies that showed sensitivities of 100%, 87% and 100 % respectively. Lower sensitivities of 74% and 63% were stated by Barresi et al. [21] and Durand et al. [22] respectively. This difference may be because they used polyclonal antibodies in their studies, in contrast to the monoclonal antibody used in the current study.

We further analysed the expression of SSTR2A in different grades and subtypes of meningioma. The highest expression was linked to lower grades of meningioma (grade I and II) ($p < 0.05$). It was also noted that despite the positive expression of SSTR2A in all cases of grade I and II, there was still a difference in the intensity of immunostaining among the grades. Most of the cases of grade I (75%) showed strong staining intensity while only 28% of grade II cases showed strong intensity and the rest showed moderate or weak intensity. As for grade III meningiomas, only one case was positive for SSTR2A, and the intensity of staining was weak.

Our findings are in concordance with those reported by Durand et al. [22] who analysed the expression of SSTR2A in meningiomas by both immunohistochemistry and RT - PCR. By immunohistochemistry, the expression of SSTR2A was negative in grade III meningiomas. By RT - PCR, the SSTR2A mRNA was detected in all grades of meningioma with higher levels expressed in grade I more than in grade II and III.

Since the expression of SSTR2A was more intense in grade I meningiomas and became lost in most of grade III cases, we suggest that detection of strong immunohistochemical staining of SSTR2A may predict a better outcome. Previous studies were done on other types of tumours also reached the same conclusion. For example, Sestini et al. [23] and Raggi et al. [24] studied SSTR2A expression in neuroblastoma and found out that it was inversely related to the tumour stage and was shown to be a good independent prognostic factor. Similarly, in colorectal carcinoma, SSTR2A expression was increased in well and moderately differentiated tumours and with lower proliferation indices [25].

In all cases of schwannoma and solitary

fibrous tumor/hemangiopericytoma selected for the present study, SSTR2A showed negative staining. Accordingly, the specificity of SSTR2A for meningioma is 100%. This was statistically highly significant. Bacchi et al. [18] and Menke et al. [20] reported slightly lower specificities of 90% and 88% respectively.

Regarding Claudin - 1, its sensitivity for meningioma was 49% in our study. The previous study done by Rajaram et al. [7] included anaplastic (grade III) meningiomas only and showed a sensitivity of 54%. Hahn et al. [14] included grade I and II meningiomas and showed a sensitivity of 53 %, which is relatively close to our results. Slightly lower sensitivity (22%) was reported by Soini et al. [26] who included all grades of meningioma. This difference may be because they used tissue micro-array blocks with a 2 mm diameter.

Despite its low sensitivity for meningiomas, Claudin - 1 did not stain any of the schwannomas or solitary fibrous tumours/ hemangiopericytomas included in our study, denoting a very high specificity (100%) for meningioma. Similar to our results, Singh et al. [27] reported negative Claudin-1 staining in the 50 cases of schwannoma included in their study. Hahn et al. [14] also reported negative Claudin - 1 staining in all the studied cases of meningeal solitary fibrous tumour/ hemangiopericytoma and schwannoma. Rajaram et al. [7] studied Claudin - 1 expression in 15 cases of meningeal solitary fibrous tumour/ hemangiopericytoma and found positive staining in 2 cases.

We detected positive expression of Claudin - 1 in the different grades of meningioma without a significant difference in positivity (50% of grade I, 43% of grade II and 60% of grade III). Soini et al. [26] also reported no difference in the Claudin - 1 expression among the three grades of meningioma. However, we detected that the intensity of staining was significantly higher in grade III than in grades I and II ($p > 0.05$).

In the current study, we found out that 34 of 35 meningiomas expressed either SSTR2A or Claudin - 1, or both of them, i.e. the sensitivity of both markers combined is 97%. Interestingly, the cases of grade III meningiomas that showed positive Claudin - 1 staining was negative for SSTR2A. On the other side, the single case of grade III meningioma (papillary subtype) that was positive for SSTR2A did not stain for Claudin - 1. Thus SSTR2A and Claudin - 1 can be used as complimentary markers with high sensitivity.

Therapeutic strategies in meningiomas include mainly surgery and radiotherapy, while chemotherapy has been used for a patient with the progressive disease, and patients with histologically malignant meningioma as an adjuvant for radiotherapy, however, the response to chemotherapy was disappointing; so the targeted therapy in such cases can be a new hope [28]. In vitro studies proved that somatostatin analogues have a cytostatic effect on tumor cells and inhibits the tumor growth [10][29]. However, the efficacy of the use of somatostatin analogues in a clinical setting is still debatable with some trials showing benefit for their use and others do not [30][31][32]. The loss of expression of SSTR2A in malignant meningioma, as shown in the present study, may explain the failure of some clinical trials to prove the efficacy of somatostatin analogues in treating recurrent high-grade meningioma [33].

Recently, Hashimoto and his colleagues generated mouse anti - Claudin-1 monoclonal antibodies and assessed their activity on mice bearing human Claudin - 1 expressing tumours. They concluded that one of these antibodies might be of benefit in cancer therapy [34]. So Claudin - 1 can be one of the targeted therapy lines in meningioma therapy.

In summary, our study demonstrates that SSTR2A is highly sensitive and specific for meningioma. Claudin - 1 is highly specific for meningioma; however its sensitivity is low. The adjunctive use of both markers can be very helpful in the diagnosis of meningioma and its distinction from schwannoma and solitary fibrous tumor/ hemangiopericytoma. Further clinicopathological studies are recommended to correlate the pattern of SSTR2A and Claudin - 1 expression in meningiomas with their potential prognostic and predictive roles in such tumors, specifically aggressive and recurring ones.

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Basic Science

Preclinical Assessment of the Proliferation Capacity of Gingival and Periodontal Ligament Stem Cells from Diabetic Patients

Mostafa Assem[1], Samia Kamal[2], Dina Sabry[3], Nadia Soliman[1], Riham M. Aly[1*]

[1]National Research Centre, Cairo, Egypt; [2]Cairo University Faculty of Oral and Dental Medicine, Cairo, Egypt; [3]Cairo University, Kasr Alainy Faculty of Medicine, Cairo, Egypt

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***Correspondence:** Riham M Aly. National Research Centre Cairo, Egypt. E-mail: riham.aly@gmail.com

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Abstract

BACKGROUND: Stem cells have recently received great interest as potential therapeutics alternative for a variety of diseases. The oral and maxillofacial region, in particular, encompasses a variety of distinctive mesenchymal (MSC) populations and is characterized by a potent multilineage differentiation capacity.

AIM: In this report, we aimed to investigate the effect of diabetes on the proliferation potential of stem cells isolated from controlled diabetic patients (type 2) and healthy individuals.

SUBJECTS & METHODS: The proliferation rate of gingival and periodontal derived stem cells isolated from diabetic & healthy individuals were compared using MTT Assay. Expression levels of Survivin in isolated stem cells from all groups were measured by qRt - PCR.

RESULTS: There was a significantly positive correlation between proliferation rate and expression of Survivin in all groups which sheds light on the importance of Survivin as a reliable indicator of proliferation. The expression of Survivin further confirmed the proliferation results from MTT Assay where the expression of stem cells from non - diabetic individuals was higher than diabetic patients. Conclusion: Taking together all the results, it could be concluded that PDLSC and GSC are promising candidates for autologous regenerative therapy due to their ease of accessibility in addition to their high proliferative rates.

Introduction

The capacity of mesenchymal stem cells to self - renew and to differentiate into multiple cells strongly suggests their role as a promising candidate for future cell-based therapies [1][2][3]. The oral and maxillofacial region encompasses a variety of distinctive mesenchymal (MSC) populations, isolated from the tooth and its supporting structures, and is characterised by a potent multilineage differentiation capacity and immunomodulatory properties [1][2][3][4][5]. Of those are the gingival mesenchymal stem cells (GMSCs) which offer a more appealing alternative to other dental originated MSCs regarding their ease of extraction and isolation from the clinically respected gingival tissues with minimal harm to the donor. GMSCs are considered an excellent source of MSCs for cell-based regenerative therapies with maximum stability for the longest period in addition to its uniformly homogenous property and stable phenotype and telomerase activity during prolonged culture time [3][6][7][8]. Periodontal ligament stem cells (PDLSCs) also represent typical properties of mesenchymal stem cells (MSCs) regarding self - renewal and expression of mesenchymal stem cells surface markers. PDLSCs also possess multilineage differentiation potential into various types of cells such as osteoblasts, adipocytes, chondrocytes and neurocytes in - vitro. Additionally, it is suggested that PDLSCs might belong to a unique population of somatic stem cells due to its distinctive potential to form cementum and PDL - like tissues in - vivo. 9 It is believed that PDLSCs are a suitable candidate cellular source for PDL regeneration as they play an endogenous role in maintaining PDL cell numbers due to their periodontal ligament derivation and their vast differentiation capacity [10]. In the current study, GMSCs and PDLSCs were recruited since both are currently considered ideal cell source for tissue engineering and repair [11]. Previous studies confirmed that GMSCs transplanted into the periodontal defects had been demonstrated to contribute to the periodontal repair and regeneration [12]. Novel studies had demonstrated the great

potential of bone healing when GMSCs were implemented into the mandibular defects in animal models [13]. In recent years, several research groups have attempted to use MSCs as a treatment option for diabetes and its complications [1][2][3][4][5][6][7][8]. However, autologous cell-based therapeutics may be unlikely to succeed if the true impact of diseases like diabetes on stem cells and progenitor cell population is not investigated thoroughly. It is thought that diabetes negatively affects the stem cell niches thus altering their dynamics and disrupting the repair and homeostasis. Predominantly, changes in reactive oxygen species (ROS) and hypoxia from neighbouring cells in case of hyperglycemia (high blood glucose level) is the reason for the alteration of the signalling to the stem cells [14][15]. Diabetes produces a surge in ROS which results in generating a prolonged inflammatory and oxidative environment that leads to the inhibition of stem cell proliferation which in turn causes stem cell senescence impairing innate tissue repair mechanisms and regeneration [16][17].

Diabetes also causes stem cell aberrations resulting in direct implications on tissue function that seem to persist even after return to normoglycemia [18]. These impairments might include changes in migration, recruitment, survival, self - renewal and differentiation capacity. Diabetes-induced in mice was found to alter the intrinsic properties of stem cells and impair their function and their regenerative ability [19][20]. Stem cells investigated in these reports were isolated from tissues other than dental tissues like adipose and bone marrow and has provided accumulating evidence that implies differences in proliferation and differentiation abilities between different sources of stem cells [18][20][21][22][23]. However, most of these reports have either investigated stem cells from healthy individuals without comparing the results to diabetic individuals or has used diabetic induced models.

In this report, we investigate the effect of diabetes on the proliferation potential of stem cells isolated from controlled diabetic patients (type II) and healthy individuals.

Subjects and Methods

Periodontal ligament stem cells were isolated from (PDLSCs) of healthy subjects and controlled diabetic patients (DM type II). Additionally, gingival mesenchymal stem cells (GMSCs) were isolated from the gingiva of healthy subjects and controlled diabetic patients (DM type II). Procedures were performed at the National Research Centre, Cairo, Egypt according to the recommendations and approval of its ethics committee. Informed consent was obtained from all subjects and patients before undergoing teeth extractions.

Sample Size Calculation

Based on previous studies, the expected average of cell viability was 26.2% with variance percentage 1.86% [24][25]. A total sample size of 116 (29 in each of the four groups) was calculated to be sufficient to detect a significant difference between the groups with a power of 80% and a significant level of 5%. The number was then increased to a total sample size of 144 (36 in each group) to allow for losses of around 25%. The sample size was calculated using nQuery Advisor.

Inclusion and Exclusion Criteria

The sample represents a population of Egyptian patients with type 2 diabetes of both sexes, ≥ 18 years of age. Participants represent a consecutive series of patients fulfilling the exclusion/inclusion criteria. Patients were recruited among those referred to the hospital of the faculty of dentistry and oral medicine, Cairo University for dental treatment. Inclusion Criteria included; clinical diagnosis of diabetes mellitus according to American Diabetes Association criteria (ADA) criteria [26], whose age ranged from 18 to 69 years, with duration of diabetes diagnosis over 12 months, and requiring oral antidiabetic drugs for optimal glycemic control in a dose of ≥ 0.7 U/kg/day at least for 1 year in addition to willingness to participate in the study.

Control diabetes was described as proven normal glucose tolerance according to ADA criteria where uncontrolled diabetes was deemed present in diabetics who had fasting blood glucose (FBG) level ≥ 7.0 mmol/L and controlled diabetes in diabetics who had fasting blood glucose (FBG) level ≤ 7.0 mmol/L [26][27]. At the time of entry into the study, all patients had a fasting blood glucose (FBG) level ≤ 7.0 mmol/L and HbA1c ≤ 7%. The exclusion criteria included the following: acute or chronic infections; any malignancies; haematological diseases; known immunosuppressive disease (for example, acquired immunodeficiency); acute or chronic pancreatitis; and a history of thoracic or abdominal aorta diseases. After recruitment, all follow - up visits were performed at the same hospital. All patients gave written informed consent.

Cell Isolation and Culture

Human third molars, which were removed for impact reason from both systemically healthy and diabetic patients, were used for tissue biopsy and PDL cell isolation. Meanwhile, gingival tissues surrounding the tooth sockets were collected immediately after tooth extraction for gingival cell isolation and subsequent investigations. Periodontal ligament stem cells (PDLSCs) were acquired from the periodontal ligament tissues by scraping the middle third of the root of extracted impacted third molars. Teeth

surfaces were rinsed with phosphate buffered saline (PBS) under aseptic conditions. The collected tissues were then digested with 3 mg/ml collagenase at 37°C for 15 min. The cell suspension was then transferred to dishes and cultured in RPMI culture media supplemented with 10% Fetal Bovine Serum (FBS) and streptomycin at 37°C in a humidified atmosphere with 5% CO_2. The culture medium was changed every three days.

In parallel, a small of biopsy of gingiva comprised of epithelium and connective tissues was harvested from the extracted third molar's cervical ridge, and the tissue was washed with PBS. The excised gingival tissues were and treated aseptically and incubated overnight at 4°C with 2 mg/ml Dispase to separate the epithelial and the spinous layers. The tissues were then minced into fragments and digested with 4 mg/ml collagenase at 37°C for two h. The dissociated cell suspension were filtered through a 70 µm cell strainer, plated on Petri dishes RPMI culture media supplemented with 10% Fetal Bovine Serum (FBS) and streptomycin at 37°C in a humidified atmosphere with CO2. The culture medium was changed every three days. Cells from all groups were passaged at a ratio of 1:2 when reaching 70 - 80% confluence. The collected stem cells were grouped as follows; periodontal ligament stem cells from healthy individuals, periodontal ligament from controlled diabetic patients, gingival stem cells from healthy individuals and gingival stem cells from controlled diabetic patients.

Flow Cytometric Analysis

The identity of isolated stem cells was confirmed by analysing surface antigen expression. Isolated stem cells from all groups at passage three were detached by 0.05 % trypsin - EDTA, centrifuged for 5 min at 2000 rpm and resuspended in PBS containing 2 % FBS. Next, they were incubated with antibodies against CD45, CD90 and CD105 for 30 min at 4°C. Isotypes were used as negative control. After washing with PBS, the cells were resuspended in 500 µl PBS containing 2 % FBS and subjected to flow cytometric analyses using CYTOMICS FC 500 Flow Cytometer (Beckman Coulter, FL, USA) and analysed using CXP Software version 2.2.

Assessment of Cell Proliferation by MTT Assay

The proliferation of isolated stem cells was evaluated using MTT assay. For MTT assay, the cells from passage three were seeded in 96 - well plates (2 x 103 cells per well) and cultured for 72h in DMEM supplemented with antibiotic and 10% FBS. Then, MTT solution (at the final concentration of 0.05 %) was added to each well, and the cells were incubated under the atmosphere of 5 % CO_2 at 37°C. After 3h, the supernatant was discarded, and the formazan precipitate was dissolved in dimethyl sulfoxide. The MTT Reagent and Detergent Solution were obtained from TACSTM TREVIGEN1 8405 Hegerman Ct. Gaithersburg, supplied ready for use. The optical density (O.D) values were measured at a range from 490 to 630 nm using an ELISA reader (Dynatech MRX 5000; Dynex, Chantilly, VA).

Real-time Quantitative PCR for Survivin Gene Expression

Total RNA of cells of all studied groups was isolated with RNAeasy Mini Kit (Qiagen) and further analysed for quantity and quality with Beckman dual spectrophotometer (USA). The mRNA expression level was quantified by qRT - PCR (Real-time PCR). 1000 mg of the total RNA from each sample was used for cDNA synthesis by reverse transcription using High Capacity cDNA Reverse Transcriptase kit (Applied Biosystem, USA). The cDNA was subsequently amplified with the Syber Green I PCR Master Kit (Fermentas) in a 48 - well plate using the Step One instrument (Applied Biosystem, USA) as follows: 10 min at 95°C for enzyme activation followed by 40 cycles of 15 seconds at 95°C, 20 seconds at 55°C and 30 second at 72°C for the amplification step. Changes in the expression of each target gene were normalized relative to the mean critical threshold (CT) values of beta actin housekeeping gene by the $\Delta\Delta$Ct method. We used 1 mM of both primers specific for each target gene. Survivin primer sequence was 5' - ACCCACACTGTGCCCATCTAC - 3' and antisense 5' – TCGGTGAGGATCTTCATGAGGTA - 3' (gene bank accession number: NG_026370.2) and β - beta actin primer sequence was 5' - GGC GGCACCACCATGTACCCT - 3' and antisense 5' - AGG GGCCGGACTCGTCATACT - 3' (gene bank accession number: NM_001101.3).

Statistical Analysis

Data were coded and entered using the statistical package SPSS (Statistical Package for the Social Sciences) version 23. Data was summarized using median and interquartile range in quantitative data. Comparisons between quantitative variables were done using the non - parametric Kruskal - Wallis and Mann - Whitney tests. Correlations between quantitative variables were done using Spearman correlation coefficient. P - values less than 0.05 were considered as statistically significant.

Results

MSCs morphology

MSCs were identified in culture by adopting a

fusiform adherent fibroblast like cells in both types PDLSCs and GMSCs for healthy subjects and diabetic patients (Figure 1).

Flow Cytometric Analysis

Surface antigens of all groups were analysed by flow cytometry. GSCs & PDLSCs isolated from healthy & diabetic patients demonstrated relatively high positivity for mesenchymal stem cell-associated markers CD 90 and CD 105, and negative for hematopoietic markers CD45 (Figure1).

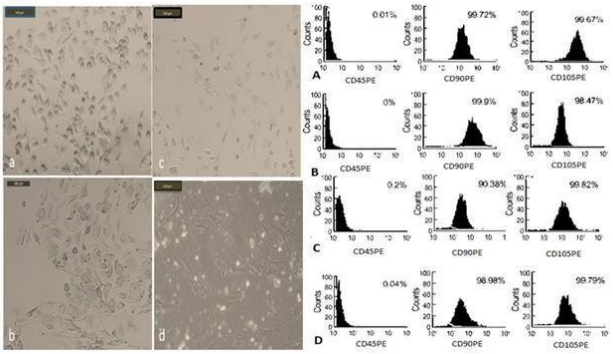

Figure 1: MSCs isolated from all groups in culture; A: PDLSCs isolated from healthy subjects, B: GMSCs isolated from healthy subjects, C: PDLSCs isolated from diabetic patients and D: GMSCs isolated from diabetic patients (Left).FACS analysis for characterisation of isolated MSCs; A: PDLSCs isolated from healthy subjects, B: GMSCs isolated from healthy subjects, C: PDLSCs isolated from diabetic patients and D: GMSCs isolated from diabetic patients. Analysis for all groups revealed high positivity of CD 90 and 105 and negativity of CD 45 for all types of isolated MSCs (Right)

Cell Proliferation by MTT Assay

Figure 2 illustrates the proliferation of PDLSCs and GSCs from normal & diabetic patients as evaluated by MTT assay.

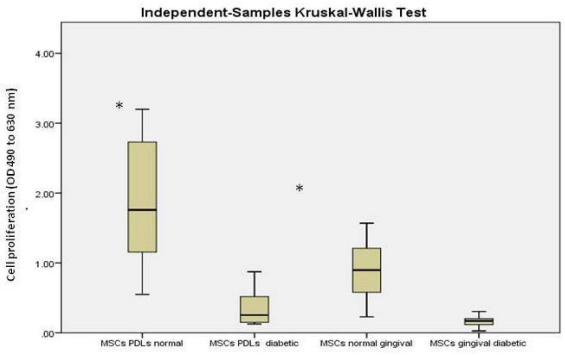

Figure 2: Proliferation of periodontal ligament stem cells (PDLS) and gingival stem cells (GSCs) from normal and diabetic individuals by MTT assay (indicates statistically significant differences between normal and diabetic individuals in each cell type P < 0.001)*

Both in normal and diabetic individuals PDLSCs exhibited greater proliferation rate than GSCs nearly two-fold higher. When comparing proliferation of each cell type about diabetes, it was revealed that the proliferation rate of PDLSC derived from normal individuals was significantly higher than those derived from diabetic individuals. The same pattern was noticed in GSCs where the proliferation rate was significantly higher in normal cells. The increase in normal PDLSCs was seven folds higher than that in diabetics and 5.29 folds higher in case of GSCs (P < 0.001).

Real-time Quantitative PCR

The expression of Survivin in PDLSCs & GSCs from normal and diabetic patients by Rt - PCR is illustrated in Table 1. Statistical analysis revealed that Survivin expression in PDLSCs isolated from normal individuals was significantly higher than those from diabetic patients (P < 0.05). Similarly, GSCs isolated from normal individuals exhibited expression that is significantly higher than GSCsc from diabetic individuals (P < 0.001).

Table 1: Survivin expression when comparing PDLSCs normal group with diabetic group, and GSCs normal group with diabetic group using multiple comparison post hoc tests (* P - value when comparing PDLSCs group versus PDLSCs diabetic group, ** P - value when comparing GSCs group versus GSCs diabetic group)

	MSCs PDLs Normal	MSCs PDLs Diabetic	MSCs Normal Gingival	MSCs Normal Diabetic	P - value
Survivin Gene Expression	1.24*	0.54	0.84**	0.26	< 0.001
SD	0.74	0.15	0.32	0.07	

Assessment of the relation between proliferation rate and Survivin Gene expression

Next, we analysed the correlation between Proliferation rate & Survivin Gene Expression. There was a significantly positive correlation between proliferation rate and expression of Survivin in all groups illustrating the importance of Survivin as a reliable indicator of proliferation (Figure 3 and Table 2).

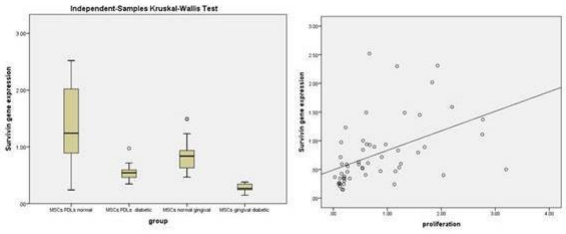

Figure 3: A plot of Survivin gene expression in all groups by RT - PCR (Left) and the relation of its expression with the proliferation potential of studied stem cells groups

Table 2: Correlation between Proliferation and Survivin expression

		Proliferation
Survivin Gene Expression	Correlation Coefficient	*.621*
	P value	*<0.001*
	N	*56*

Discussion

Stem cells have recently received great interest as a potential therapeutic alternative for a variety of diseases. This is especially true for a disease like diabetes. The majority of mesenchymal stem cells previously studied for therapeutic purposes have been from sources like bone marrow and adipose tissue [23][28]. Both, PDLSCs and GSCs demonstrated promising potential for use in tissue regeneration [29][30][31]. In the present study, we investigated stem cells isolated from two different oral tissues. Moreover, to address whether oral stem cells derived from different oral tissues have different proliferation ability and whether controlled type II diabetes influences these abilities, we compared the growth rate of gingival and periodontal derived stem cells isolated from diabetic & healthy individuals.

Our results showed that both PDLMSCs and GMSCs demonstrate satisfactory proliferation rates. We observed a significant difference in the proliferation pattern of both tissues. Stem cells isolated from PDL exhibited higher proliferation than those isolated from gingival tissues both in normal and diabetic patients. This was in contrast to multiple reports that showed GMSCs to have a higher proliferation compared to PDLSCs [29][32][33][34]. This pattern of proliferation described in the diabetic patient was in contrast to previous reports that demonstrated that early after the induction of diabetes with streptozotocin, the proliferative capacity of bone marrow-derived stem cells increased [23]. The inclusion of a greater number of diabetics enrolled in our study in contrast to using diabetes-induced models may help explain this result. Our results were in consistence with Stolzing et al who attributed the diminished proliferative capacity of bone marrow cells to the long-term incidence of diabetes [35].

It is worthy to note that in the present study both PDLMSCs and GMSCs were phenotypically similar in diabetic and non - diabetic groups as demonstrated by FACS analysis. The homogeneity of the results in all groups suggests that diabetes has not altered the stemness of either population confirming that the changes associated with diabetes mainly affect the proliferation capacity of the cells.

To further assess the effect of diabetes on PDLSCs and GSCs, we measured the expression levels of Survivin in isolated stem cells from all groups and correlated its expression with the proliferation patterns studied. Survivin is a critical inhibitor of apoptosis-inducing proteins that are up-regulated in many types of malignant diseases. Additionally, it has been demonstrated previously that Survivin expression is correlated with the expression of cell proliferation and survival markers [36]. In the present study, there was a significantly positive correlation between proliferation rate and expression of Survivin in all groups which sheds light on the importance of Survivin as a reliable indicator of proliferation. The expression on Survivin further confirmed the proliferation results from MTT Assay where the expression of stem cells from non - diabetic individuals was higher than diabetic patients and PDL stem cells demonstrated significantly higher expression than GSCs. Taking together all the results, it could be concluded that PDLSC and GSC are promising candidates for autologous regenerative therapy due to their ease of accessibility in addition to their high proliferative rates. However, it could also be concluded that stem cells from diabetic donors exhibit decreased proliferative potential while maintaining their stemness properties. Thus it is essential to improve methods and factors that would increase the proliferation capacity of stem cells isolated from diabetic patients to ensure the success of regenerative medicine options especially when autologous cells are intended for use.

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Basic Science

Ki-67 Expression as a Predictive Factor of Muscle Invasion in Bladder Cancer

Syah Mirsya Warli*, Dhirajaya Dharma Kadar, Ginanda Putra Siregar

Universitas Sumatera Utara, Fakultas Kedokteran, Urology, Medan, Sumatera Utara, Indonesia

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***Correspondence:** Syah Mirsya Warli. Universitas Sumatera Utara Fakultas Kedokteran Ringgold standard institution - Urology Medan, Sumatera Utara, Indonesia. E-mail: war_uro@yahoo.com

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Abstract

BACKGROUND: Bladder cancer is the 9th most frequent cancer worldwide. Ki-67 is immunohistochemistry marker that is predictive of cancer cell proliferation. The expression of Ki-67 is associated with poor prognosis in several types of malignancy, yet the value of Ki-67 as the prognostic factor in bladder cancer remains controversial.

AIM: This study is aimed to investigate the association between Ki-67 expression with muscle-invasive bladder cancer (MIBC) and non-muscle invasive bladder cancer (NMIBC).

METHODS: This was a case-control study with a retrospective design. The study was conducted at the Department of Pathology, University of Sumatera Utara, Indonesia. Samples were paraffin blocks from patients diagnosed with bladder cancer and agreed to be put in the study. The samples were stained with Immunohistochemistry Staining (IHC), and then we quantitatively counted the number of the Ki-67 stained nucleus on a microscope.

RESULTS: A total of 54 samples were obtained in this study. Samples consisted of 27 samples with NMIBC and 27 samples with MIBC. The cut-off point was 20%, we found 17 patients with MIBC and 14 patients with NMIBC presented with biomarker > 20%. Biomarker ≤ 20% was found in 10 patients with MIBC and 13 patients with NMIBC. On statistical analysis with Chi-Square test, no significant association found (p = 0.583) between KI-67 and muscle - invasiveness with OR of 1.579, 95% CI (0.533-4.678).

CONCLUSION: There is no association between expression of Ki-67 and muscle invasiveness in bladder cancer.

Introduction

Bladder cancer is a type of neoplasia in which the cell aligning the bladder lost its ability to control growth and cell division. Worldwide, bladder cancer ranked 9th commonly found neoplasia [1]. There are two known histopathological staging, **non-muscle invasive bladder cancer** (NMIBC), in which the mass was limited to mucous with no muscle involvement, and **muscle-invasive bladder cancer** (MIBC).

Majority of cases found was NMIBC [1][2]. Although this type of cancer can be managed easily by trans-urethral resection, the recurrence rate was 15%-75%, with 10% of cases progress into MIBC. MIBC tend to have a poor prognosis. It is imperative to develop an early diagnosis, as well as appropriate treatment in a patient with bladder cancer [3].

Immunohistochemistry is an established method that supports histopathologic assessment in the diagnosis of various benign and malignant diseases. In 1982, Nathrath et al. were the first to experiment on bladder cancer paraffin sample and documenting a set of keratin and carcinoembryonic antigen [3]. Since then, the prognostic value of immunohistochemistry marker becomes important. In the last two decades, immunohistochemistry evaluation, specifically regarding cell cycle and apoptosis, was intensively pursued to obtain a better understanding of prognosis in patient with bladder cancer. A prognostic biomarker is essential in which they can provide information on disease progression, regardless of intervention. Two types of prognostic biomarker were recognised, the biomarker which can give information of recurrence and biomarker which contain information of disease progression (progression-free survival). A predictive biomarker can provide information about the effect of an intervention, as well as able to be used as targeted

therapy [4]. Ki-67 is immunohistochemistry marker known to be present on an actively proliferating cell. Evidence has shown that Ki-67 is a predictive factor of cancer cell proliferation, expression of Ki-67 correlate with poor prognosis in several types of cancer [5][6]. Nevertheless, the value of

Ki-67 as the prognostic factor in bladder cancer remains controversial [7]; few studies result show that Ki-67 can predict progression and recurrence, while other studies show no significant correlation. One meta-analysis study by Tian et al. concludes that Ki-67 expressions correlate significantly with recurrence, progressivity, and lower survival only in Caucasian [8]. This research aims to evaluate the association between Ki – 67 expressions with MIBC and NMIBC.

Methods

This study was conducted at the Department of Pathology, University of Sumatera Utara, Indonesia. Samples were obtained from bladder cancer patient primary specimen paraffin blocks that were analysed in Pathology laboratory in 2013-2015. The patients have been confirmed agreed to be put in the study. Each group contained 27 samples. Samples were then stained with Immunohistochemistry Staining (IHC), and then quantitatively count the number of the Ki-67 stained nucleus on a microscope. The interpretation was made by calculating the amount of stained cell from 100 tumour cells, with cut-off point 20% as in Otto et al. study (≤ 20% counted as "low expression" and > 20% count as "overexpression") [9].

Results

Fifty-four samples, which consists of 27 samples with NMIBC and 27 samples with MIBC were obtained. Sample characteristics are shown in Table 1.

Table 1: Sample characteristics of NMIBC and MIBC

Variable	Group		p value
	NMIBC	MIBC	
	N = 27	N = 27	
Age	56.1 ± 11.43	60.5 ± 9.85	0.413*
Gender			
Male	25 (92.6%)	23 (85.2%)	0.669*
Female	2 (7.4%)	4 (14.8%)	
Grading			
High Grade	12 (44.4%)	27 (100%)	<0.001*
Low Grade	15 (55.6%)	0 (0%)	

*Chi-Square Test.

In NMIBC group, the mean age was 56.1 ± 11.43 years, while in MIBC was 60.5 ± 9.85 years. Male (twenty-three in MIBC and twenty-five in NMIBC) was more common than female (four in NMIBC and two in NMIBC) in both categories. In NMIBC group, more than half of samples were low grade (55.6%), and 44.4% high grade (p < 0.001). On the above table, sample characteristic on both groups from age and gender perspective didn't show a significant difference (p = 0.669).

Table 2: KI-67 Test Result on NMIBC and MIBC

Biomarker	Group		p-value	OR	95% CI
	NMIBC	MIBC			
	N = 27	N = 27			
KI 67 > 20	17 (63.0%)	14 (51.9%)			
			0.583*	1.579	(0.533-4.678)
< 20	10 (37.0%)	13 (48.1%)			

*Chi-Square Test.

We divided biomarker with the cut-off point of 20%, Ki-67 > 20% and Ki-67 ≤ 20%. For KI – 67 > 20%, we found 17 patients with MIBC and 14 patients with NMIBC. Biomarker ≤ 20% was found in 10 patients with MIBC and 13 patients with NMIBC. On statistical analysis with Chi-Square test, there was no significant association (p = 0.583) between KI-67 and muscle-invasiveness with OR of 1.579, 95% CI (0.533-4.678).

Discussion

Usage of the marker in tissue to help clinicians decision was successfully done in few malignancy cases. In a research conducted by Jonat and Arnold, evaluating Ki-67 and its function in clinical practice [10] significantly proves the importance of the utilisation of marker mentioned above to detect the proliferation of a tumour. Also shown in the research done by Heslin et al. [11], those in patients with soft tissue sarcoma, the increase in Ki-67 marker expressions proved as an independent prognostic tool to predict the metastasis and the mortality of a tumour. Several studies suggest the effectivity of Ki-67 as a prognostic marker.

Ki-67 is one of many biomarkers that can be detected by the monoclonal antibody as a proliferation marker. The usage of this immunohistochemistry is highly useful due to its rapid and accurate result to indicate the presence of ongoing proliferation rather than a solid tumour [12] In the recent years, studies about proliferation biomarkers are high in demand. Studies show that Ki-67 correlated significantly with tumour cells of bladder cancer, and is capable of calculating the prognostic factor of the disease [13].

The value of Ki-67 as a prognostic biomarker of urothelial malignancies in urinary tract system is depicted in the meta-analysis study conducted by Lei et al. [14]. Contraries to that, a survey was done by Acikalin et al. find that there is no statistically significant correlation between the expression of Ki-67 and tumour recurrence progressivity and mortality [8]. Instead, they detect the presence of Ki-67 correlate with tumour size and grading. The research above is congruent with the fact that our study did not find a statistically significant relationship between the presence of Ki-67 and bladder cancer progression. Several factors may have affected the result, such as incomplete biomarker data and a limited number of samples due to patient's reluctance to join our study.

This study concludes that there is no association between expression of Ki-67 and muscle invasiveness in bladder cancer. This result can be used as a reference for further research, as biomarker nowadays proved to be valuable in clinically to predict prognosis in the patient, as well as provide better intervention for the patient with prostate cancer.

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Prognostic Significance of Vascular Endothelial Growth Factor (VEGF) and Her-2 Protein in the Genesis of Cervical Carcinoma

Arshad H. Rahmani[1], Ali Yousif Babiker[1,2*], Mohammed A. Alsahli[1], Saleh A. Almatroodi[1], Nazik Elmalaika O. S. Husain[3]

[1]Department of Medical Laboratories, College of Applied Medical Sciences, Qassim University, Buraidah , Saudi Arabia; [2]Department of Histopathology and Cytology, College of Medical Laboratories Science, University of Sciences and Technology, Omdurman, Sudan; [3]Department of Pathology, Faculty of Medicine, Omdurman Islamic University, Omdurman, Sudan

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***Correspondence:** Ali Yousif Babiker. Department of Medical Laboratories, College of Applied Medical Sciences, Qassim University, Buraidah , Saudi Arabia. E-mail: alibabkr99@gmail.com

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Abstract

BACKGROUND: Angiogenesis plays a pivotal role in the progression of tumours through the formation of new blood vessels. Vascular endothelial growth factor (VEGF) is a chief factor responsible for inducing and regulating angiogenesis. Additionally, the human epidermal growth factor receptor family of receptors also plays an important role in the pathogenesis of tumours.

AIM: This study aimed to examine the association between VEGF and Her-2 protein expression and its correlation with clinic-pathological characteristics; in particular, prognosis.

METHODS: A total of 65 cases of cervical carcinoma and 10 samples of inflammatory lesions were evaluated for VEGF and Her-2 protein expression.

RESULTS: Expression of VEGF and Her-2 was detected in 63.07% and 43.07% in cervical carcinoma cases respectively whereas control cases did not show any expression. The difference in the expression pattern of both markers comparing cancer and control cases was statistically significant ($p < 0.05$). However, no significant difference in the expression pattern of VEGF protein was observed among the different grades and stages of tumours ($p > 0.05$). Comparing different grades of a tumour, expression of Her-2 was detected in 31.8% of well-differentiated tumours, 36.0 % in moderately differentiated tumours and 66.66 % in poorly differentiated cancers. The expression of Her-2 was increased in high-grade tumours, and the difference of expression level between tumour grades was statistically significant ($p < 0.05$). The expression level of Her-2 protein was not correlated with the stage of a tumour ($p > 0.05$).

CONCLUSION: The present study supports earlier findings that over-expression / up-regulation of VEGF and Her - 2 is linked with poor prognosis and may play a vital role in the development and progression of cervical cancer.

Introduction

Cervical cancer is the most common form of female genital malignancy and is a principal cause of cancer-associated mortality. Despite numerous studies, the exact carcinogenetic events in the development and progression of cervical cancer are not entirely understood. In this regard, hormonal imbalance, smoking and obesity are suggested to be the chief causative agents in the development and progression of cervical cancer. Additionally, Human Papillomavirus (HPV) has been recognised as an etiological agent in the pathogenesis of uterine cervix cancer [1][2] and epidemiological studies have shown that HPV infection is commonly identified in invasive cervical cancer [3].

Angiogenesis has also been shown to play a vital role in tumour development and progression through the formation of new blood vessels. Vascular endothelial growth factor (VEGF) acts as the chief mediator of tumour angiogenesis and stimulates the growth of new blood vessels [4]. It has been shown that VEGF plays an important role in inducing angiogenesis in some physiological and pathological processes [5]. Moreover, VEGF expression may be induced by various factors such as altered expression of tumour suppressor genes [6], oncogenes [7][8], insulin-like growth factor – 1 [9] and altered VEGF expression has been associated with

both the advanced pathological stage of cancer and lymph node metastasis [10].

The human epidermal growth factor receptor 2 (Her2) is a transmembrane receptor tyrosine kinase [11][12] and has been shown to play a significant role in the pathogenesis of tumours. Her2-gene amplification appears to be an early event in the development of cancer [13]. Up-regulation of Her-2 has been noticed in the genesis of numerous cancers including cervical cancer.

The current study aimed to examine the expression patterns of VEGF and Her-2 proteins in cervical cancer and correlate the expression pattern of both markers with clinic-pathological features observed in patients.

Materials and Methods

Patients and tissues

Tissue from a total of 65 patients (mean age 65 ± 14 years, range 26 – 98 years) diagnosed with cervical carcinoma was collected from the Histopathology Department at the National Health Laboratory (and other histopathology laboratories) in Sudan. A total of 10 cases of inflammatory lesions of the cervix were included in the study as a control group. The study was approved by the institutional Research Ethics Committee, and written consent was obtained from each patient. Hematoxylin and Eosin (H&E) staining were performed on each case to evaluate the histopathology of tissue samples, including grade and stage of a tumour (Figure 1). The detailed clinic-pathological feature of the patients is presented in Table 1. A series of 5 – µm -thick sections were prepared from each formalin-fixed, paraffin-embedded block and immunohistochemical staining was performed to evaluate the expression pattern of VEGF and Her-2 protein to allow its interpretation and correlation with clinical outcome.

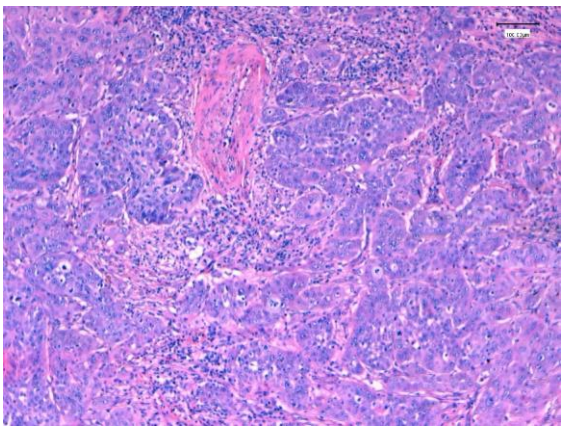

Figure 1: Section from cervical carcinoma grade II(H & E X40)

Table 1: Correlation between VEGF and clinicopathological features in cervical carcinoma

Variables	Total cases	VEGF positive	VEGF negative	the
Cervical carcinoma	65	41 (63.07%)	24 (36.92%)	< 0.05
Inflammatory lesion	10	0 (0%)	10 (100%)	
Age (In years)				
≤ 55 years	28	16 (57.14%)	12 (42.85%)	> 0.05
> 55 years	37	25 (67.56%)	12 (32.43%)	
Histological grades				
Well differentiated	22	12 (54.54%)	10 (45.45%)	
Moderately differentiated	25	17 (68.00%)	14 (32.55%)	
Poorly differentiated	18	12 (66.66%)		> 0.05
Histologic type				
Squamous Cell Carcinoma	59	38 (64.4%)	21 (35.59%)	
Adenocarcinoma	6	3 (50.0%)	3 (50.0 %)	> 0.05
Clinical stage				
I	14	7 (50.0%)	7 (50.0%)	> 0.05
II	17	11 (64.7%)	6 (35.29%)	
III and IV	34	23 (67.64%)	11 (32.35%)	

Immunohistochemical staining

Five µm sections from each paraffin-embedded tissue block were prepared from cervical cancer and inflammatory lesions. Immunohistochemical staining was performed on paraffin-embedded tissue sections as described by Rahmani et al., 2015 [14]. In brief, deparaffinization of all sections was made through a series of xylene solutions, and rehydration was performed through graded ethanols. Also, endogenous peroxidase activity was blocked by pre-treatment of sections with 0.3% hydrogen peroxide for 20 minutes. Antigen retrieval was performed using a sodium-citrate buffer (pH 6.0), in a microwave oven for 30 minutes. Additionally, protein blocking agent (Abcam, USA) was applied for 10 minutes to reduce nonspecific binding. VEGF and Her-2 antihuman monoclonal antibodies (Abcam, Cambridge, MA, USA) were used as primary antibodies followed by the secondary biotinylated antibody (Abcam, USA). Detection of immunostaining for VEGF and Her-2 protein was performed using the streptavidin-biotin method. Diaminobenzidine (DAB) chromogen (Abcam, USA) was applied then sections were counterstained and mounted with DPX. Negative controls (omission of primary antibody) and positive controls were used to verify the quality of staining.

Staining Interpretation

Expression of VEGF and Her-2 protein was evaluated, and the mean percentage positivity was calculated. Five fields from each section were selected, and positive cells for each marker (lowest to the highest number of positive cells) were counted at 40 X magnification. Specimens were considered as positively stained when more than 10% of the tumour cells showed cytoplasmic expression for VEGF and membranous or cytoplasmic stain for Her2 protein. If 10% or less than 10 % of cells showed cytoplasmic expression, they were classified as negative cases.

Statistical analysis

Expression of markers was correlated with clinicopathological features of the patients. Chi-square $(\lambda)^2$ test was used to make the correlation of marker with grade, age and stage of the tumour. Statistical significance was defined as $p < 0.05$.

Results

Association of VEGF protein expression with clinicopathological characteristics

The expression pattern of VEGF was examined in uterine cervix cancer including squamous cell carcinoma (SCC), adenocarcinoma and inflammatory lesions of the cervix. The expression of VEGF was detected only in the cytoplasm of cancer cells (Figure 2, a-c) whereas control cases did not show any expression. VEGF protein showed expression in fewer than 10% of cells or no expression in all ten inflammatory lesions and these were thus considered as negative for VEGF expression.

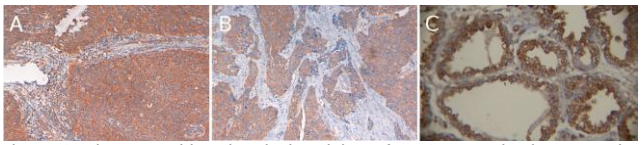

Figure 2: a) Immunohistochemical staining of VEGF protein demonstrating strong cytoplasmic expression in a cervical carcinoma (Orig mag. 40X); b) Immunohistochemical staining of VEGF protein showing moderate cytoplasmic expression in a cervical carcinoma(Orig mag. 40X); c) Expression of VEGF protein in section from carcinoma of cervix(Orig mag. 40X)

Conversely, greater than 10% positivity for VEGF staining was observed in 41/65 (63.07%) cases of cervical cancer patients, and these were considered VEGF positive. The difference in VEGF expression between cancer cases and control cases was statically significant *($p \leq 0.05$).* Moreover, the expression level for VEGF was correlated with tumour grade was found to be positive in 12/22 (54.54%) well-differentiated tumours, 17/25 (68.00%) samples of moderately and 12/18 (66.66%) poorly differentiated carcinoma. The difference in VEGF expression between different grades of a tumour did not reach statistical significance ($p > 0.05$) (Table 1), and intensity of expression was high in poorly differentiated carcinoma as compared to well and moderately differentiated carcinoma.

Additionally, the expression of VEGF was evaluated according to the stage of cancer, and it was found that VEGF expression was positive in 7/14 (50%) Stage I samples, 11/17 (64.7%) stage II samples and 23/43 (67.64%) stage III and IV samples (Table 1). The difference of VEGF expression observed in the different tumour stages was also found to be statically insignificant *($p > 0.05$)*. The expression of VEGF was also correlated with the age of the patients (younger than 55 years and equal or older than 55 years). Again, no significant difference was observed between samples from different age groups.

Expression pattern of Her-2 protein in cervical cancer and inflammatory lesions

Her-2 protein was not detected in inflammatory lesions of the cervix while there were strong expression observed in 28/65 (43.07%) carcinoma cases (Figure 3) ($p < 0.05$). Expression analysis of Her-2 protein in different tumour grades showed that Her-2 expression was increased with a grade of a tumour. Her-2 expression was detected in 7/22 (31.8%) well-differentiated tumours, 9/25 (36.0 %) moderately differentiated tumours and 12/18 (66.66 %) poorly differentiated cancers (Table 2). Her-2 expression showed a significant difference in expression between different grades of cancer ($p < 0.05$).

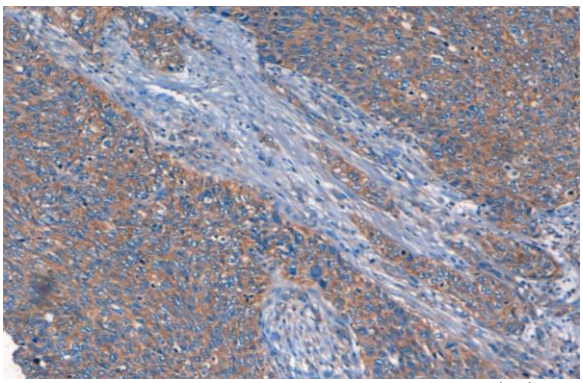

Figure 2: Immunohistochemical expression of Her-2 protein in poorly differentiated cervical carcinoma (Orig Mag. 40X)

The immunoreactivity of Her-2 was not found to be correlated with stage of a tumour, Her-2 expression observed in 6/14 (42.85%) Stage I, 11/17 (64.7%) Stage II and 17/34 (50.0%) Stage III/IV tumours (Table 2). Moreover, the expression profile of Her-2 protein also showed no significant difference between different age groups of patients (≤ 55 years and > 55 years).

Table 2: Correlation between Her-2 protein and clinicopathological features of cervical carcinoma

Variables	Total cases	Her-2 positive	Her-2 negative	p - value
Cervical carcinoma	65	28 (43.07%)	37 (56.92%)	< 0.05
Inflammatory lesion	10	0 (0%)	10 (100%)	
Age (In years)				
≤ 55 years	28	11 (39.28%)	17 (60.71%)	> 0.05
> 55 years	37	17 (45.94%)	20 (54.05%	
Histological grades				
Well differentiated	22	7 (31.8%)	15 (68.18%)	
Moderately differentiated	25	9 (36.0%)	16 (64%)	< 0.05
Poorly differentiated	18	12 (66.66%)	6 (33.33%)	
Histologic type				
Squamous Cell Carcinoma	59	25 (42.37%)	34 (57.62%)	
Adenocarcinoma	06	3 (50.0%)	3 (50%)	> 0.05
Clinical stage				
I	14	6 (42.85%)	8 (57.14%)	> 0.05
II	17	11 (64.7 %)	06 (35.29%)	
III and IV	34	17 (50.0%)	17 (50.0%)	

Relationship between VEGF protein expression, and Her 0-2 expression with clinicopathologic features

Both proteins VEGF and Her-2 showed high levels of expression in cancer cases while none of the control lesions that were studied showed positive expression. The difference in expression for both markers comparing cancer cases and control cases were thus statically significant. Furthermore, the immunoreactivity of Her-2 marker increased according to grades of a tumour whereas VEGF did not show a significant difference in expression when comparing tumour grade. Both markers did not show any significant difference in the level of expression when comparing different age groups of patients.

Discussion

Angiogenesis is the process of formation of new blood vessels from sprouting of existing vessels. Extensive laboratory data suggests that new vessel formation is an important step in driving tumour growth and progression [15][16][17]. Vascular Endothelial Growth Factor (VEGF) is one of the chief angiogenic factors that stimulate the formation of new blood vessels and thus can influence tumour growth [18]. Previous studies have confirmed that over-expression of VEGF can be correlated with tumour growth, metastasis and patient survival [19][20][21]. Altered expression of VEGF has been observed in cancers and strongly suggesting that angiogenesis participates in tumour development and progression. In the current study, it was found that VEGF expression was high in 41 (63.07%) in cervical carcinoma cases while inflammatory lesions did not show detectable expression. The difference in expression of VEGF in cancer cases and control cases was found to be statistically significant ($p < 0.05$). These findings are consistent with an earlier study on cervical cancer, which reported that VEGF was not expressed in control tissue samples whereas VEGF expression was high in patients with high-grade squamous intraepithelial (HSIL) lesions (33.33%) and patients with cervical cancer (60.87%) [22], showing a statistically significant difference between tumour samples and control [22].

Another study also reported that VEGF expression progressively increased along a continuum from normal epithelium to invasive SCC [23]. Our findings showed that immunostaining was high in high-grade tumours and slightly increased according to the grade of a tumour, this difference was not statistically significant ($p > 0.05$). A study examining transitional cell carcinoma of the urinary bladder reported that VEGF immunostaining was positively correlated with grade, stage, and recurrence of transitional cell carcinoma but the findings were not statistically significant [24] while another study on urinary bladder carcinoma showed that the rate of VEGF expression increased significantly with the progression of tumour grade [25]. An earlier report looking at cervical cancer stated that high VEGF immunostaining was observed in grade III cervical intraepithelial neoplasia whereas expression was found to be low in grade I cervical intraepithelial neoplasia and in control group samples [26]. Lastly, an earlier study also found that there was no significant difference between VEGF expressions in tumours of various grades of differentiation [27].

In the current study, the expression of VEGF protein was analysed in different age groups of the patients (≤ 55 years and > 55 years), and it was found that there was no significant difference between age groups ($p > 0.05$). A previous study also reported no correlation between age and VEGF positive tumours, with the number of VEGF positive tumours in patients <50 years 58.0 %, while 50-55 years showed 63.75%, and >55 years was 52.9%, all of these differences showing no statistical significance [27].

Her-2 belongs to the family of *HER* genes that control cell growth, survival, differentiation and migration [28][29]. Her-2 over-expression has been noticed in invasive breast cancers [30], gastric cancer [31] and previous studies examining cervical cancer reported that 29.7% tumours expressed the c – erbB-2 (Her-2) protein [32]. The present study was also aimed to measure the expression of Her-2 in cervical cancer, and positive immunostaining was observed in 28 (43.07%) cases while the control group did not show any expression. This is similar to a previous study examining lesions of the uterine cervix, which reported that higher expression of Her-2/neu was noticed in malignant lesions as compared to benign lesions [33]. Moreover, expression of the Her-2 protein was found to be higher in high-grade tumours when compared to low-grade tumours. The difference of Her-2 expression between the tumour grades was found to be statistically significant ($p < 0.05$) in this study. Furthermore, an earlier study reported that malignancies exhibited a variable degree of Her-2 expression with significantly higher expression observed in high-grade squamous cell carcinomas [33]. Lastly, another study reported similar findings where benign, premalignant and malignant cervical tissues showed a highly significant correlation between the expression of the epidermal growth factor receptor (EGF-R), the oncogene product of c–erbB-2 and the histological grade of the lesion [34]. In the current study, immunoreactivity of Her-2 was not correlated with stage of a tumour. This differs from two previous reports that showed Her-2 positivity was higher in Stage III (87.50%) and stage IV (100%) tumors as compared to stage I (48.28%) and Stage II

(72.22%) [33]; and that stronger Her-2/neu expression were observed in higher stage tumors [35].

The present study supports earlier findings that overexpression/up-regulation of VEGF and Her-2 protein in cervical cancer are linked to poor prognosis and that expression of these proteins may play vital roles in the development and progression of cervical cancer.

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Basic Science

Effect of a Histone Deacetylases Inhibitor of IL-18 and TNF-Alpha Secretion in Vitro

Zlatka Georgieva Dobreva*, Boncho Grigorov Grigorov, Spaska Angelova Stanilova

Department of Molecular Biology, Immunology and Medical Genetics, Medical Faculty, Trakia University, Armeiska 11, Stara Zagora 6000, Bulgaria

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Keywords: HDAC; IL-18; SAHA; TNF-α; PBMC

***Correspondence:** Zlatka Georgieva Dobreva. Department of Molecular Biology, Immunology and Medical Genetics, Medical Faculty, Trakia University, Armeiska 11, Stara Zagora 6000, Bulgaria. E-mail: zdobreva@mf.uni-sz.bg

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Abstract

BACKGROUND: Interleukin-18 (IL-18) and Tumor Necrosis Factor-alpha (TNF-α) are proinflammatory cytokines that increased the development of Th1 immune response, but have a different type of regulation of the gene expression. Whereas TNF-α has an inducible expression, IL-18 is translated as an inactive protein and required proteolytic cleavage by Casp-1 in inflammasome complexes.

AIM: To investigate the effect of the histone deacetylases inhibitor Suberoylanilide Hydroxamic Acid (SAHA) on the gene expression and secretion of both cytokines, IL-18 and TNF-α, according to their contribution to the cancer development and anticancer immunity.

METHODS: Isolated peripheral blood mononuclear cells (PBMC) were stimulated with LPS and C3bgp with or without SAHA. Cytokine production was assessed by ELISA at 6 and 24h.

RESULTS: IL-18 and TNF-α secretion was significantly increased at 6h and 24h in response to stimulation. TNF-α production from stimulated PBMC was downregulated by SAHA at 6 and 24h. Treatment with SAHA does not inhibit the secretion of IL-18 significantly either at 6 or 24h of stimulation.

CONCLUSION: The inhibition of histone deacetylases by SAHA does not influence the inflammasome-dependent production of immunologically active IL-18. In contrast, the production of proinflammatory TNF-α in cultures was mediated by the activity of HDAC class I and class II enzymes.

Introduction

The inducible cytokine release is regulated at several levels started from chromatin remodelling allowing gene expression and finalised with protein secretion. Before transcription, the gene region must be accessible to the transcriptional factors binding. The key role in this process has the histone modification mainly acetylation and deacetylation. Two classes of enzymes drive the acetylation status of the chromatin. Acetyltransferases open the chromatin conformation by histone acetylation and allow the transcriptional process. Histone deacetylases (HDACs) compress the chromatin structure triggering the gene silence by deacetylation [1][2]. HDACs are divided into four classes. Of them, class I are resident to the nucleus, where they act as histone modifiers and repressors of the transcription. Histone deacetylases class II is moving between the nucleus and cytoplasm. They can regulate the gene expression also by the changes in acetylation/deacetylation status of other proteins [3][4].

Recently, a new class of small organic molecules-HDAC inhibitors (HDI), which abolish the action of HDACs, are intensively studied, especially about cancer and inflammatory diseases treatment [5][6]. They regulate the expression up to 10% of the cellular genes by affecting enzymes including in chromatin remodelling complex and recruiting of the transcription factors [7]. Although the histone acetylation is linked to an increased transcription; HDI also can increase the expression of some genes by the still unclear mechanism. Suberoylanilide Hydroxamic Acid (SAHA) is the HDI interacting with

class I and class II histone deacetylases [4]. SAHA was the first HDI approved by U. S. Food and Drug Administration for the treatment of some malignant disease as CTCL [8].

During the early phase of the immune response, binding of microbial antigens (especially those referring to pathogen-associated molecular patterns-PAMPs) to pattern recognition receptors (PRR) of the immune cells, activate intracellular signalling pathways, which in turn lead to alteration in cell behaviour and gene expression. As a consequence soluble mediators are synthesised and secreted by the activated immune cells including proinflammatory and immunoregulatory cytokines like TNF-α and IL-18. Tumor necrosis factor-alpha (TNF-α) was first discovered as mediating cell death of some malignant cells. Recently, a growing body of evidence showed that TNF-α has a tumour-promoting role as a key mediator of chronic inflammation which drives the cancer development [9]. TNF-α is secreted as a soluble 17-kDa molecule after processing of membrane-bound TNF-α by constitutive expressed membrane TNF-α converting enzyme [10], mainly by the activated macrophages/monocytes. Interleukin-18 (IL-18) is also a proinflammatory cytokine, but it is included in Th1 polarisation. It's inducible gene expression after recognition of PAMPs by PRR lead to the synthesis of an inactive protein (pro – IL-18). Pro – IL-18 is converted into biologically active IL-18 by another activation pathway in inflammasome complexes through caspase 1-mediated cleavage [11]. IL-18 is involved in the development of successful antitumor immunity through its ability to induce IFN-γ secretion [12]. Unlike TNF-α, the mechanisms regulating IL-18 processing and secretion remains not well understood.

In this regard, our study was designed to investigate the SAHA effect on protein synthesis and release of TNF-α and IL-18 from stimulated healthy human PBMC.

Methods

Isolation of PBMC

Peripheral venous blood was taken by venipuncture from 10 healthy donors after the approval of the Ethics Board of Medical Faculty, Trakia University. Each volunteer was informed and signed informed consent. The samples (10 ml) were collected in sterile tubes with EDTA. Peripheral blood mononuclear cells (PBMC) were harvested after density gradient centrifugation over Histopaque-1077.

In vitro culturing

PBMC (1 x 10^6 cells/ml) cultures were prepared as described previously by Dobreva et al. [13]. They were stimulated with: 30 μg/ml C3 binding glycoprotein, (C3bgp) [14]; or 1 μg/ml Lipopolysaccharide (LPS) from Escherichia coli (Sigma-Aldrich-Merck, Darmstadt, Germany). PBMC cultures were incubated at 37^{o}C for 6 and 24h. After incubation the separated supernatants were stored at -70^{o}C.

HDAC inhibition

SAHA (Sigma-Aldrich-Merck, Darmstadt, Germany) (5 μM) was used for the inhibition of histone deacetylases. The inhibitor was added one h before stimulation.

Cytokine evaluation

Assessment of IL-18 and TNF-α was performed by ELISA, under to the manufacturer's instructions. For the detection of TNF-α, R&D Systems Qantikine ELISA kit (Minneapolis, MN 55413, USA) was used. IL-18 production was measured using commercially available kits purchased from MBL International Corporation (Woburn, MA 01801, USA). The colour reaction was measured as OD units and expressed in pg/ml. The sensitivity of the ELISA kits was 12 pg/ml for IL-18 and 15 pg/ml for TNF-α.

Statistical analysis

The data was presented as means and standard error of the mean. Evaluation of the statistical differences between cultures was performed by Student's t-test. Differences were significant when the P value was equal or less than 0.05.

Results

TNF-α production was suppressed by SAHA

Results presented in Figure 1 demonstrated that C3bgp and LPS increased significantly TNF-α production at 6 and 24 h in comparison with nonstimulated controls ($p < 0.05$). The addition of SAHA to stimulated cultures leads to significantly decreased TNF-α production at 6 and in higher degree at 24 h. TNF-α quantity secreted by PBMC cultured with HDAC inhibitor was 4 to 6-fold less than in cultured PBMC without SAHA at six h and 5 to 8 fold less at 24 h.

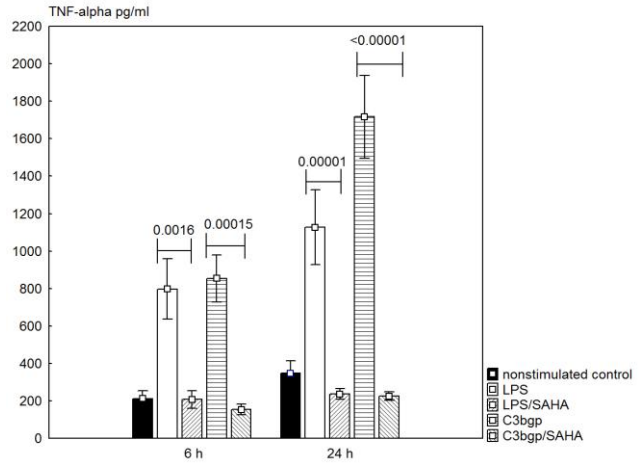

Figure 1: TNF-α production from stimulated with LPS and C3bgp PBMC after inhibition of HDAC by five μM SAHA at 6 and 24 h. P value between stimulated cultures without SAHA and stimulated cultures with SAHA is indicated on the figure

SAHA did not modulate significantly IL-18 production

The addition of C3bgp and LPS leads to significantly more IL-18 production in comparison with nonstimulated cultures ($p < 0.05$). Moreover, we did not observe significant differences between 6 and 24 h in the secretion of IL-18 from stimulated cultures. The inhibition of HDAC slightly decreased IL-18 production. However, we did not detect significant differences between stimulated cultures treated with SAHA and cultures without SAHA as shown in Figure 2.

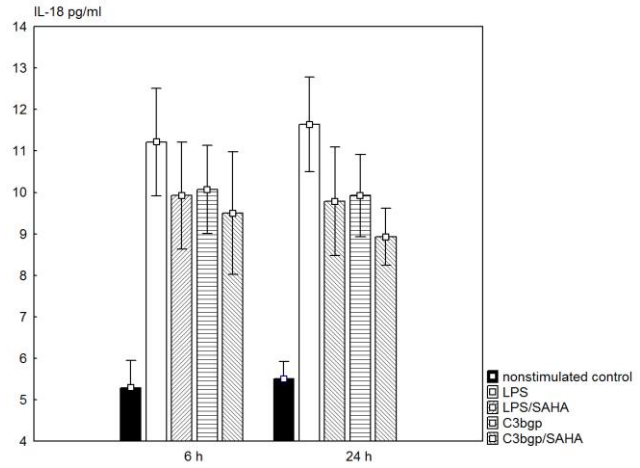

Figure 2: IL-18 production from stimulated with LPS and C3bgp PBMC after inhibition of HDAC by five μM SAHA at 6 and 24h

Discussion

IL-18 and TNF-α are proinflammatory cytokines, with a different effect on the acquired immunity and different production and regulatory mechanism as well. Whereas TNF-α gene expression and secretion is mediated through the TLR signalling pathway, IL-18 required proteolytic processing by Casp-1 in inflammasome before its secretion [11]. In the current study, we evaluated the effect of the histone deacetylases inhibitor SAHA on the gene expression and secretion of both cytokines, IL-18 and TNF-α, according to their contribution to the cancer development and anticancer immunity.

TNF-α is a main proinflammatory cytokine identified for the first time because of its rapid cytolytic effect on some experimental cancers [15]. Recently, new studies showed that TNF-α has protumorigenic activity and is involved in all key points of tumorigenesis – tumour promotion, malignant transformation, tumour cell proliferation, angiogenesis and malignant cell spreading [9]. This investigation showed that the addition of the HDAC inhibitor SAHA downregulated the production of TNF-α released by the stimulated mononuclear cells. The same results were obtained from other authors [16]. Today is accepted that histone deacetylation is linked to the decreased gene transcription. Nevertheless, there was experimental evidence demonstrating that the treatment with HDI downregulated the expression of some proinflammatory cytokines by a mechanism which currently is being studied extensively. For example, Takada et al. demonstrated that HDI SAHA did not affect the binding of NF-kB transcription factor to the promotors of the target genes, but inhibited IkBα kinase activation, IkBα phosphorylation and translocation of p65 to the nucleus [17]. It is widely accepted that TNF-α production in response to LPS is mediated by TLR4 followed by the activation of NF-kB. Our study demonstrated that SAHA downregulated TNF-α production after stimulation with LPS. Therefore, our results are in accordance with decisions of the other investigators [17][18] that the downregulating effect of SAHA on the proinflammatory cytokine production is mediated by the suppression of NF-kB transduction pathway. Previously we showed that C3bgp activated JNK and p38 intracellular transduction pathways [19]. Furthermore, our results showing that SAHA inhibited C3bgp-mediated TNF-α production are in concordance with the study of Ajizian et al., demonstrating that the suppression of p38 MAPK leads to downregulated TNF-α production [20] and with the study of Choo and coauthors, showing that SAHA affected p38 activation [21].

Currently, it is widely accepted that IL-18 drives the Th1 immune response, because induces IFN-γ secretion from T cells and natural killer cells [12]. There is evidence that treatment with IL-18 of experimental animals has significant antitumor action [11]. Moreover, in vivo IL-18 administration in experimental mice inoculated with tumour cell line stimulated IFN-γ production and IL-12 independent antitumor response [22]. Its elevated levels have been

observed in several types of cancers, especially in advanced cancers with metastasis [23][24][25]. Our study indicated that SAHA did not influence significantly IL-18 production from PBMC. There are few studies about SAHA and its effect on IL-18 secretion. However, our results contradict the study of Choo et al., which demonstrated that SAHA inhibits the production of IL-18 in E11 and THP-1 cell lines in a dose-dependent manner [26]. One explanation for this discrepancy may be the different cell sources of IL-18. Our experiments were done with PBMC from healthy donors. In their study Choo et al., used E11 cell line of human rheumatoid synovial cells, transformed with simian virus 40 large T antigen expression vector or a THP-1 monocytic cell line derived from a patient with acute myeloid leukaemia. Moreover, it is well known that the tumour cell lines had specific regulation of the gene expression in comparison with normal human cells [27].

Recently, it is widely accepted that chronic inflammation has a crucial role in the tumour-promoting and survival, regardless of its origin. In this process, proinflammatory cytokines play a key role [28]. Many studies indicated that SAHA suppresses proinflammatory cytokines like IL-12, IFN-γ, TNF-α and IL-1β expression [13][16]. However, not always the inhibition of IL-12 and IFN-γ has positive effects, because of their immunoregulatory function as cytokines triggering cell-mediated anticancer immunity. Our study has shown that treatment with SAHA leads to decreased TNF-α and unmodified IL-18 production by PBMC-a surprising effect on the synthesis and secretion of both cytokines. Considering the protumorigenic activity of TNF-α, the downregulation of its production is a desirable effect of treatment with various histone deacetylase inhibitors including SAHA. However, IL-18 like IL-12 has antitumorigenic activity because of its property to induce Th1 cell-mediated type of the immune response. Therefore, under immunosuppression triggered by SAHA, an unaffected IL-18 production may have a crucial role in the realization of antitumor immunity. Thus, this SAHA-specific modulation of the TNF-α and IL-18 production may provide a further clinical advantage for the treatment of various malignant conditions.

In summary, our study showed that HDAC inhibitor SAHA downregulated TNF-α production and did not affect IL-18 secretion from activated PBMC. Therefore, we conclude that the production of TNF-α is mediated by HDAC class I and class II enzymes, but they are not involved in inflammasome-dependent regulation of biologically active IL-18 secretion. This different regulation of both cytokines by histone deacetylases and their inhibitors may be used in the development a new approach in the therapy of certain types of cancers.

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Association Study of the *ATP - Binding Cassette Transporter A1 (ABCA1)* Rs2230806 Genetic Variation with Lipid Profile and Coronary Artery Disease Risk in an Iranian Population

Habib Ghaznavi[1], Ehsan Aali[2], Mohammad Soleiman Soltanpour[3*]

[1]Health Promotion Research Center, Zahedan University of Medical Sciences, Zahedan, Iran; [2]Department of Pharmacology, School of Medicine, Qazvin University of Medical Sciences, Qazvin, Iran; [3]Department of Medical Laboratory Sciences, School of Paramedical Sciences , Zanjan University of Medical Sciences, Zanjan, Iran

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Keywords: ATP - binding cassette transporter A1; Polymerase chain reaction-restriction fragment length polymorphism; R219K mutation; Coronary artery disease

***Correspondence:** Mohammad Soleiman Soltanpour. Department of Medical Laboratory Sciences, School of Paramedical Sciences , Zanjan University of Medical Sciences, Zanjan, Iran. E-mail: soltanpour86@gmail.com

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Abstract

BACKGROUND: *ATP - binding cassette transporter A1 (ABCA1)* plays essential roles in the biogenesis of high - density lipoprotein - cholesterol. Variations in the ABCA1 gene may influence the risk of coronary artery disease (CAD).

AIM: Present study aimed to investigate the association of rs2230806 (R219K) polymorphism of *ABCA1* gene with the development and severity of CAD in an Iranian population.

MATERIALS AND METHODS: Our study population consisted of 100 patients with angiographically confirmed CAD and 100 controls. The genotyping of *R219K* mutation of *ABCA1* gene was determined by PCR - RFLP method. Lipid profile was determined using routine colourimetric assays. Statistical analysis was done by SPSS - 16.

RESULTS: The genotypic (P = 0.024) and allelic (P = 0.001) distribution of the *ABCA1 R219K* polymorphism were significantly different between the two groups. In a univariate analysis (with genotype RR as the reference), the *RK* genotype (OR = 0.46, 95%CI = 0.25-0.86, P = 0.020) and *KK* genotype (OR = 0.27, 95%CI = 0.11 – 0.66, P = 0.005) was significantly associated with a decreased risk of CAD. A multiple logistic regression analysis revealed that smoking (0.008), diabetes (P = 0.023), triglyceride (P = 0.001), HDL - cholesterol (P = 0.002) and *ABCA1 KK* genotype (P = 0.009) were significantly and independently associated with the risk of CAD. The association between different genotypes of *R219K* polymorphism with lipid profile was not significant in both groups (P > 0.05). The *R219K* polymorphism was significantly associated with severity of CAD (P < 0.05).

CONCLUSION: The carriage of K allele of *ABCA1 R219K* polymorphism has a protective effect on CAD risk and correlates with a decreased severity of CAD. This protective effect seems to be mediated independently of plasma lipid levels.

Introduction

Coronary artery disease (CAD) is considered as one of the main leading causes of morbidity and mortality in Iranian population. Several epidemiological studies have indicated a strong inverse association between high-density lipoprotein cholesterol (HDL - C) levels and CAD occurrence [1][2]. Low levels of HDL-C in plasma are considered as an independent risk factor for CAD development [3]. The circulating levels of HDL - C are determined by the interaction of both environmental and genetic factors. Genetic factors contribute considerably (up to 60%) for plasma HDL - C levels and some genes including ATP - binding cassette transporter A1 (ABCA1) have been associated with its plasma concentration [4][5].

The ABCA1 is a membrane transporter protein that plays an essential role in the efflux of cholesterol from peripheral tissues back to the liver.

The ABCA1 transporter functions in the initial step of revers cholesterol transfer and transfers cholesterol and phospholipid from peripheral tissues to lipid-poor apolipoprotein AI, creating nascent high - density lipoprotein particles [6]. Reduced activity of the ABCA1 transporter leads to disturbances in plasma lipid composition and levels which are common findings in patients with CAD [6].

Reduced HDL - C level is the most common lipoprotein abnormality observed among CAD patients and is a major determinant of morbidity and mortality rate in these patients [7][8]. Genetic and molecular biology studies have shown that common polymorphisms in the *ABCA1* gene can influence the function of ABCA1 transporter resulting in the altered biosynthesis of HDL - C particles [9][11]. Reduced function of the ABCA1 transporter may be involved in the pathogenesis of CAD. The *R219K* (rs2230806, 107620867C>T) common polymorphism in the *ABCA1* gene is located in the two major extracellular loops of ABCA1 protein, which has a critical role for interaction with apoA - I and for cholesterol efflux [12][13]. Therefore, it is likely that the *R219K* variant acts as a functional mutation to modulate HDL - C level [12]. The *R219K* polymorphism may affect the development and severity of CAD via altering the lipid profile [14]. Numerous studies have investigated the association of R219K mutation of *ABCA1* gene and the development of CAD in different populations [9][10][15]. However, in-consistent results have been reported [9][10][14][15][16]. The aim of the present study was to investigate the role of *ABCA1* rs2230806 genetic polymorphism in the development of CAD from an Iranian population. Also, the effect of the *ABCA1* rs2230806 common polymorphism on lipid profile was investigated in CAD patients and control subjects.

Material and Methods

Study Population

Our study population consisted of 200 subjects including 100 patients (50 male and 50 female) with a confirmed diagnosis of CAD and 100 matched controls (50 male and 50 female). The mean age of CAD patients and controls were 58.96 ± 11.54 and 57.53 ± 16.1, respectively. The CAD diagnosis was made by angiography conducted by an expert cardiovascular specialist. The minimum required criteria for including of CAD patients in the study were the presence of more than 50% stenosis in at least one major coronary vessel. The severity of CAD was determined based on the number of the stenotic vessel showing ≥ 50% stenosis. Accordingly, patients were classified as single, double and triple vessel stenosis patients. Patients showing fewer than 50% stenosis or taking lipid-lowering drugs were excluded from the study. Patients suffering from valvular heart disease, cardiomyopathy, autoimmune disorders, inflammatory disease, infectious disease, renal, heart and liver failures and cancer were excluded from the study. Control subjects were selected randomly after careful inspection of a cardiovascular specialist. Control subjects were included in the study if they had no personal or family history of CAD or other reasons to suspect CAD. Also, control subjects with overt concomitant diseases such as malignant diseases, autoimmune disease, infectious disease and organ failure were excluded. For all subjects, complete medical history including questions about smoking habits, history of hypertension and diabetes and family history of heart disease was obtained by questionnaire. Diabetes was defined by fasting blood glucose > 126 mg/dL, and hypertension was defined by systolic blood pressure >140 mmHg and/or diastolic blood pressure >90 mmHg. All of the study subjects participated voluntarily in the study and written informed consent was obtained from all participants. The study was approved by ethical committee of Zahedan University of Medical Sciences (Ethical committee code: IR.ZAUMS.REC.1394.383), Zahedan, Iran.

Blood Samples Collection

From each participant, 5 ml fasting blood were collected in EDTA containing tubes and instantly centrifuged. Plasma fraction was separated and stored at -40ºC and the cellular fraction was used for DNA extraction.

ABCA1 R219K Polymorphism Analysis

Genomic DNA was purified from blood leukocytes using a commercially available DNA extraction kit (Viogene, Poland). The genotyping of *ABCA1* gene R219K polymorphism was conducted by PCR reaction at an annealing temperature of 62ºC. The sequence of primers were obtained from previous studies [17] and was as follows: forward 5' – AAAGACTTCAAGGACCCAGCTT - 3' and reverse 5' - CCTCACATTCCGAAAGCATTA - 3'. After amplification, a seven microliter aliquot of PCR product was digested with 5u EcoN1 restriction enzyme (Fermentas, Inc, Germany) for at least 8 hours at 37 °C. The obtained fragments after restriction digestion were separated on 2.5% agarose gel and stained with Sybr green dye. After digestion, the 309bp PCR amplicon cleaves into 184 and 125 bp fragments in the presence of K allele whereas the R allele produces a single non - digested 309 bp fragment. Genotyping results from ten percent of samples determined by PCR - RFLP method was randomly confirmed by direct sequencing of PCR products (Figure 1).

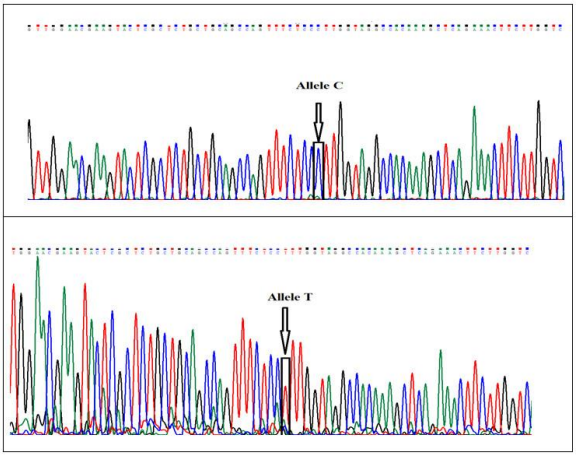

Figure 1: Results of direct sequencing of PCR products showing wild (C) and mutant (T) allele of ABCA1 R219K polymorphism

Biochemical Methods

Plasma total cholesterol (TC), Triglycerides (TG), high-density lipoprotein- cholesterol (HDL - C), low-density lipoprotein cholesterol (LDL - C) and fasting glucose levels were measured with commercially available enzyme assay kits (Pars Azmon Co, Tehran, Iran) using Mindray autoanalyser (BS-200).

Statistical Analysis

In descriptive statistics, numerical variables presented as mean ± SD and were compared using Student t-test. Categorical variables were compared with Chi-square test or Fisher exact test (in case of small sample size). The deviation of genotype distribution from the Hardy - Weinberg equilibrium (HWE) was assessed in both patients and controls by Chi-square test. Multivariate logistic regression analysis was performed to determine the independent association of each covariate with the risk of a CAD. The statistical analysis with a P value < 0.05 was considered significant. All statistical analysis was performed using SPSS 16 software.

Results

The comparison of clinical and biochemical parameters of the CAD patients and control subjects revealed no statistically significant differences in the mean ages and sex distribution between CAD group and control group (P = 0.471; P = 1.000, respectively). However, plasma levels of TC, TG and LDL - C were significantly higher while the plasma levels of HDL - C were significantly lower in CAD group compared with control group. Moreover, the presence of diabetes (P = 0.014), hypertension (P = 0.009) and smoking habit (P = 0.002) were significantly more common in CAD group than control group (Table 1). Also, statistical analysis using Chi-square test indicated that the genotype distribution of *ABCA1* R219K polymorphism was significantly different between the two groups (P = 0.024, χ^2= 13.6) (Table 1).

Table 1: Clinical characteristics of the CAD group and control group included in the study

Variables	CAD group n=100	Control group n=100	P value
Age (years)	58.96 ± 11.54	57.53 ± 16.1	0.471
Sex (M/F)	50/50	50/50	1.000
TG (mg/dl)	202.21 ± 90.02	162.17 ± 69.67	0.001
TC (mg/dl)	200.78 ± 58.13	173.57 ± 40.13	<0.001
HDL-C (mg/dl)	39.17 ±8.45	45.39 ± 12.62	<0.001
LDL-C (mg/dl)	114.98± 44.43	96.97 ± 34.51	0.002
Hypertension (n.%)	23 (23%)	8 (8%)	0.009*
Diabetes (n.%)	24 (24%)	10 (10%)	0.014*
Smoking (n.%)	33 (33%)	12 (12%)	0.002*
R219K genotypes RR:RK:KK	50:40:10	29:50:21	0.024

CAD: Coronary artery disease, TC: total cholesterol, TG: Triglyceride, HDL: High - density lipoprotein - cholesterol, LDL: Low - density lipoprotein- cholesterol. * P value determined by Fisher exacts test.

The frequency of RR, RK and KK genotypes among the CAD group was 50.0%, 40.0%, and 10.0%, respectively, and among the control group was 29.0%, 50.0% and 21.0%, respectively (Table 2). The χ2 test showed that the genotype distribution of *ABCA1* gene R219K polymorphism were in coincidence with the Hardy - Weinberg equilibrium in both CAD patients (P = 0.63) and controls (P = 0.98). In a univariate analysis (with genotype RR as reference) the RK genotype (OR = 0.46, 95% CI: 0.25 - 0.86; P = 0.020) and KK genotype (OR = 0.27, 95% CI: 0.11-0.66; P = 0.005) was significantly correlated with a decreased risk of CAD (Table 2). Also, the frequency of minor K allele of *ABCA1* R219K polymorphism was significantly lower in CAD group compared with control group (30% vs. 46%; OR = 0.50; 95% CI: 0.33-0.75; P = 0.001).

Table 2: Prevalence of *ABCA1* R219K allele and genotypes in CAD group and control group

ABCA1 R219K Polymorphism	CAD group (n=100)	Control group (n=100)	OR (95%CI)	P value*
R allele	140 (70.0%)	108 (54.0 %)	Ref	-
K allele	60 (30.0%)	92 (46.0%)	0.50 (0.33-0.75)	0.001
RR	50 (50%)	29 (29%)	Ref	-
RK	40 (40%)	50 (50%)	0.46 (0.25-0.86)	0.020
KK	10 (10%)	21 (21%)	0.27 (0.11-0.66)	0.005

CAD: Coronary artery disease, RR: Wild-type, RK: Heterozygote; KK: Homozygote; * P values were determined by Fisher's exact test.

In a multiple binary logistic regression analysis using the study group (CAD group vs. control group) as the dependent variable and using age, sex, TG, TC, HDL - C, LDL - C, smoking, diabetes, hypertension and R219K genotypes as covariates, the KK genotype of *ABCA1* R219K polymorphism was independently associated with a decreased risk of CAD (OR = 0.212, 95% CI: 0.069 - 0.653; P = 0.009) but the RK genotype was not (OR = 0.430, 95% CI: 0.194-0.954; P = 0.074). Also, TG levels (P = 0.001), HDL - C levels (P = 0.002), diabetes (P = 0.023) and

smoking (P = 0.008) were significant risk factor for CAD development. However, TC levels (P = 0.245), LDL - C levels (P = 0.644) and hypertension (P = 0.279) didn't show any significant association with CAD risk in regression analysis (Table 3).

Table 3: Multiple Logistic regression analysis of association between the *ABCA1* R219K genotypes and risk of coronary artery disease

Variable	Wald	Odd ratio	95% CI	P value
Age	0.075	0.997	0.973-1.021	0.785
Sex	0.622	1.321	0.662-2.636	0.430
Hypertension	1.198	1.740	0.639- 4.741	0.279
Diabetes	5.097	2.881	1.154-7.175	0.023
Smoking	6.889	3.134	1.349-7.281	0.008
TG	10.181	1.009	1.003-1.014	0.001
TC	1.353	1.009	0.994-1.023	0.245
HDL-C	7.959	0.951	0.918-0.985	0.002
LDL-C	0.001	1.000	0.982-1.019	0.644
RK vs. RR	4.313	0.430	0.194-0.954	0.074
KK vs. RR	7.302	0.212	0.069-0.653	0.009

Furthermore, the association of different genotypes of *ABCA1* R219K polymorphism with lipid profile was presented in Table 4. As shown, no significant differences were observed in the lipid distribution according to the different genotypes of *ABCA1* R219K polymorphism in CAD group and control group.

Table 4: Lipid profile distribution among different genotypes of *ABCA1* R219k polymorphism

Parameter	Controls*			Cases†		
	RR	RK	KK	RR	RK	KK
TG mean	162.2	162.4	161.6	192.1	215.1	185.9
(SD)	(91.7)	(42.6)	(88.3)	(95.3)	(91.3)	(38.5)
TC mean	181.9	173.8	160.6	196.9	197.6	232.7
(SD)	(33.6)	(43.4)	(39.0)	(53.1)	(57.5)	(79.3)
HDL-C mean	43.3	46.6	45.3	38.4	40.5	37.6
(SD)	(12.7)	(13.4)	(10.5)	(8.1)	(9.2)	(6.6)
LDL-C mean	108.4	94.7	86.5	123.0	107.3	105.4
(SD)	(32.6)	(33.9)	(35.6)	(39.7)	(47.4)	(51.4)

*One way ANOVA test showed insignificant differences in TG (P = 0.99), TC (P = 0.21), HDL-C (P = 0.53) and LDL - C (P = 0.06) levels according to different genotypes of R219K polymorphism. †One way ANOVA test showed insignificant differences in TG (P = 0.31), TC (P = 0.18), HDL - C (P = 0.44) and LDL - C (P = 0.19) levels among different genotypes of R219K polymorphism.

However, investigating the prevalence of different genotypes of ABCA1 R219K polymorphism between patients with one and two or three stenotic vessels revealed significant differences in the genotype distribution between them (P < 0.05) (Table 5).

Table 5: The association of severity of CAD with the number of stenotic coronary vessels

ABCA1 R219K genotypes	1 SV N = 36	2 SV N = 42	3 SV N = 22	P value* 2 SV vs. 1SV	P value* 3 SV vs. 1SV
RR	12 (33.33%)	24 (57.14%)	14 (63.63%)	Ref	Ref
RK	17 (47.22%)	17 (40.48%)	6 (27.27%)	0.225	0.080
KK	7 (19.44%)	1 (2.38%)	02 (9.1%)	0.013	0.135
RK+KK	24 (66.67%)	18 (42.86%)	08 (36.36%)	0.042	0.031*

SV: Stenotic vessel, ABCA1: ATP - binding cassette transporter A1, RR: Wild-type, RK: Heterozygote; KK: Homozygote. * P values were calculated using Fisher's exact test,

Discussion

We found a significant association between the K allele of *ABCA1* R219K polymorphism and a decreased susceptibility to CAD development in an Iranian population. Based on the present study, the carriers of K allele exhibited lower risk of CAD development than the carriers of R allele (OR = 0.50, 95% CI = 0.33-0.75, P = 0.001) Also, the severity of CAD was significantly lower in K allele carriers of R219K polymorphism relative to homozygous carriers of R allele. However, the association of R219K polymorphism with the lipid profile didn't show any significant association.

The association of an R219K polymorphism of *ABCA1* gene with CAD risk and lipid profile has been investigated in several studies. The study by Abd El-Aziz et al. reported that the K allele of ABCA1gene confers protection against CAD development [10]. In another study by Yin et al., it was shown that carriers of K allele relative to carriers of R allele of R219K polymorphism of *ABCA1* gene had a significantly lower risk for progression of atherosclerosis (P < 0.01) [18]. Also, the study by Benton et al. conducted in the United States indicated that the KK genotype of R219K polymorphism was associated with slightly higher HDL - C and thus may protect against subclinical cardiovascular disease [19]. Interestingly, in a recently published study by Au et al., it was shown that *ABCA1* R219K K variant allele may associate with decreased risk of cerebrovascular disease, and thus provided further evidence for the protective effects of *ABCA1* R219K K variant allele on the development of another vascular disease such as cerebrovascular disease [20].

By these studies, the current study demonstrated a lower risk of CAD development in a carrier of K allele than carriers of R allele of ABCA1 R219K polymorphism. On the contrary, Rejeb et al. didn't find any significant association between R219K polymorphism and CAD risk in a Tunisian population [9]. Moreover, Li et al concluded that the R219K polymorphism does not seem to influence CAD risk in a China population [13]. Also, the study by Cyrus et al in a Saudi Arabians population identified that the KK genotype of *ABCA1* R219K polymorphism acts as a promoting risk factor for CAD development [16]. There are several possible speculations for the inconsistent results of association studies. Some of these controversies may be related to different genetic background and genetic diversity among ethnicities. Ethnic differences may influence the impact of this polymorphism on CAD risk [11]. Indeed, a recently published study by Liu et al indicated interethnic differences in the genotype distribution of R219K genetic polymorphism and demonstrated that this genetic variant acts as a significant protective factor against CAD development in Asian population but not in some Caucasians populations [21]. Also,

population-specific linkage disequilibrium between markers loci and disease-susceptibility loci, confounding sampling bias, sample selection criteria, variation in study design, misclassification of phenotypes, gene-gene and gene-environment interactions are other factors that influence genetic association results [22]. Moreover, a great difference in the K allele frequency of R219K polymorphism was seen in various ethnic groups that may influence the consistency of association studies [19][23].

Increased circulating level of HDL - C is a significant protective factor against CAD development. HDL - C deficiency is the most common lipid abnormality observed among patients with premature CAD [10]. At least 50% of HDL - C levels are determined by genetic factors [4]. ATP - binding cassette transporter A1 (*ABCA1*) is shown to play an essential role in the HDL-C biosynthesis by efflux of cellular cholesterol to apolipoprotein - A1. The effect of R219K polymorphism of *ABCA1* gene on lipid profile has been studied extensively with discrepant results [9][14][17][24][25]. Our study didn't find any significant association between this common polymorphism and TG, TC, HDL - C and LDL - C levels. In accordance with present study, some other studies also reported no significant association between the K allele of *ABCA1* R219K polymorphism and serum HDL - C levels and TG levels [17][23][25] However, some other studies reported this common polymorphism as a significant contributing factor for the plasma lipid levels [9][19][24]. Recently, a study by Rejeb et al. reported that the K allele was significantly associated with a higher mean HDL - C concentration [9]. Also, another study by Mokuno et al. reported that the mean HDL - C level was slightly higher in KK genotype than in RR genotype in both men and women [24]. Moreover, the study by Benton et al. demonstrated higher HDL - C level and lower LDL - C and TG levels in carriers of homozygote KK genotype [19].

The causes for the discrepant association of *ABCA1* R219K polymorphism with lipid profile in different studies remains to be determined. However, the interaction between genetic and environmental factors plays an essential role in determining the lipid profile. So, the variation in environmental factors such as geographic distribution, climate, diet, lifestyle and socioeconomic status may modify the effect of *ABCA1* R219K polymorphism on lipid profile which can explain the inconsistencies among different studies [4][11].

On the other hand, some other studies including the current study have reported a significant association of K allele of *ABCA1* R219K polymorphism with a decreased risk of CAD development independent of serum lipid levels [21][26]. According to some studies, cholesterol efflux capacity has been associated with carotid intima-media thickness and the risk of cardiovascular disease independent of HDL-C levels [27, 28]. So, the independent association of K allele of *ABCA1* R219K polymorphism with decreased susceptibility to CAD development may be attributed to increased cholesterol efflux capacity seen in K allele carriers of R219K polymorphism [29]. Recently, in a study by Villard et al., it was shown that cholesterol efflux capacity is significantly and inversely associated with an incident of coronary heart events independent of established cardiovascular risk factors including the HDL cholesterol or apoA-I concentrations [30].

The present study bears some limitations as follows (i) The cholesterol efflux capacity and its association with different genotypes of *ABCA1* R219K polymorphism were not determined (ii) The plasma concentration of Apo-A and Apo-B were not measured (iii) The other polymorphisms of *ABCA1* gene and their interaction with R219K polymorphism were not determined.

In conclusion, the carriage of K allele of the *ABCA1* R219K polymorphism has a protective effect on CAD development and correlates with a decreased severity of CAD. This protective effect of the K allele seems to be mediated independently of plasma lipid levels.

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Basic Science

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism as a Confirmatory Test for Onychomycosis

Nova Zairina Lubis*, Kamaliah Muis, Lukmanul Hakim Nasution

Universitas Sumatera Utara, Dermatology and Venereology, Medan, North Sumatera, Indonesia

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Keywords: Onychomycosis; Culture; Diagnostic tests; PCR – RFLP; Alternative examination

***Correspondence:** Zairina Lubis, Universitas Sumatera Utara Ringgold standard institution - Dermatology and Venereology Medan, North Sumatera, Indonesia. E-mail: novazairina_2011@yahoo.com

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Abstract

BACKGROUND: Onychomycosis is a fungal infection of one or more units of the nail caused by dermatophytes, or mould and nondermatophytes yeast. Investigations are needed to establish the diagnosis of onychomycosis before starting treatment. Several investigations methods for diagnosing onychomycosis are microscopic examination with 20% KOH, fungal culture, histopathology examination with PAS staining (Periodic acid Schiff) and PCR (Polymerase Chain Reaction). Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) is a method after PCR amplification allowing more specific results.

AIM: To determine the diagnostic value of PCR - RFLP in the diagnosis of onychomycosis using fungal culture as the gold standard and to find out the majority fungal species that cause onychomycosis.

METHODS: This study is a diagnostic test for the diagnosis of onychomycosis by using culture as the gold standard.

SUBJECTS: Thirty - five patients suspected of having onychomycosis from history and dermatological examination.

RESULTS: PCR - RFLP in the diagnosis of onychomycosis has a sensitivity of 85.71%, specificity of 28.57%, positive predictive value (PPV) of 82.76% and negative predictive value (NPV) of 33.33%. The positive and negative likelihood ratios are 1.20 and 0.5 with an accuracy of 74.29%.

CONCLUSIONS: PCR - RFLP may be considered for a faster and more accurate alternative examination in the diagnosis of onychomycosis.

Introduction

Onychomycosis is a fungal infection of one or more units of the nail caused by dermatophytes, or mould and nondermatophytes yeast [1]. 50 % of nail infections and 30 % of superficial fungal infections are caused by onychomycosis [1][2][3][4]. Factors that may influence the onychomycosis prevalence rate include age, predisposing factors, social class, occupation, climate, environment and travelling frequency [3][4]. 50% of world population suffer from onychomycosis [4].

There are 3 onychomycosis - related fungal groups: dermatophytes, non - dermatophytes/ mould and yeast [1][2][3][4]. Dermatophytes include *Trichophyton rubrum, Trichophyton mentagrophytes*, *Epidermophyton floccosum* [1] [2] [4] [7] [8] [9] [10] [11] [12]. Nondermatophytes/mould include *Acremonium sp., Alternaria sp., Aspergillus sp., Botryodiplodia theobromae,* and *Fusarium sp.* among others [4] [6] [7] [8] [9]. *Candida Albicans* is the most commonly found yeast [1] [2] [3] [4] [5] [6] [7].

The diagnostic test is needed to confirm onychomycosis diagnosis before starting the antifungal therapy. Known diagnostic tests for onychomycosis include microscopic examination with 20% KOH, PAS (Periodic Acid Schiff) - staining examination, microscopic immunofluorescence with calcofluor - stain. PCR (Polymerase Chain Reaction) and fungal culture [3] [4] [11] [12] [13].

Microscopic examination with 20% KOH and fungal culture are the two most important tests used to confirm fungal infection diagnosis. Fungal culture needs around four weeks to identify the etiological agent of onychomycosis [3] [14].

Specific and sensitive molecular techniques such as Polymerase Chain Reaction (PCR) can also be used to diagnose myriads of microorganism agents

including pathogenic fungi [3] [15]. PCR is an in - vitro DNA synthesis and amplification technique [18]. The technique was first proposed by Karry Mullis in 1985 [15] [16]. PCR can be used to amplify the DNA within hours exponentially. This discovery has revolutionised the medical science and technology especially for its high diagnostic value [15]. In this current study, considering the capability of PCR technique to do early and accurate identification of pathogenic microorganisms and viruses, we'd like to evaluate the diagnostic technique capability for onychomycosis and comparing the results to the culture as the golden standard [14].

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR - RFLP) is a PCR method with enzymes addition after the DNA amplification. Thus it may give a more specific result [18][19]. Previous study my Monod et al. in 2006 found that PCR - RFLP results are fast and reliable enough to identify nondermatophytes as the aetiology of onychomycosis [20]. A study by Elavarashi et al. in 2013 found that PCR - RFLP with Internal Transcribed Spacer (ITS) primer, *Mva*I and *Dde*I enzymes may give promising results [21]. Therefore, this study was conducted to evaluate PCR as a diagnostic test to diagnose onychomycosis.

Methods

This study was conducted from April 2014 until reaching the minimum sample requirement in the Mycology Outpatient Clinics of RSUP H. Adam Malik, Medan Dermatovenereology Department. Twenty-five nail samples were taken in Mycology Outpatient Clinic of RSUP H. Adam Malik Medan Dermatovenereology Department, and fungal cultures were done in Microbiology laboratory of the University of Sumatera Utara, Faculty of Medicine. PCR - RFLP was done in Integrated Laboratory of the University of Sumatera Utara, Faculty of Medicine. Instruments used include scalpels, envelopes, ice bags, PCR tubes (Biologix), microcentrifuge tube (Sorenson), white tip (Biologix), yellow tip (Biologix), blue tip (Sorenson), micropipet (Rainin), cold storage, centrifuge (Biofuge, Germany), incubator (Mammert), thermocycler (applied biosystem type Veriti 96 well thermal cycler, Singapore), electrophoresis apparatus with power supply (Scie - plans, UK) and vortex (Biosan). Perishables used include nail speciments, Saboraud's dextrose agar medium, buffer Tris - EDTA (Sigma), EDTA (Sigma), DNA extraction kit (Promega), lyticase enzyme (Sigma), PCR kit (Promega), Internal Transcribed Spacer 1 (ITS1) primer and Internal Transcribed Spacer 4 (ITS 4) (1st Base), 2 % agarose gel (Promega), isopropanol (Merck), ethanol 70 % (Merck), ethidium bromide (Promega), DNA marker (Promega) and restriction enzyme *Mva*I and *Hae* III (Fermentas).

Basic data (including history - taking and dermatology examination) inputs were done in RSUP H. Adam Malik, Medan, Dermatovenereology Department. Nail sampling was done by the researcher. Taken nail samples were divided into two envelopes in which the first was taken to the microbiology laboratory for the fungal culture and the second was taken to the integrated laboratory for the PCR - RFLP. The collected data were summarised in 2 x 2 table and were analysed. Sensitivity and specificity of PCR - RFLP then compared with the gold standard, which is culture. Moreover, we compare the accuracy, negative predictive value (NPV) and positive predictive value in both modalities.

Results

Female is the gender group with most counts at 25 people (71.4%) as seen in Table 1. From Table 2 we can see that *Candida* onychomycosis (14 people, 40%) are the most commonly found onychomycosis clinical appearance followed by distal and lateral subungual onychomycosis (10 people, 28.5%) and total dystrophic onychomycosis (11 people, 31.4%). Table 1 shows most subjects' onychomycosis are located in foot nails (21 people, 60 %) with hand nails location at 14 people (40%).

Table 1: Characteristics of subjects in RSUP H. Adam Malik Medan in 2014

	Frequency	Percentage (%)
Gender		
Female	25	71.4
Male	10	28.6
Total	35	100.0
Clinical Appearance		
Candida Onychomycosis	14	40,0
Total Dystrophic Onychomycosis	10	28,5
Distal and Lateral Subungual Onychomycosis	11	31,4
Total	35	100,0
Location		
Foot nails	21	60
Hand Nails	14	40
Total	35	100,0

The most common fungal species identified from the cultures was *Candida albicans* (15 people, 42.8%) with *Phaecylomyces sp.*, *Epidermophyton floccosum, Trichophyton tonsurans, Candida tropicalis* and Culvularia were the least common at one person each (2.9%) as shown in Table 2.

Table 2: Onychomycosis fungal culture frequency distribution in RSUP H. Adam Malik Medan in 2014

No.	Species	Frequency	Percentage (%)
1.	No Growth	7	20,0
2.	*Candida albicans*	15	42,8
3.	*Aspergillus niger*	5	14,3
4.	*Cladosporium sp*	3	8,6
5.	*Phaecylomyces sp*	1	2,9
6.	*Epidermophyton floccosum*	1	2,9
7.	*Trichophyton tonsurans*	1	2,9
8.	*Culvularia*	1	2,9
9.	*Candida tropicalis*	1	2,9
Total		35	100,0

The most common fungal species identified from the PCR - RFLP technique was *Candida* albicans at 15 people (42.8%) with *Epidermophyton floccosum, Candida tropicalis,* and *Trichophyton tonsurans* was the least common at one person each (2.9%) as shown in Table 3.

Table 3: Onychomycosis PCR - RFLP Fungal Species Frequency Distribution in RSUP H. Adam Malik Medan in 2014

No.	Fungi Detected from PCR-RFLP	Frequency	Percentage (%)
1.	Not detected	11	31,4
2.	*Candida albicans*	15	42,8
3.	Negatif	6	17,1
4.	*Epidermophyton floccosum*	1	2,9
5.	*Trichophyton tonsurans*	1	2,9
6.	*Candida tropicalis*	1	2,9
Total		35	100,0

Onychomycosis detection using PCR - RLP yields a sensitivity value at 85.71% when compared to fungal culture results as the golden standard which means that 85.71 % of onychomycosis patients in this study were detected using this method and this shows that the instrument yield a high sensitivity (Table 4).

Table 4: Analysis and statistical tests results

Sensitivity	$=\frac{a}{a+c}$	x 100 %	$=\frac{24}{28}$	x 100 %	= 85.71%
Specificity	$=\frac{d}{b+d}$	x 100 %	$=\frac{2}{7}$	x 100 %	= 28.57%
Accuracy	$=\frac{a+d}{a+b+c+d}$	x 100 %	$=\frac{26}{35}$	x 100 %	= 74.29%
PPV	$=\frac{a}{a+b}$	x 100 %	$=\frac{24}{29}$	x 100 %	= 82.76%
NPV	$=\frac{d}{c+d}$	x 100 %	$=\frac{2}{6}$	x 100 %	= 33.33%

Discussions

This study found that PCR - RFLP method yield 85.71% sensitivity value and 28.71 % specificity value. The results were lower than the PAS - staining method but the invasiveness of PAS - staining compared to PCR - RFLP method should be put into consideration.

Kardjeva et al. in 2004 done a study in Germany and the study found out that from 261 onychomycosis cases, PCR method as a confirmatory diagnostic test yield 84% sensitivity compared to the fungal culture at 22% sensitivity. This shows that molecular methods yield better results and less time-consuming at 2 - 3 days when compared to a fungal culture that may take time from 2 to 4 weeks [12].

Litz et al., in 2010 compared the PCR method with KOH test, fungal culture, and PAS - staining from 559 nail specimens with the results was 37%, 40%, 22%, and 54% respectively [14]. A study by Rizal in 2010 at RSUP Haji Adam Malik Medan found that PAS - staining yield better results than fungal culture in onychomycosis diagnosis with 96.8% sensitivity and 50% specificity [5]. Mirzahoseini et al., in 2009 in Iran showed that PCR - RFLP method is fast and reliable enough to identify most of the pathogenic fungal species [19].

A study by Arca et al. in 2004 in Turkey found 40 positive results (77%) using 20% KOH test, 12 positive results (23%) using fungal culture, and 20 positive results (38%) using PCR method within 44 onychomycosis nail samples [22]. PCR is a selective and highly valued diagnostic tool to detect fungal species especially in cases where they can't be detected using conventional methods [17]. Baek et al., from Korea stated that PCR - RFLP is a highly sensitive method to detect and identify onychomycosis, having a higher diagnostic value when compared to conventional methods [22].

Specificity test aims to evaluate the capability of an instrument / a method to bring negative results among people who are without the disease. The specificity value of 28.57% means that 28.57% of onychomycosis patient suspects who are without the disease can be excluded using the PCR - RFLP method.

A study by Mohamed LM et al in 2007 in Egypt showed that out of 30 onychomycosis cases 13 (43.3%) were found positive, and 17 (56.7%) were found negative using the fungal culture whereas 16 (53.3%) were found positive and 14 (46.7%) were found negative using the PCR method [13].

The ability of the PCR method to detect the genome of infectious fungi on onychomycosis patients may explain the high sensitivity of the method [14]. Presence of contaminants during sampling and sample processing may explain the low sensitivity of the PCR - RFLP method [13] [17].

Positive Predictive Value (PPV) gives the estimated probability of subjects with positive results. This study found the PPV was at 82.76%. This suggests that this tool has a high strength to determine true positive results. Negative Predictive Value (PPV) gives the estimated probability of subjects with negative results. This study found the NPV was at 33.33%. This suggests that this tool has a low strength to determine true negative results thus not suitable as an onychomycosis screening tool.

Accuracy is an ability of an instrument to give the correct results out of the subjects. The PCR - RFLP method in this study was found with an accuracy of 74.29%, which showed the high ability of the tool to detect onychomycosis correctly.

Positive Likelihood Ratio (PLR) is the ratio between true positive results with false negative results. The PLR value of the PCR - RFLP method in this study is 1.20. Negative Likelihood Ratio (NLR) is the ratio between false negative results with true negative results. The NLR value of the PCR - RFLP method in this study is 0.5. A diagnostic test with a great positive strength usually gives a ratio value much more than 1 and is deemed as significant if the ratio is

more than 10. A diagnostic test with a great negative strength will give a likelihood ratio closer to 0.

This study concludes that PCR - RFLP can be deemed as a good tool to diagnose onychomycosis. The high sensitivity value suggests that this tool may be used as an alternative diagnostic tool for onychomycosis.

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Total Antioxidant Capacity, Haematological and Coagulation Parameters after Orthodox Christian Fast

Kali G. Makedou[1*], Eleni Vagdatli[2], Evangelia Patziarela[2], Vasiliki Konstantinidou[2], Eirini Poimenidou[2], Evgenia Lymperaki[2]

[1]Aristotle University of Thessaloniki, Faculty of Health Sciences, Laboratory of Biological Chemistry, Thessaloniki, Greece; [2]Alexandrion Technological Education Institute of Thessaloniki, Department of Medical Laboratory Studies, School of Health Care Professions, Thessaloniki, Greece

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Keywords: Orthodox Christian fast; Haematological parameters; Coagulation parameters; Antioxidant capacity

***Correspondence:** Kali G Makedou. Aristotle University of Thessaloniki, Faculty of Health Sciences - Laboratory of Biological Chemistry, Thessaloniki, Greece. E-mail: kmakedou@auth.gr

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Abstract

BACKGROUND: Orthodox Christian believers fast abstaining from meat, eggs, dairy products or even fish and oil in certain days of the fasting period, three times a year.

AIM: The present study aimed to investigate the impact of a 48-day fast before Easter in blood count parameters, coagulation and antioxidant status.

MATERIAL AND METHODS: A total of 35 healthy volunteers, 19-66 years old, were included in the study. White blood cells (WBC), lymphocytes (Lymph), granulocytes (Gran), haemoglobin (Hb), hematocrit (Ht), red blood cells (RBC), mean erythrocyte volume (MCV), platelets (Plt), were measured. Blood coagulation parameters, such as PT, aPTT, fibrinogen concentration, factor VII activity were also determined, and INR (PTsample/PTcontrol) and aPTT ratio (aPTTsample/aPTTcontrol) were calculated. Total antioxidant capacity (TAC) was assayed.

RESULTS: Levels of all parameters remained within normal. By the end of the fasting period, lymphocytes and TAC levels were significantly increased ($p = 0.011$), whereas all the other parameters, except fibrinogen, were significantly decreased.

CONCLUSION: Orthodox Christian fast impairs all haematological and coagulation parameters and seems to be beneficial in the body antioxidant protection.

Introduction

Many religions, including Orthodox Christian religion, require fasting as part of the preparation of believers for certain periods of the year [1]. Greek Orthodox Christian believers fast abstaining from meat, eggs, dairy products, even from fish and oil in certain days of the fasting period; protein and fat intake decrease, saturated and trans-fat is eliminated. The main constituents of this fast include fruits, vegetables, grains, cereals and legumes.

Fasting periods for a Greek Orthodox Christian believer begin 40 days before Christmas, 48 days before Easter and 15 days before Assumption in August.

The present study aimed to investigate the impact of Orthodox Christian fast during a 48 - day period before Easter in blood count parameters, coagulation and antioxidant status.

Methods

A total of 35 healthy volunteers, aged 19 - 66 years old, were included in the study. All of the participants were followers of the Orthodox Christian fast, and none of them was taking medication that could affect coagulation parameters or antioxidant status. All subjects signed a consent form for the participation in the study.

Blood samples (whole blood with EDTA,

serum and citrate plasma) were collected, after overnight fast, before and after the pre - Easter fasting period for those participants that followed the fast, whereas only one blood sample was collected from each non - fasting adult. For the purpose of the study, whole blood parameters, such as count of white blood cells (WBC), lymphocytes (Lymph), granulocytes (Gran), haemoglobin (Hb), hematocrit (Ht), red blood cells (RBC), mean erythrocyte volume (MCV), platelets (Plt), were measured by the analyzer Cell - Dyn 1800 (Abbott Laboratories, Chicago, Illinois, USA). Blood coagulation parameters, such as PT, aPTT, fibrinogen concentration, factor VII activity were also determined by the analyzer ACL Elite, (Instrumentation Laboratory, Milano, Italy – Beckman Coulter, Brea, California, USA), and INR (PTsample/PTcontrol) and aPTT ratio (aPTTsample/aPTTcontrol) were calculated. All these parameters were measured immediately after the blood collection, whereas serum was stored in - 80ºC until total antioxidant capacity (TAC) was assayed by ELISA (Sigma - Aldrich Inc., PO Box 14508, St Louis, MO, USA).

Data were analysed with IBM SPSS Statistics 20 for Windows. The normality of distribution of values was investigated with Kolmogorov-Smirnov test. Within-group comparisons before and after fasting were performed with paired samples T-test, as long as the distribution of values was normal. Statistical significance was set at $P < 0.05$.

Results

Mean values of blood count parameters, coagulation parameters and TAC in the study group, before and after the fasting period, are shown in Table 1.

Table 1: Differences of blood count parameters, coagulation parameters and TAC in the study group (n=35), before and after the fasting period

Parameter	Before fast	After fast	*P*
	Mean (SD)		
WBC (*$10^3/\mu L$)	5.685 (1.24)	4.876 (0.89)	<0.001
Lymphocytes	1.846 (0.45)	2.073 (0.57)	0.004
Granulocytes	3.485 (0.97)	2.439 (0.58)	<0.001
RBC (*$10^6/\mu L$)	4.64 (0.41)	4.56 (0.37)	0.036
Hemoglobin (g/dL)	13.4 (1.10)	12.9 (0.91)	<0.001
Hematocrit (%)	41.4 (3.20)	38.6 (2.62)	<0.001
MCV (fL)	89.14 (7.70)	85.05 (6.49)	<0.001
Platelets (*$10^3/\mu L$)	238.50 (41.80)	225.60 (34.30)	0.005
INR	0.94 (0.048)	0.95 (0.049)	0.028
aPTT ratio	1.07 (0.097)	0.91 (0.096)	<0.001
Fibrinogen (mg/dL)	355.23 (75.65)	341.06 (61.71)	0.135*
Factor VII (%)	104.8 (18.9)	97.7 (19.4)	0.003
TAC (mmol/L)	0.84 (0.28)	0.98 (0.04)	0.011

* Non – significant. Abbreviations: WBC, white blood cells; RBC, red blood cells; MCV: mean erythrocyte volume; INR: international normalised ratio; TAC, total antioxidant capacity.

All mean values were within normal range before the initiation of fast. Significant alterations, within normal values, were observed after 48 days of fast in most of the parameters, except fibrinogen. In particular, the parameters that decreased significantly were WBC, granulocytes, haemoglobin, RBC, MCV, platelets, INR, aPTT and FVII activity, whereas lymphocytes presented significant elevation.

About TAC levels, there was statistically significant increase by the end of the fasting period ($P = 0.011$) (Table 1, Figure 1).

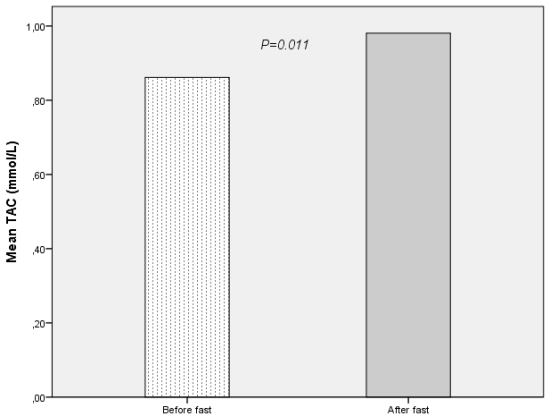

Figure 1: Changes in mean TAC values before and after fast

Discussion

Although many studies have investigated the impact of nutrition on the levels of the above-mentioned parameters, on coagulation [2][3][4] and, some, to the plasma TAC levels [5][6][7], very little evidence exists on the impact of Orthodox Christian fasting on the above mentioned parameters. The present study states that there is a significant modification of blood parameters after a 48 - day fasting period.

Orthodox Christian fast is very similar to the Mediterranean diet, with olive oil as the only oil used and with deprivation even of oil in certain days of the week. Milk and dairy products are excluded from this dietary pattern, as well as meat and eggs. A significant increase of INR and decrease of aPTT ratio shows an impact of the fast on both the exogenous and the endogenous coagulation pathway. This study shares some common findings with that of Liali et al. [8], but the mechanism of the activation of both pathways remains to be clarified. Moreover, there was a significant reduction in the platelets count after the fast in the study group. Fibrinogen levels were not altered significantly, in contrast to the study of Mezzano et al., who studied the impact of Mediterranean diet. The significant reduction of FVII activity after the fast can be attributed to the use of

olive oil, rich in monounsaturated fatty acids, then other oils and the abstention from saturated fat, as shown by the study of Allman - Farinelli MA et al. [9]. Moreover, Orthodox Christian fast is enriched in nuts, vegetables and seeds, which have shown to exert beneficial effects on coagulation parameters [10], something that is stated by the results of the present study, as well.

About white blood cells, to our knowledge so far there is no other study investigating the impact of Orthodox Christian fast on white blood cell count. Nevertheless, the study of Latilynia et al., [11] showed that Ramadan has a beneficial effect on neutrophil phagocytic function. In contrast to these results, the present study showed a significant decrease in the count of granulocytes and total white blood cells, with a significant increase in lymphocytes' count, after the 48 - day pre - Easter fasting period. Nevertheless, the mechanism for these alterations is not yet known.

Furthermore, it seems that Ht, Hb and MCV are significantly decreased after a 48-day fast, within the normal range, in contrast to the study of Sarri et al., who showed non - significant reduction of Hb after a 40 - day pre - Christmas Orthodox Christian fast [12]. Another parameter investigated in the present study was antioxidant status, in the study group before and after fast. The findings, presented in Table 1 show that members of the study group had significantly higher TAC serum levels after the fast. This can be attributed to the constituents of the Orthodox Christian fast, such as olive oil, nuts, red wine, vegetables and seeds, all previously shown to be rich in flavonoids, carotenoids and other antioxidants [13].

In conclusion, the findings of the present study suggest that the Orthodox Christian fast, a vegetarian type of diet followed for three periods of time each year, has a significant impact on most parameters studied. In particular, blood count and coagulation parameters were significantly impaired by the fast, but within normal range. Hematocrit, haemoglobin, MCV and RBC were significantly decreased, as well. Endogenous and exogenous coagulation pathways were both significantly impaired, whereas there may not be an impact on blood coagulation due to contradictory effects on the two pathways. On the whole, the Orthodox Christian fast might not have a serious impact on the fasters' health, due to its short duration, that keeps the alterations within normal range. On the other hand, it seems that this fast enhances the antioxidant system and might have long-term health benefits. The results of the present study, in addition to the results of previous studies [12][14][15] show that Orthodox Christian fast might help in the prevention of cardiovascular diseases. Nevertheless, further studies are needed to establish the findings of the present study.

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Increasing Atherosclerosis in Streptozotocin-Induced Diabetes into Four Groups of Mice

Kiking Ritarwan[1], Aznan Lelo[2], Yunita Sari Pane[2], Nerdy Nerdy[3*]

[1]Department of Neurology, Adam Malik General Hospital, Faculty of Medicine, University of Sumatera Utara, Medan, Indonesia; [2]Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Sumatera Utara, Medan, Indonesia; [3]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Sumatera Utara, Medan, Indonesia

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***Correspondence:** Nerdy Nerdy, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Sumatera Utara, Medan, Indonesia. E-mail: nerdy190690@gmail.com

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Abstract

AIM: To study the protective effect of medicines on the formation of atherosclerosis in mice, it is needed to conduct the study in mice which is not genetically diabetic mice induced by streptozotocin (STZ) to produce hyperglycemia and atherosclerosis, compared with mice treated by yolk or its combination.

MATERIAL AND METHODS: Fifty-six mice, Double Deutch Webster strain, male, receive 10 weeks, 20 - 30 gr bodyweight were divided into 4 groups (n = 14) i.e. control (do not received any agents), STZ (45 mg/kg/BW was injected intraperitoneally for 5 days), yolk (0.2 cc orally daily for 6 weeks), and combination of STZ and yolk (STZ: 45 mg/kg/BW intraperitoneally add 0.2 cc yolk orally). All animals were executed in the 42nd day. Then, the aorta of the mice's heart tissue was histopathology examined. Blood glucose and cholesterol levels were determined every week.

RESULTS: Hyperglycemia occurred in mice induced by STZ injection with the highest BGL (521.8 ± 48.2 mg/dl; 188.4%) in the 4th-week observation; after that BGL decrease. We found that, except the control, all treatment groups with STZ, egg yolk, and combination underwent atherosclerosis.

CONCLUSION: The present study was able to demonstrate the occurrence of atherosclerosis in mice treated by STZ accompanied with increasing blood glucose and cholesterol level.

Introduction

Diabetes mellitus is a chronic metabolic disorder, identified by the increase in blood glucose level (BGL, hyperglycemia) [1]. Chronic hyperglycemia is the initial cause of microvascular complication, such as retinopathy, neuropathy and nephropathy, as well as macrovascular complications, especially cardiomyopathy [2]. The atherosclerosis risk factors, explain just of a minor part of the excess incidence of vascular disease among diabetic patients, diabetogenic factors themselves must contribute to the development the arterial disease. In particular, hyperglycemia, the primary clinical manifestation of diabetes, is thought to contribute, to diabetic complication by altering vascular cellular metabolism, vascular matrix molecules, and circulating lipoproteins. For instance, hyperglycemia increases diacylglycerol levels and activates protein kinase C activity in the aorta of streptozotocin (STZ) induced diabetic rats and dogs [2]. Thickening of the basement membranes in renal glomeruli and peripheral capillaries has been observed in STZ - induced diabetic rats and diabetic patients [3].

Hyperlipidemia is a feature of drug-induced diabetes in rats and rabbits, as well as poorly controlled diabetes in humans. Alterations in lipoprotein–cell interactions are also seen in vitro upon glycation of circulating lipoproteins. The role of each of these mechanisms in the pathogenesis of macro - and microangiopathy needs to be clarified [2][3]. The data from the World Health Organization in 2004 showed that 30% of mortality rate was caused by heart and blood vessel diseases, and 60% of that percentage was caused by coronary heart disease [4].

In Indonesia, the mortality rate caused by coronary heart disease has increased significantly: from 16% (in 1986) to 26.4% (in 2001) and then up to 59.5% (in 2007) [4][5][6]. This has highlighted the importance and urgency of studying the mechanism of diabetic atherosclerosis and exploring therapeutic options [5][7][8].

Due to its unique advantages over other animal models, the mouse is the most used model for studying the mechanism of diabetes-accelerated atherosclerosis and exploring effective therapeutic approaches. Their advantages are a small size, short generation time, and ease of induction of diabetes and atherosclerosis by diet, drug treatment (streptozotocin or alloxan) or a genetic approach and cost-effectiveness. In the past decade, several diabetic atherosclerosis mouse models have been established [7].

Because in Indonesia it is difficult to obtain transgenic mice, the present study focused on to make healthy mice become diabetes by injecting STZ. Kunjathoor et al., (1996) had shown accelerated atherosclerosis in response to hyperglycemia in STZ - induced diabetic mice [3]. Kostogrys et al. (2012) found that the blood cholesterol level (BCL) was significantly increased in egg yolk diet mice and promoted atherosclerosis [9].

Prior studies have found a positive relationship hypercholesterolemia in STZ - induced diabetic rats as a result from increased intestinal absorption and synthesis of cholesterol. This was followed by the formation of atherosclerosis, which resembles that occurs after high cholesterol diet (egg yolk diet) [10][11]. Canadian experts had been suspicious of the involvement of egg yolk in atherosclerosis formation and then warned people not to consume egg yolk [12]. On the contrary, Voutilainen et al., found that egg consumption was not associated with increased risk of myocardial infarct, where the relative risk (RR = 0.87 (95% CI 0.71 - 1.07, p = 0.73) in the highest (> 46 g/day) vs the lowest (< 15 g/day) cholesterol diet [13].

Various studies have been conducted to prevent and treat diabetic atherosclerosis by using antioxidants [14][15][6] and angiotensin receptor blockers [18][19][20].

Therefore, prior to conducting a study on the benefits of natural materials, such as caffeic acid phenethyl ester (CAPE) and other antioxidants in preventing and treating the formation of atherosclerosis, it is necessary to conduct a study on the effect of STZ injection in mice on the incidence of hyperglycemia, hypercholesterolemia, and atherosclerosis formation compared to that occurs in mice ingested with egg yolk or its combination.

Material and Methods

Fifty-six male mice (*Mus musculus*) of certified Double Dutch Webster lineage, 8 to 10 weeks old, and 20 - 30 gram of body weight were used. Before conducting the study, they were adapted to a place with light controlling of 12 hour - daylight (6:00 A.M. – 6:00 P.M.) and 12 dark hours (6:00 P.M. – 6:00 A.M.) with standard diet (eat and drink ad libitum). Their food came from Chroen Phosphate. The mice were placed in the natural temperature and humidity for 6 weeks. They were weighed once in a week to avoid stress. The study began after approved by the Committee of Research Ethics of the Faculty of Mathematics and Science University of Sumatera Utara Medan.

After an adaptation period, the mice were randomly divided into four groups with each group consisted of 14 mice. Group I did not get any treatment as the control, group II obtained STZ injection (45 mg/kg weight, intraperitoneal for five days), group III obtained egg yolk (0.2 cc per - oral for six weeks), and group IV obtained the combination of STZ injection (45 mg/kg weight, intra-peritoneal for five days) and egg yolk (0.2 cc per-oral for six weeks). STZ was obtained from Nicalai - Japan (Batch no. 32238 – 91/2).

Laboratory Analysis

The execution was performed each week by cervical dislocation in each group which consisted of two mice until the end of the study (six weeks). The mice's tails were cut off each week to get their blood for examining glucose level by using digital glucometer (Accu-check® Advantage, Roche Diagnostic, Germany) and for cholesterol level by using digital cholesterol test (Easy touch®GCU, Taiwan). The incidence and the severity level of atherosclerosis were proved by examining its result, using lighting microscope with the magnification of 40-400 times. The parameter was the thickness of aorta wall, the present of foam cells and sclerosis in mice's aorta tissues. The severity of atherosclerosis was differentiated into four groups according to histopathology findings (Figure 1):

1. Normal, when the following pathological matters were not found;

2. Mild, when monocyte adherent, foam cells, lipid core, proliferation of smooth muscle cells were found;

3. Moderate, when the above matters were found plus fibrous plague, sclerosis, and fibrous cap;

4. Severe, when all the matters above were found, followed by rupture and thrombus.

All data were presented in the Mean ± SEM.

The difference of the average of Blood Glucose Level (BGL) and Blood Cholesterol Level (BCL) was analysed with ANOVA, followed by a post-hoc Bonferroni multiple comparisons, correlation of BGL and CBL with Pearson's regression, and the difference of the incidence of atherosclerosis among the groups with χ^2 test. While for data not normally distributed were analysed by Kruskal Wallis. A difference or correlation was stated significant when $p < 0.05$.

Results

In this present study, the change in the BGL (Table 1), BCL (Table 2), and the incidence of atherosclerosis (Table 3) were documented.

Table 1: The profile of blood glucose level (BGL mg/dl; mean ± SEM) of all treatment groups, related to time (week)

Groups	Week (wk)							
	0 N =14	1 N = 12	2 N = 10	3 N = 8	4 N = 6	5 N = 4	6 N = 2	P (Sig)
Control	157.1 ± 10.2	148.1 ± 6.9	151.6 ± 9.6	144.3 ± 7.5	149.2 ± 9.3	146.0 ± 11.25	192.0 ± 15.0	0.515
STZ	180.7 ± 7.2	192.3 ± 13.5	258.2 ± 41.3	302.8 ± 47,2	521.8 ± 48.2	310.0 ± 86.4	**	0.000
Egg Yolk	174.9 ± 7.5	156.0 ± 4.2	164.2 ± 8.7	145.9 ± 4.9	146.0 ± 14.3	145.0 ± 14.0	145.0 ± 5.0	0.000
Combination	167.3 ± 6.5	181.5 ± 18.5	277.2 ± 39.2	294.3 ± 73.9	392.3 ± 119.9	367.0 ± 108.3	190.5 ± 1.5	0.014
P	0.123*	0.096	0.006	0.001	0.000	0.115	0.291	

*Kruskal Wallis; ** death mice.

Table 1 showed no significant change of BGL in control group until the 5th week, except in the last week (in the 6th week BGL is 192.0 ± 15.0 mg/dl), BGL slightly increased (22.3%). In the STZ group, BGL increased significantly (188.4%) in the 4th week; it then decreased after the 4th week, and before the 6th week (the 39th day) the mice died before the execution. In the groups where the mice obtained egg yolk, BGL significantly decreases to 17.1% until the end of the study. As demonstrated in STZ group, in the combination group, BGL increased to 134.7% until the 4th week, and it then decreased again by the end of the study, this BGL alteration is statistically significant.

Table 1 also showed the difference of BGL among the groups, related to time, where significant difference started from the 2nd week up to the end of the study. The most significant difference of BGL was found in the 4th week. However, the difference between BGL in STZ group and the combination group was not statistically significant in all observed weeks. Table 2 showed that the average of BCL in all treatment groups tended to increase, including in the control groups. In the control group, BCL increased from 150.2 ± 15.5 mg/dl in the 1st week to 216.0 ± 37.0 mg/dl in the 6th week, where the highest CBL in the 4th week (227.0 ± 22.7 mg/dl; 33.9%).

Table 2: The profile of blood cholesterol level (mg/dl; mean ± SEM) of all treatment groups, related to time (week)

Groups	Week (wk)							
	0 N = 14	1 N = 12	2 N = 10	3 N = 8	4 N = 6	5 N = 4	6 N = 2	P
Control	152.5± 13.3	150.2 ± 15.5	156.5 ± 11.6	178.4 ± 15.6	227.0 ± 22.7	178.0 ± 5.7	216.0 ± 37.0	0.039
STZ	120.8± 6.0	122.1 ± 6.8	148.3 ± 7.2	175.5 ± 17.5	215.0 ± 13.8	119.8 ± 8.3	**	0.000
Egg Yolk	139.8± 7.4	141.2 ± 8.5	151.0 ± 11.6	181.1 ± 9.4	165.3 ± 13.7	145.0 ± 21.0	135.5 ± 122.5	0.289
Combi-nation	168.8± 12.7	176.3 ± 13.5	152.6 ± 8.6	169.8 ± 13.8	181.8 ± 4.7	163.3 ± 23.1	166.5 ± 7.5	0.895
P	0.244*	0.188	0.220	0.421	0.461	0.461	0.433	

*, Kruskal Wallis; **, death mice.

In the STZ group, the highest increase of BCL (76.2%) occurred in the 4th week; after that, it was decreased. The same profile to the egg yolk group which the highest increase of BCL (17.9%) was occurred in the 4th week, while in the combination group, the highest increase of the CBL (6.8%) occurred in the 4th week, and after that, it decreased lower than the initial level. However, the changes of BCL in the yolk and combination groups were not significant. Furthermore, based on ANOVA statistical analyses there was no significant difference in CBL between groups for each weeks observation, with an exception for the data of BCL at week-0 was analysed by Kruskal Wallis.

Table 3: The incidence of atherosclerosis

Severity	Control	STZ	Egg yolk	Combination
Normal	13	0	4	0
Mild	1	3	4	1
Moderate	0	11	6	12
Severe	0	0	0	1

$\chi^2 = 45.05$; df = 9; $P < 0.0001$.

Table 3 showed that the incidence of atherosclerosis was statistically different ($p < 0.0001$). Atherosclerosis was hardly found in the control group. In the STZ group, all mice underwent atherosclerosis, and the most serious incident was found in the combination group. In the egg yolk group, some mice did not undergo atherosclerosis.

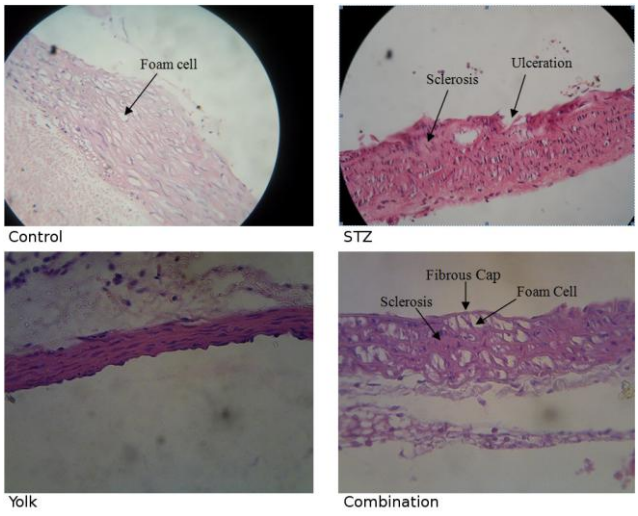

Figure 1: Microscopic Classification of The Atherosclerosis Severity between groups; i.e. Control, STZ, Egg yolk and Combination by magnification 400 times

Furthermore, in the present study, there was a significant positive correlation ($p < 0.05$) between BGL and CBL in STZ group (r = 0.8469), while in egg yolk group there was a significant negative correlation (r = -0.8476). On the contrary, there was no significant correlation between BGL and BCL in the combination group (r = 0.1170).

Discussion

The present study was successful in demonstrating that hyperglycemia occurred in mice induced by STZ injection (Table 1) with the highest BGL (521.8 ± 48.2 mg/dl; 188.4%) appeared in the 4th-week observation; after that BGL decrease. Unfortunately, before the 6th week (i.e. the 39th day), the mice died before the execution. This might be probably caused by the inability to consume food and drink by them. The condition of dehydration accompanied by high BGL in mice may lead to the occurrence of ketoacidosis followed by death [21].

In the present study, we used STZ (45 mg/kg BW intraperitoneally for 5 consecutive days) to induce diabetes in mice. Streptozotocin enters the pancreatic cell via a glucose transporter - GLUT2 and causes alkylation acid and then destruct DNA [22]. Twenty years ago, Kunjathoor et al. (1996) have demonstrated that injection of STZ (40 mg/kg BW intraperitoneally for 5 consecutive days) in mice may increase BGL (250 -420 mg/dl), as the result of the decrease in plasma insulin level [3]. Arora et al. (2009) demonstrated that single injection of 180 mg/kg BW STZ will induce diabetes type 1 mice, and 100 mg/kg bw failed to produce diabetes except for sustained hyperglycemia. However, in multiple low doses STZ 40 mg/kg, BW for 5 days constitutively will induce diabetes type 2 [23]. While Mansor et al. (2013) demonstrated that administration STZ intraperitoneally in various doses 15, 20, 25 mg/kg BW gave no effect on BGL and did not change insulin concentration in the rat. In contrast dose of 30 mg/kg, BW STZ induced hypoinsulinemia, hyperketonemia and weight loss. It appears there is a positive dose-dependent relationship between the dose of STZ and BGL [24].

In the control group, there was no significant change of BGL, except in the last week of the study (the 6th week) there was a slight increase of the BGL (22.3%). The similar profile of the BGL happened in the egg yolk group, but BGL tends to decrease lower than the initial BGL (17.1%) at the end of the study. Jung et al. have given fatty diet to mice and found that BGL, BCL and insulin increased [8]. Theoretically, an increase of insulin level should be followed by the decrease in BGL. Although our study did not examine insulin level, the report of Jung et al. could explain why in the present study the decrease of BGL was found in the egg yolk group as the result of the increase of insulin secretion. [8]. As happened in the STZ group and combination group there was a significant increase of BGL (134,7%) in the 4th week, after that BGL decrease although still higher than basal level (13.8%) at the end of the study.

There was a contradictory of the highest percentage increase of BGL at the 4th week of STZ group (188.4%) and combination group (134.7%), the effect of the increasing BGL by STZ was hampered by egg yolk which decreased BGL. Mansor et al. (2013) demonstrated that high-fat diet or in combination with STZ did not increase BGL. High cholesterol diets produce hyperinsulinemia mice [24]. A combination of various doses of STZ (15, 20, 25 and 30 mg/kg/BW) with high - fat diet, demonstrated that combination of 25 mg/kg STZ was able to rise in BGL [25].

The present study demonstrated that the BCL tended to increase, related to time, in all treatment groups, including the control group. In the control group, BCL increased up to 33.9%, while in the STZ and egg yolk group the increase in of BCL was twice as much as that in the control group. The BCL increased up to 76.2% in the STZ group was occurred in the 4th week; after that, BCL decreased. In the egg yolk group, the highest BCL of 70.9% occurred in the 6th week. Unfortunately, the alteration of BCL was not statically significant. On the contrary in the combination group there was a slight increase of BCL (2.8%), and at the last week, BCL decreases lower than initial level. This data demonstrated that egg yolk diet has minimal effect on BGL although co-administration with STZ. The mechanism of negative interaction should be investigated.

Spence et al. found a significant difference of the area of atherosclerosis plague ($p < 0.0001$) in patients who consumed a different amount of egg yolk. People who consume fewer than two eggs a week (125 ± 129 mm^2) will have a smaller area than those who consumed three eggs or more a week (132 ± 142 mm^2) [12]. They recommended that the patients with the risk for cardiovascular disease do not consume egg yolk continuously. Consuming cholesterol-rich diet should be consumed less than 200 mg per day while egg yolk of a big egg contains about 275 mg cholesterol which is more than daily need. From the study on the animal experiment, it was found that cholesterol-rich diet will weigh down the accumulation of macrophage in adipose and atherosclerosis tissues in mice and increase systemic inflammation [12].

Although egg yolk was rich in cholesterol, consuming it in some subjects did not have any effect on BCL. Nevertheless, oxidised cholesterol could increase atherosclerosis although BCL was normal. Oxidized LDL plays an important role in initiation and progress of atherosclerosis. Meanwhile, a Finland researcher reported that consuming egg yolk regularly would not affect the area of carotid plague or acute

myocardium infarct in Finland males [13]. Even though the previous investigators gave controversial information, the finding in the present study showed that, except the control, all treatment groups with STZ, egg yolk, and combination underwent atherosclerosis. The incidence of atherosclerosis was hardly found in the control group although one mouse was executed in week 0. This incidence might be probably caused by a genetic factor. In the STZ group, all mice underwent atherosclerosis in which the most serious incidence was found in the combination group of STZ and egg yolk. In the egg yolk group, there were some mice which did not undergo atherosclerosis; they were executed in week 0 and the 1st week. The combination of STZ and egg yolk cholesterol-rich diet gave synergic effect in forming atherosclerosis. Chono et al. had researched the benefit of an anti-atherosclerotic medicine which was tested on atherogenic mice, not on usual mice. In fact, Chono study took longer time (14 weeks) to form atherosclerosis after cholesterol-rich diet[25] compared with the findings in the present study (4 weeks) [25].

This present study showed significant positive between BGL and BCL in the STZ group at R-value = 0.8469 which indicated the increasing of BGLwill followed by the increase in blood cholesterol level. However, in the egg yolk group, there was a negative correlation at R-value = -0.8476 in which BGL decreased will follow by the increase in BCL. On the contrary, there was no significant correlation between BGL and BCL in the combination group (r = 0.1170).

Based on the data obtained in the present study, the next study of the benefit of antioxidant CAPE and angiotensin receptor blocker telmisartan on the incidence of atherosclerosis in diabetic mice induce by STZ could be conducted for 4 week observation. In the 4th week treatment, there was a significant change in histopathology (atherosclerosis) and laboratories (hyperglycemia, and hypercholesterolemia) after giving of STZ to non-transgenic mice.

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Clinical Science

Mean Blood Pressure Difference among Adolescents Based on Dyssomnia Types

Krisnarta Sembiring*, Oke Rina Ramayani, Munar Lubis

Department of Child Health, Medical School, University of Sumatera Utara, Medan, Indonesia

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***Correspondence:** Krisnarta Sembiring. Department of Child Health, Medical School, University of Sumatera Utara, Medan, Indonesia. E-mail: krisnarta@gmail.com

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Abstract

BACKGROUND: Dyssomnia is the most frequent sleep disturbance and associated with increased blood pressure. There has been no study determining the difference in mean blood pressure based on dyssomnia types among adolescents.

OBJECTIVE: To determine the difference in mean blood pressure among adolescents based on dyssomnia types.

METHODS: Cross-sectional study was conducted in SMP Negeri 1 Muara Batang Gadis in April 2016. Samples were students having sleep disturbance based on Sleep Disturbance Scale for Children (SDSC) questionnaire. Stature and blood pressure data were collected along with demographic data and sleep disorder questionnaire. Analyses were done with Kruskal-Wallis test and logistic regression. P - value < 0.05 was considered significant.

RESULTS: Seventy-six samples were obtained with mean age 13.9 (SD 1.14) years - old. Dyssomnia proportion and hypertension were 72/76 and 20/76 respectively. Mean systolic (SBP) and diastolic blood pressure (DBP) was 111.1 (SD 16.46) mmHg and 70.3 (SD 11.98) mmHg respectively. Mean SDSC score was 49.7 (SD 8.96), and the most frequent dyssomnia type was disorders of initiating and maintaining sleep. Age and sex were not the risk factors of hypertension in dyssomnia. There was a significant difference in mean SBP (P = 0.006) and DBP (P = 0.022) based on dyssomnia types. Combination dyssomnia type had the highest mean blood pressure among dyssomnia types.

CONCLUSION: There is a significant difference in mean blood pressure among adolescents based on dyssomnia types.

Introduction

Adolescent phase is an important phase in human's growth and development and associated with alteration in cognitive, behaviour, social, and emotional functions. Along with the alteration, adolescents frequently experience sleep disturbances such as dramatically changing in duration, pattern, and amount of sleep [1][2].

Sleep disturbance in adolescents is a neglected health condition. On the other hand, sleep disturbance has a massive negative impact on adolescents later in their life [3][4]. There are three types of sleep disturbance in adolescents: dyssomnia, parasomnia, and secondary sleep disturbance. The classification is described further by Bruni et al. in Sleep Disturbance Scale for Children (SDSC) questionnaire [4][5].

Sleep disturbance affects adolescent's daily life and health status. The impacts on health are disruptions in growth, cardiovascular, cognitive functions, and daily behaviour [6][7]. Several studies have shown that sleep disturbance might cause elevation of blood pressure in adult, but a study in adolescent population is still scarce. Recent studies in adolescent population also showed controversies in the relationship between sleep disturbance and blood pressure [8][9].

Even there are still controversies, the relationship between sleep disturbance and blood pressure in adolescents is more frequently reported. But there has been no study determining the difference in blood pressure based on sleep

disturbance classification, especially dyssomnia which is the most frequent sleep disturbance in adolescents. It is important to know which type of dyssomnia that mostly affects blood pressure so that clinicians can be more aggressive in managing the sleep disturbance. This will minimise the impact of dyssomnia in the future.

Methods

Study Design

A cross-sectional study was conducted to determine the difference in mean blood pressure among adolescents based on dyssomnia types in SMP Negeri 1 Muara Batang Gadis on April 2016. Samples were students having sleep disturbance based on SDSC questionnaire. The exclusion criteria were students with secondary diseases and medications affecting blood pressure. Samples were obtained by total sampling method. Stature and blood pressure data were collected along with demographic and sleep disturbance data. Data analysis was done with statistical software, and the result will be presented in tables. This study was approved by the Health Research Ethical Committee, Medical School, University of Sumatera Utara.

Sample Recruitment

All students who fulfilled the inclusion criteria were enrolled in this study. SDSC questionnaires were distributed to each of them and collected in the following day. The questionnaire was filled by student's parents. SDSC score was then calculated, and dyssomnia types were determined. We interviewed each student to obtain demographic data. Student's stature was measured using microtome. Blood pressure was measured using mercury sphygmomanometer three times with interval 15 minutes. Students were allowed to rest for 10 minutes before measurement. Mean blood pressure was categorised into hypertension and non - hypertension.

Statistical Analysis

The relationship between demographic factors and blood pressure in dyssomnia students was analysed using logistic regression test. Kruskal - Wallis test was used to determine the difference in mean blood pressure based on dyssomnia types. Statistical calculation was done at 95% confidence interval and P - value < 0.05 was considered significant.

Results

Of 205 students in the school where the study was conducted, 76 fulfilled the inclusion criteria. Table 1 shows the baseline characteristics of students, including mean age, sex, and school grade. We found that mean students stature was 146.3 (SD 7.2) cm with mean systolic and diastolic blood pressure was 111.1 (SD 16.3) mmHg and 70.3 (SD 11.8) mmHg respectively. Mean SDSC score was 49.4 (SD 8.8).

Table 1: Baseline characteristics of students

Characteristics	n = 76
Mean age, years (SD)	13.9 (1.1)
Sex, n (%)	
Male	26 (34)
Female	50 (66)
School grade, n (%)	
Grade 7	34 (45)
Grade 8	29 (38)
Grade 9	13 (17)

Table 2 shows students distribution based on sleep disturbance types. The most frequent sleep disturbance type was disorders of initiating and maintaining sleep (37%) while the rarest was disorders of arousal/nightmares. Students were divided based on their sleep disorder type into dyssomnia and non - dyssomnia. There were 11 students with a combination of two sleep disturbance types, but both of the types were classified as dyssomnia type. Therefore all of them were grouped into dyssomnia type. Based on the information, it was known that the proportion of dyssomnia among all sleep disturbance types was 95%. Students were also divided into hypertension and non - hypertension based on mean blood pressure according to their age, sex, and stature. We found that the proportion of hypertension in this study was 26%.

Table 2: Distribution of students based on sleep disturbance types

Sleep disturbance type	Percentage
Disorders of initiating and maintaining sleep	37
Sleep breathing disorders	8
Disorders of arousal/nightmares	1
Sleep-wake transition disorders	25
Disorders of excessive somnolence	10
Sleep hyperhidrosis	4
Combination	14

Logistic regression test was conducted to determine the relationship between demographic factors and hypertension in students with dyssomnia (Table 3). According to the test's result, demographic factors such as age and sex were not the risk factors for hypertension in dyssomnia students.

Table 3: Risk factors for hypertension in student with dyssomnia

	Constant	Wald	P*
Age	0.003	0.000	0.991
Sex	-1.387	3.636	0.057

* Logistic regression test

Normality test was done using Kolmogorov-Smirnov test toward systolic and diastolic blood pressure. The result showed that systolic and diastolic blood pressure were not normally distributed (Table 4).

Table 4: Normality test for mean systolic and diastolic blood pressure

	Z	P*
Mean systolic blood pressure	0.178	0.0001
Mean diastolic blood pressure	0.167	0.0001

* Kolmogorov - Smirnov test.

Statistical analysis was then continued to Kruskal - Wallis test. There was the statistically significant difference in mean systolic based on dyssomnia types (P = 0.006) (Table 5). Combination dyssomnia type had the highest mean systolic blood pressure (126.0, SD 13.2 mmHg).

Table 5: Mean systolic blood pressure difference based on dyssomnia types

	Mean (mmHg)	SD (mmHg)	P*
Disorders of initiating and maintaining sleep	108.6	15.5	
Sleep breathing disorders	116.3	9.6	
Sleep-wake transition disorders	107.0	17.2	0.006
Disorders of excessive somnolence	104.7	13.2	
Combination	126.0	14.9	

* Kruskal-Wallis test.

The test also showed a difference in mean diastolic blood pressure based on dyssomnia types (P = 0.022) and the highest mean diastolic pressure was found in students with combination dyssomnia type (80.1, SD 11.3 mmHg).

Table 6: Mean diastolic blood pressure difference based on dyssomnia types

	Mean (mmHg)	SD (mmHg)	P*
Disorders of initiating and maintaining sleep	69.5	11.8	
Sleep breathing disorders	74.6	7.2	
Sleep-wake transition disorders	66.4	12.7	0.022
Disorders of excessive somnolence	65.6	6.4	
Combination	80.1	11.3	

* Kruskal-Wallis test.

Discussion

Sleep disturbance is a neglected health condition mainly in children and adolescent population. Sleep disturbance is a group of disorders associated with alteration in amount, quality, and duration of sleep [5]. The prevalence of sleep disturbance is increasing in the recent decades [3][10]. Sleep disturbance is classified into dyssomnia, parasomnia, and secondary sleep disturbance. Dyssomnia correlates with problems in the amount of sleep, initiating sleep, and maintaining sleep and is the most frequent sleep disorder in children [3][4][6][11][12]. Bruni et al. divided dyssomnia in their Sleep Disturbance Scale for Children questionnaire into disorders of initiating and maintaining sleep, sleep breathing disorders, disorders of arousal/nightmares, and disorders of excessive somnolence [3].

The prevalence of sleep disorder based on Sleep Disturbance Scale Disorders questionnaire in this study is 37.1%. The most frequent sleep disorder type is disorders of initiating and maintaining sleep (37%). Overall, the proportion of dyssomnia among all sleep disturbance types is 95%. These results are by the study by Ohida, et al. in Japan, which reported a range of sleep disturbance prevalence from 15.3% to 39.2% [10]. Liu et al. did a study in Beijing and reported a prevalence of 21.1%, a value that is close to the prevalence in this study [3]. A study in Jakarta showed a higher prevalence than this study (62.9%) [13]. Among all of the studies, Bruni, et al. reported the highest prevalence of sleep disturbance (73.4%) [10]. Disorders of initiating and maintaining sleep were known as the most frequent sleep disorder type in the study held by Chevrin, et al. The prevalence ranged from 10% to 20%. Another multicenter study in France, Great Britain, Germany, and Italy showed that 25% of sleep disorders in adolescents was insomnia, which is also a part of dyssomnia [3]. The number from the last study is not very different with the result in this study.

The incidence of hypertension in adolescents is increasing significantly, changing the opinion that hypertension is found exclusively in the adult population. This tendency may be caused by changes in lifestyle including dietary pattern, sedentary life, and mentally or physically exhaustion [14][15]. This study shows that the prevalence of hypertension in dyssomnia students is 26%. This is relevant to the result of a study conducted by Kuchiene et al. where the prevalence of hypertension in adolescents with sleep disturbance is 22.5% [16].

According to Ewald, et al. there were several risk factors for hypertension in adolescents including age, sex, race, medical condition, dietary intake, and lifestyle [17]. On the other hand, Tavasoli et al. reported a contrary result. They reported that body mass index, age, sex, and family predisposition were not related to hypertension in adolescents [8]. In our study, we found that that age and sex are not risked factors for hypertension in adolescents with dyssomnia.

Dyssomnia will increase vasoactive hormone secretion that causes vessel constriction. It also activates the renin-angiotensin aldosterone system followed by raising in intravascular volume. Cortisol secretion and sympathetic nervous system are stimulated, resulting in increasing cardiac contractility. The combination of those mechanisms will elevate the blood pressure [6][8]. Au et al. reported that there was a relationship between sleep disturbance and blood pressure [9]. Narang et al. confirmed that finding in

the study, they conducted [18]. In contrast, Tavasoli et al. found no relationship between sleep disturbance and blood pressure. Additionally, they found no difference in blood pressure between normal children and children with sleep disturbance. None of the studies has determined the difference in blood pressure based on dyssomnia types. The result of this study shows that there is a difference in blood pressure among adolescents based on dyssomnia types. Combination dyssomnia type has the highest mean blood pressure, suggesting that addition of dyssomnia types will increase blood pressure in adolescents.

This is a pilot study. Therefore further investigation is mandatory to determine risk factors for hypertension in adolescents with dyssomnia including socioeconomic status, salt consumption, and sedentary life which are not analysed in this study. A larger study involving more samples and better method is needed to confirm the result of this study.

This study shows that there is a statistically significant difference in blood pressure among adolescents based on dyssomnia types. Combination dyssomnia type has the highest mean blood pressure compared to the other dyssomnia types. Age and sex are not the risk factors for hypertension in adolescents with dyssomnia.

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Electrocardiographic Parameters as Predictors of Response to Cardiac Resynchronization Therapy

Lidija Poposka*, Vladimir Boskov, Dejan Risteski, Jane Taleski, Filip Janusevski, Elizabeta Srbinovska, Ljubica Georgievska-Ismail

University Clinic of Cardiology, Faculty of Medicine, Ss Cyril and Methodius University of Skopje, Skopje, Republic of Macedonia

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Keywords: Cardiac resynchronization therapy; Responders; non-responders; non-LBBB; Electrocardiography parameters

***Correspondence:** Lidija Poposka University Clinic of Cardiology, Faculty of Medicine, Ss Cyril and Methodius University of Skopje, Skopje, Republic of Macedonia. E-mail: lidijapoposka@gmail.com

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Abstract

INTRODUCTION: Although strict selection criteria are used to select patients for cardiac resynchronisation therapy, up to 30% of patients do not have a positive clinical response.

PATIENTS: A total of 102 consecutive patients who had biventricular pacemaker/defibrillator (CRT-P or CRT-D) implanted were enrolled in this prospective observational study.

RESULTS: During the average follow-up period of 24.3 months 5 patients died and 17 (16.7%) patients were hospitalised with the symptoms of heart failure; 75 (73.5%) patients were responders based on the previously defined criteria. Responders in the group of LBBB patients kept the significant difference in a computed variable (S1 + R6) - (S6 + R1) and R6/S6 ratio. Responders in non-LBBB patients kept the significant difference only in the height of R waves in V6. The R6/S6 ratio tended to be higher, but it did not reach a statistical significance.

CONCLUSION: None of the tested ECG parameters stands out as an independent predictor of response to cardiac resynchronisation therapy, but some of them were different in responder-compared to the non-responder group. The amplitude of R wave in V6, higher R/S ratio in V6 and higher computed variable (S1 + R6) - (S6 + R1) may predict the likelihood of response to CRT therapy in both LBBB-patients and non-LBBB patients.

Introduction

Cardiac resynchronization therapy (CRT), according to ESC Guidelines for diagnoses and treatment of acute and chronic heart failure (HF) is recommended for symptomatic patients in sinus rhythm, LBBB-QRS morphology and left ventricular ejection fraction (LVEF) ≤ 35%, despite optimal medical treatment, in order to improve symptoms and reduce morbidity and mortality [1][2]. If the QRS duration is ≥150 ms, it is class 1 indication for CRT implantation, LOE A, or if the QRS duration is 130-149 ms it is class 1 indication, LOE B. CRT should be considered for symptomatic patients with HF in sinus rhythm with a QRS duration ≥150 ms and non-LBBB QRS morphology and may be considered for symptomatic patients with HF in sinus rhythm with a QRS duration of 130-149 ms and non-LBBB QRS morphology [1]. For HF patients with atrial fibrillation (AF), the cut-off point for QRS duration is ≥ 130 ms (class IIa, LOE B) [1]. According to the same Guidelines CRT is contra-indicated in patients with a QRS duration < 130 ms, compared to 2013 guidelines on cardiac pacing and cardiac resynchronisation therapy where the cut-off point for QRS duration was 120 ms [3].

Despite very strong selection criteria and recommendation, up to 30% of patients after CRT implantation do not have a positive clinical response [4]. Responders are defined as patients who do better with the treatment rather than without it, regarding symptoms, physical activity, less frequent hospitalisations for HF etc. However, there is a poor correlation between clinical improvement and prognosis in heart failure patients. Variable response may be also due to differences in the underlying heart disease. In the literature, there are few definitions of "responders". Patients with NYHA-functional class III or IV could experience symptoms reduction, whereas

the REVERSE trial showed no symptoms improvement in patients with functional class I or II compared to optimal drug therapy [5]. If we choose mortality as an endpoint or ventricular remodelling, or hospitalisation rate we will get a completely different picture of responders. Daubert C. *et al*. [6], in their practical guide to CRT, have proposed a definition of positive response as being alive with a sustained improvement in well-being, which for patients with moderate or severe HF means fewer hospitalisations and for patients with less advanced HF means no sign of disease progression. They have also suggested that remodelling of the left ventricle should be included in the global composite score, especially in patients with mild HF [6].

As predictors of non-response, several clinical and echocardiographic variables have emerged from previous studies. Ischemic aetiology, male gender, NYHA functional Class IV, severe mitral regurgitation, left atrial dilatation, and a short interventricular mechanical delay has been associated with worse clinical or echocardiographic outcomes [7][8].

Guidelines indicate that the QRS morphology is an important predictor of the therapeutic response to cardiac resynchronisation, giving a higher class to the patients with LBBB morphology in comparison to non -LBBB morphology patients. However, conventional criteria for diagnosing LBBB, including QRS duration > 120 msec, QS or rS in lead V1, and broad R waves, without Q waves in the lead I or V6 in the resynchronisation era seemed insufficient. ACC / AHA / HRS added notched, or slurred R wave in the lead I, aVL, V5 and V6, and occasional RS pattern in V5 and V6 attributed to the displaced transition of QRS complex [9]. It seems that prolonged duration of the QRS complex serves only as an indicator of the severity of the conduction disturbance [3].

Our study aimed to define more ECG criteria which can predict response to cardiac resynchronisation.

Patients and Methods

A total of 102 consecutive patients who had biventricular pacemaker/defibrillator (CRT-P or CRT-D) implanted at the University Clinic of Cardiology in Skopje, were enrolled in this prospective observational study. The indications for CRT were according to ESC Guidelines 2013 [3]: heart failure symptoms despite optimal medication; New York Heart Association (NYHA) functional class II-IV; LVEF ≤ 35%; and QRS duration ≥ 120 ms. Patients were followed for a mean of 24.3 months.

Surface 12-lead ECGs were acquired at a paper speed of 25 mm/s and a scale of 10 mm / mV at baseline and immediately after CRT device implantation. All ECGs were individually reviewed by two investigators. To assess the reproducibility as well as the reliability of the ECG measurements, we calculated the intra-and interclass correlation coefficient (ICC) by assessing 20 randomly selected images seen in two different occasions by the same or two investigators. The ICC for intra-observer variability was in the range 0.959 – 0.983 and for inter-observer variability was in the range 0.974 – 0.987. QRS duration was measured from its first deflection to its end. Left bundle branch block (LBBB) was defined as QRS duration > 120 msec, QS or rS form in V1 and broad R waves, without Q waves in the lead I or V6. Right bundle branch block (RBBB) was defined as QRS duration > 120msec, with qR or rSR form in V1 and deep S waves in the lead I and V6. Every other wide QRS without typical LBBB or RBBB morphology was classified as undetermined bundle branch (BB) morphology. From pre-implantation ECG we also analysed: R amplitude in V1 and V6, S amplitude in V1 and V6 and R6/S6 ratio and (S1 + R6) - (S6 + R1).

All patients underwent complete echocardiography examination at baseline, at 3-6 months and 12 months after CRT device implantation. Complete M-mode, 2-D, and Doppler evaluations were performed. Images were obtained in the parasternal and apical views. LV end-systolic volume, LV end-diastolic volume, and LVEF were calculated using the biplane Simpson's technique.

CRT device implantation was performed by the standard transvenous procedure. The left ventricular (LV) lead was advanced to a lateral vein or, when it was unattainable, to a postero-lateral vein. The right ventricular (RV) lead was implanted in the apex of the right ventricle. In patients with the indication of ICD – implantation CRT-D device was implanted. The right atrial lead was implanted in the right auricular. All devices were programmed at a standard atrioventricular delay after implantation with optimisation using echocardiography usually performed 3-6 months after implantation. Medications were recorded immediately before implantation of the CRT device with titration of medications made at the discretion of the responsible cardiologist.

Definition of CRT Responder

Patients and implanted devices were followed up in the outpatient clinic at 1 month after implantation and then at 3-6 months. To define if the patient is responder at 6 months follow-up we used as the following parameters: increase in left ventricular ejection fraction (LVEF) more than 10%, lowering of NYHA class and on the other site hospitalisation for heart failure in 6 months after implantation. As non – responders were defined all patient who were not alive at 6 months follow-up and patients who have hospitalisation for heart failure in this period and responders were all patients free of hospitalisation

either with lowering of NYHA class or increase in LVEF more than 10%

Statistical analysis

Categorical parameters were summarised as percentages and continuous parameters as mean ± SD. Comparisons between the two groups were performed using the Student's t-test for continuous parameters and Pearson's chi-square test for categorical parameters. Assessment of correlation was done using Spearman's correlation analysis. Multiple logistic regression analysis was performed in stepwise order to determine independent predictors of OAC use.

All data analysis was performed using SPSS version 22.0 (IBM SPSS, Inc., Chicago, Illinois) and a p-value ≤ 0.05 was considered significant.

Results

Patients included in this study were with a mean age of 62.1 ± 9.6 years, predominantly male (57. 8%). Ischemic heart disease was the underlying aetiology of heart failure in 21.6% of patients and non-ischemic heart disease in 78.4% of patients. Before implantation 41.2% of patients were in NYHA functional class II, 48% in class III and 10.8% were in class IV. Baseline rhythm was sinus in 88.2% of patients, 8.8% were in permanent atrial fibrillation, and 2.9% had AV block of second or third degree. Mean duration of QRS complex was 171.9 ± 22.4 ms; 74.5% of patients had LBBB morphology, and in other/the remaining 25.5% non-typical BB morphology. None of the patients had RBBB morphology of QRS complex.

Implantation of the CRT device was successfully performed in all patients. A CRT-P device was implanted in 81.4% of patients, and CRT -D device in 18.6% of patients (74% of whom had ischemic aetiology of heart failure, P < 005).

During the average follow-up period of 24.3 months, from a total of 102 patients, 5 patients died, and 17 (16.7%) patients were hospitalised with symptoms of heart failure. Among 102 patients, 75 (73.5%) patients were responders based on the previously defined criteria.

The clinical characteristics of the responders and non-responders are summarised in Table 1.

There were no significant differences in age, sex, the aetiology of heart failure and presence of arterial hypertension. Responders were more likely to have lower BMI, lower NYHA class, less present atrial fibrillation and diabetes mellitus and lower hospitalisation rate before CRT implantation compared to non-responders.

Table 1: Clinical characteristics of patients

	Responders (n = 75)	Non-responders (n = 27)	p-value
Age	61.5 ± 9.7	63.5 ± 9.2	n.s.
Gender (male %)	58.7%	55.6%	n.s.
BMI	24.9 ± 2.4	26.2 ± 2.1	0.012
Etiology of HF (ischemic /non-ischemic)	20% / 80%	26%/74%	n.s.
Atrial fibrillation	13.3%	33.3%	0.022
NYHA functional class	2.6 ± 0.6	3 ± 0.8	0.004
Arterial hypertension	48%	44.4%	
Diabetes mellitus	21.3%	48.1%	0.008
Hospitalization for HF prior to implantation	42.7%	70%	0.013

BMI - Body Mass Index; HF – Heart Failure; NYHA - New York Heart Association

Differences in ECG variables are listed in Table 2. There was no significant difference in QRS duration, the presence of LBBB or non-LBBB morphology of the QRS complex. Responders were more likely to have taller R waves and shorter S waves in V6, and bigger R6 / S6 ratio, compared to non-responders. The computed variable (S1 + R6) - (S6 + R1) was also significantly bigger in responders. Paced QRS duration was shorter in responders than in non-responders, but it did not reach a statistical significance.

Table 2: Electrocardiographic variables in responder and non - responder group

	Responders (n = 75)	Non-responders (n = 27)	p-value
PR interval	162.2 ± 23.0	182.2 ± 32.3	0.02
QRS duration	172.4 ± 21.9	170.7 ± 24.2	n.s.
LBBB morphology	77.3%	66.7%	n.s.
R amplitude in V1	1.1 ± 0.4	1.3 ± 0.5	0.04
S amplitude in V1	14.2 ± 5.9	14.5 ± 6.8	n.s.
R amplitude in V6	6.6 ± 5.0	3.6 ± 2.9	0.01
S amplitude in V6	4.1 ± 3.9	7.3 ± 6.6	0.01
R6/S6	4.6 ± 5.4	1.7 ± 2.5	0.02
(S1+R6)-(S6+R1)	15.7 ± 10.8	9.5 ± 8.8	0.02
Paced QRS duration	127.5 ± 26.3	137.0 ± 23.2	0.08

To find out if there is a difference in ECG parameters we divided the patient group in those with LBBB and those with non-LBBB morphology of the QRS complex. The ECG parameters of the responders and non-responders are summarised in Table 3.

Table 3: Electrocardiographic variables in different QRS morphology patients

	LBBB -patients			Non LBBB -patients		
	Responders	Non-responders	p-value	Responders	Non-responders	p-value
PR interval	163.6±24.2	180.7±36.1	n.s.	157.5±18.0	185±25.63	0.02
QRS duration	176.2±21.2	173.3±18.8	n.s.	159.5±19.8	165.6±33.2	n.s.
R amplitude in V1	1.1±0.4	1.4±0.5	n.s.	1.1±0.3	1.2±0.4	n.s.
S amplitude in V1	15.0±6.0	16.0±7.3	n.s.	12.1±5.1	11.2±3.7	n.s.
R amplitude in V6	7.7±5.2	4.7±3.0	0.01	3.3±2.5	1.3±0.8	0.01
S amplitude in V6	2.8±3.2	6.7±7.5	n.s.	7.6±3.7	8.5±4.5	n.s.
R6/S6	5.9±5.6	2.4±2.8	0.04	0.72±1.2	0.17±0.1	0.07
(S1+R6)-(S6+R1)	18.8±10.1	12.6±8.3	0.03	6.6±7.2	2.8±6.1	n.s.
Paced QRS duration	128.8±25.4	136.1±20.9	n.s.	122.9±29.7	138.9±28.5	n.s.

Responders in the group of LBBB patients kept the significant difference in a computed variable (S1 + R6) - (S6 + R1) and R6 / S6 ratio, probably due to a significant difference in height in R waves in V6.

On the other hand, responders in the group of non-LBBB patients kept the significant difference only in the height of R waves in V6. R6 / S6 ratio in this group of patients tended to be higher, similar to the LBBB patients, but it did not reach a statistical significance.

In both groups of patients, PR interval was longer in non-responders, but it reached statistical significance in non-LBBB group of patients.

Multivariate logistic regression analysis identified that none of the tested ECG parameters at baseline is an independent predictor of response to cardiac resynchronisation therapy.

Representative ECG of responders and non-responders with LBBB and non-LBBB morphology before implantation are shown in Figure 1.

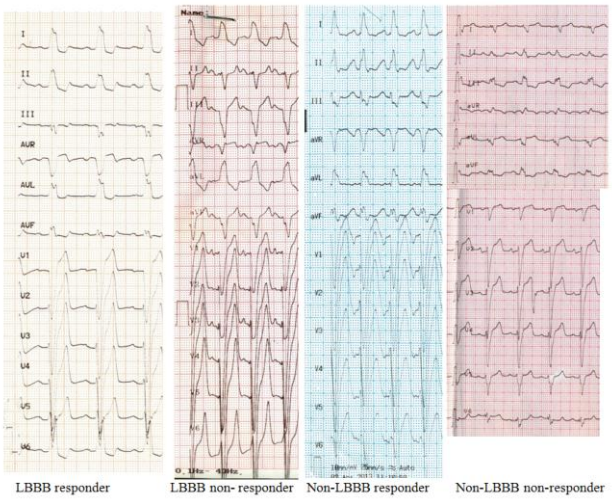

Figure 1: Representative electrocardiograms in responder and non-responder group

Discussion

The baseline QRS duration is a key part of the indication for cardiac resynchronisation therapy. It has been recognised since the publication of the Pacing Therapies in Congestive Heart Failure (PATH - CHF) I and II studies [10][11]. After publication of MADIT-CRT study in June 2009, FDA requested an additional 6 months of follow-up to see if the benefit of CRT-D persisted over time.

It was subsequently discovered and validated that the LBBB subgroup patients (approximately 70% of the total MADIT-CRT population) received substantial benefit from CRT-D. Non-LBBB patients did not show evidence of benefit [12]. During a sub-analysis, it was noticed that women more likely than men have LBBB [12]. Also, Sweeney et al. [13] have demonstrated that an LBBB-pattern of QRS is a strong predictor of response to CRT. This was further confirmed in the RAFT study [14]. LBBB configuration of the ECG was accepted as a better predictor of CRT response than any of the echocardiographic parameters [15].

However, across the studies, approximately 30% of the implanted CRT patients have non-LBBB QRS morphology: 13% have RBBB morphology and 17% IVCD [16]. The MADIT – CRT study has shown that CRT implantation has led to more frequent heart failure events and death in patients with intraventricular conduction delay who received CRT - Defibrillators compared to implantable defibrillator alone [8]. As cardiac resynchronisation therapy is largely included in the management of patients with heart failure a need for a new definition of LBBB emerged, especially to distinguish LBBB from conduction delay due to left ventricular hypertrophy (LVH). New criteria for complete LBBB have been proposed, which include a terminal negative deflection in V1, QRS duration ≥ 140 ms for men and ≥ 130 ms for women (due to the size of the heart in different genders), and also mid-QRS notching or slurring in at least 2 of the leads I, aVL, V1, V2, V5 or V6 [17]. The presence of notching is very important in establishing the diagnosis of LBBB, and it should begin after the first 40 ms of the QRS, but before 50% of QRS duration, when the activation wave-front reaches the endocardium of the LV [17]. But, if the duration of QRS is long enough even in non-LBBB patients, the number of responders to CRT increases [18].

In our study 73.5% of patients were responders, and the rest of them were classified as non -responders. In the responder group, 22.7% of patients had non-LBBB morphology of the QRS, and opposite of that 66.7% of patients in the non - responder group had LBBB morphology of the QRS complex. Some of the studies suggest that patients with IVCD did not respond to CRT therapy [18][19]. The study of Takaya *et al.* (20) showed that 40% of patients with IVCD responded to CRT. This response rate was lower compared to large major trials, and the study concluded that patients with IVCD derive fewer benefits from CRT therapy regarding symptoms relief and echocardiographic findings [21][22].

ECG predictors in patients with LBBB and non-LBBB patterns

Mean QRS duration in our patient group was 171.9 ± 22.4 ms and we found no significant difference between non-responders and responders, or between LBBB and non-LBBB pattern of QRS. In both ECG patterns, paced QRS was wider in non - responder group compared to responder group, but this difference was not statistically significant. There is evidence in the literature that shortening of the QRS after CRT implantation is a predictor of response to CRT, even better than baseline QRS duration [23][24]. Lecoq *et al.* [24] reported that the only independent predictor of the CRT response is shortening of the QRS after CRT implantation. We found no statistical

significance of this parameter, but the results were in the same direction, confirming that changes in QRS duration after implantation may reflect the quality of electrical resynchronisation and the degree of correction of electromechanical abnormalities.

The pattern of ventricular activation sequence on ECG has been very rarely analysed as a predictor. In our study responders in LBBB-group showed a significant difference in amplitude of the R wave in V6, computed variable (S1 + R6) - (S6 + R1) and R6 / S6 ratio. This finding is in concordance with the Strauss *et al.* [17] explanation of the real LBBB in comparison to EKG changes in case of left ventricular hypertrophy. Absolute and relative R wave amplitude in V6 could serve as a simple predictor of response to resynchronisation therapy in patients with LBBB morphology.

Responders in non-LBBB patients kept the significant difference only in the height of R waves in V6. R6 / S6 ratio in this group was higher, similar to LBBB patients, but it did not reach a statistical significance. Sweeney *et al*. [13] found that characterisation of ventricular activation sequence on the ECG anticipated the probability of response to CRT in patients with LBBB. They showed that QRS axis shifted from left to right, marked an increase in R-wave amplitudes in V1 through V2 on the ECG after device implantation, and predicted LV reverse remodelling.

Patients with non-LBBB pattern have delayed activation of either some or all of the right, left, or both ventricles. These patients may have less left-sided conduction delay than LBBB patients and therefore may not respond to CRT implantation. In the study of Takaya *et al.* [20], left axis deviation of QRS before implantation and QRS axis shift from left to right after implantation were found as predictors to CRT response.

Our study has clinical importance because it is one step forward in identifying patients who will respond to CRT therapy in a very simple way like an electrocardiographic recording.

Study limitations: This study is single centre experience, which is a limitation regarding treatment bias and could influence the outcome of the therapy.

The study is prospective, but with limited strength, because it is observational and has no control group. A small number of patients studied arises a need for confirmation in large prospective studies.

In conclusion, implantation of CRT device is a demandable procedure regarding sources, expertise and knowledge. The other part of the complexity is related to the disease itself, as heart failure is different and unpredictable in every single patient. Over the last decade, a majority of clinical studies have been focused on how to improve the selection of patients who will respond to this therapy-modality.

Our study gives a contribution to the proper patient selection by additional electrocardiographic criteria. Although none of the tested ECG parameters stands out as an independent predictor of response to cardiac resynchronisation therapy, some of them were clearly different in responder-group compared to the non-responder group. The amplitude of R wave in V6, higher R / S ratio in V6 and higher computed variable (S1+R6) - (S6+R1) may predict the likelihood of response to CRT therapy in both, LBBB-patients and non-LBBB patients.

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Clinical Science

CD33$^+$ HLA-DR$^-$ Myeloid-Derived Suppressor Cells Are Increased in Frequency in the Peripheral Blood of Type1 Diabetes Patients with Predominance of CD14$^+$ Subset

Mirhane Hassan[1*], Hala M. Raslan[2], Hesham Gamal Eldin[1], Eman Mahmoud[1], Hanaa Alm-elhuda Abd Elwajed[1]

[1]Clinical and Chemical Pathology Department, National Research Center, Dokki, Egypt; [2]Internal Medicine Department, National Research Center, Dokki, Egypt

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***Correspondence:** Mirhane Hassan. Clinical and Chemical pathology department, National Research Center, Dokki, Egypt. E-mail: mirhanehassan@gmail.com

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Abstract

INTRODUCTION: Type 1 Diabetes Mellitus (T1D) is an autoimmune disease that results from the destruction of insulin-producing beta cells of the pancreas by autoreactive T cells. Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of cells that can potently suppress T cell responses.

AIM: To detect the presence of MDSCs in T1D and compare their percentage in T1D versus healthy individuals.

METHOD: Thirty T1D patients were included in the study. Diabetic patients with nephropathy (n = 18) and diabetic patients without nephropathy (n = 12). A control group of healthy individuals (n = 30) were also included. CD33$^+$ and HLA-DR$^-$ markers were used to identify MDSCs by flow cytometry. CD14 positive and negative MDSCs subsets were also identified.

RESULTS: MDSCs was significantly increased in T1D than the control group and diabetic patient with nephropathy compared to diabetic patients without nephropathy. M-MDSCs (CD14$^+$ CD33$^+$ HLA–DR$^-$) were the most abundant MDSCs subpopulation in all groups, however their percentage decrease in T1D than the control group.

CONCLUSION: MDSCs are increased in the peripheral blood of T1D with a predominance of the CD14$^+$ MDSCs subset. Future studies are needed to test the immune suppression function of MDSCs in T1D.

Introduction

Autoimmune Diabetes (AID) also known as type 1 diabetes (T1D) usually develops as a result of the triad of environmental factors, genes and immune system interaction [1]. Several genes like cytotoxic T-lymphocyte antigen 4 (CLTA4), major histocompatibility complex class II (MHC II), and others drive an immune response that controls the T1D outcome. The over-reactive immune system also occurs in AID patients [2]. Subsequently, loss of insulin-producing pancreatic β – cells, caused by infiltrating immune cells, results in hypoinsulinemia, hyperglycemia and fatal complications. To date, no means for preventing or curing AID exists [3]. Numerous strategies have been developed, trying to restore physiological insulin production in diabetic patients [4]. Despite some progress, restoring self - tolerance or specifically correcting autoimmunity remains a crucial step toward reversing AID. In this respect, research and clinical interest in regulatory Treg and MDSC have increasingly grown [5][6][7].

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of cells that are defined by their myeloid origin, immature state and ability to suppress T cell responses potently. Under normal conditions, they differentiate into dendritic cells, macrophages and granulocytes. However, in pathological states such as infection, inflammation or cancer, there is an arrest of differentiation of this population of cells resulting in their accumulation [8][9][10]. The composition and percentage of MDSCs were found to vary according to disease nature. However, MDSC of different origins has potent suppressive activities [11][12].

Human MDSCs were initially defined as HLA-DR$^-$CD33$^+$ or CD14$^-$CD11b$^+$ cells, with both

phenotypes identifying cell populations with T cell suppressive activity [13][14]. Recently, other markers have defined MDSC subsets, including CD14-positive monocytic MDSC (Mo-MDSC) and CD15-positive granulocytic MDSC (G-MDSC). The expression of CD14 on MDSCs as a Mo-MDSC marker was initially controversial but has now become accepted. The CD14 expression is difficult to be used as a lineage marker as low levels of this marker are expressed on polymorphonuclear leukocytes (PMNs). Therefore, differentiation between $CD14^{bright}$ versus $CD14^{low}$ in the assessment of MDSCs is crucial [15].

Accumulative evidence shows that T- cells play a pivotal role in AID pathogenesis through the secretion of inflammatory cytokines and destruction of β-cells [16]. Accordingly, the importance of studying MDSCs emerges since several therapeutic strategies targeting MDSCs could inhibit immune responses in the setting of autoimmune disease [17].

This study aimed to determine the frequency of MDSCs and study MDSCs subsets in type1 autoimmune diabetes and to evaluate the effect of diabetic nephropathy on the frequency of MDSCs for later studying of their suppressor activities on T cells and their potential therapeutic use in AID.

Material and Methods

Subjects

This study enrolled thirty type 1 diabetic patients from the diabetes outpatient clinic of National Research Center (NRC) and thirty age - and gender-matched healthy controls. In particular, inclusion criteria for patients were age range 20 - 60 years. The first group of patients included 12 T1D without diabetic nephropathy. Diagnosis of diabetes was based on criteria of the American Diabetes Association [18]

The second group of patients included 18 T1D with diabetic nephropathy. Diagnosis of diabetic nephropathy is based on detection of abnormal levels of urinary albumin in diabetic patients combined with exclusion of other causes of albuminuria. Albumin measurements are defined as follows [19]:

1. Normal albuminuria: urinary albumin excretion < 30 mg/24h;

2. Microalbuminuria: urinary albumin excretion in the range of 30 - 299 mg/24h;

3. Macroalbuminuria: urinary albumin excretion ≥ 300 mg/24h.

Exclusion criteria included Type 2 diabetes, any febrile illness during the last three months, chronic inflammatory/rheumatic disease or other chronic kidney disease.

Clinical data were collected from patients' records. Informed consent was obtained from each subject. Approval for the study was obtained from the Ethics Committee of National Research Center, Cairo, Egypt (Number of the approval: 15160).

Samples

From each subject, 3 ml venous blood was taken and divided into three sterile tubes. Two tubes were sent to the Clinical Chemistry laboratory in NRC for the determination of fasting blood sugar level; two hours postprandial blood sugar test, glycated haemoglobin level. The third tube was sent to the flow cytometry unit in Kasr El Aini hospital for immunofluorescent staining for the determination of myeloid-derived suppressor cells.

Samples were kept at room temperature and were not shaken. They were analysed within 24 hours of venipuncture.

Microalbuminuria was defined by urinary albumin excretion of at least 30 mg in a 24 - hour period

Flowcytometry

First, isolation of human PBMCs was performed using a Ficoll - Hypaque density gradient before antibody staining. Second, 100 µl of test sample was incubated with 20 µl of specific monoclonal antibodies: CD33 PE (Beckman Coulter, USA), CD14 - FITC (Beckman Coulter, USA) and HLA - DR PC5 (Beckman Coulter, USA) at room temperature in the dark, same species isotypes served as a negative control.

After 20 min of incubation, 2 ml of Phosphate Buffer Saline were added to each tube of monoclonal treated cells. The mixtures were then centrifuged for 5 min at 150 xg at room temperature followed by discarding the supernatant and resuspending the pellet in 3 ml PBS. Cell analysis was performed using CYTOMICS FC 500 Flow Cytometer (Beckman Coulter, FL, USA) and analysed using CXP Software version 2.2.

Statistical analysis

The data were analysed using Microsoft Excel 2010 and statistical package for social science (SPSS version 24.0) for Windows (SPSSIBM, Chicago, IL). Results were expressed as mean ± SD with 95% confidence interval using the range for

quantitative variables, and using the frequencies and percentage for qualitative ones; a p- value < 0.05 was considered statistically significant. Spearman's rank correlation coefficient (r) was done to show the correlation between different parameters in this study. Diagnostic parameters of subjects were compared using the non-parametric Wilcoxon – Mann - Whitney U - test, whereas the parametric parameters were compared using the Paired samples (t) test. Also, Chi-square (χ^2) test was used for comparison of categorical data. Whenever the expected values in one or more of the cells in a 2 x 2 tables were less than 5, Fisher exact test was used instead and using linear by linear association in larger than 2 x 2 cross - tables.

Results

Characteristics and clinical data

A total number of 30 T1D patients were included in the study. Diabetic patients with nephropathy represented 60% (n = 18) and diabetic patients without nephropathy represented 40% (n = 12). A control group of healthy individuals (n = 30) were also included. Demographic and laboratory data of Patients and controls are shown in Table 1. Human MDSCs are immature myeloid cells that express the cell surface antigen CD33 and weakly express HLA-DR being a mature myeloid cell marker [20]. Accordingly, we used the surface markers $CD33^+$ within HLA-DR neg cells to identify MDSCs and compared the frequency of MDSCs in the peripheral blood of the two groups of patients - diabetes with nephropathy (Figure 1a) and diabetes without nephropathy (Figure 1b) - and of the control group as well (Figure 1c).

To determine the percentage of MDSCs in patients, acquired cells were first gated based on the expression of HLA DR (PC5). The PC5 subset was comprised of HLA DR^- cells. Within this population, the fraction of cells expressing CD33 (PE) and CD14 (FITC) was determined.

Table 1: Demographic and laboratory data of the two groups of patients and control

Descriptive parameters		Control N=30	Diabetic patients Without nephropathy N=12	Diabetic patients With nephropathy N=18
	Range	28 - 55	35 - 55	28 – 56
Age	Mean ± SD	38.3 ± 5.3	49.1 ± 5.3	40.0 ± 8.7
	Female	21 (70.0%)	9 (75.0%)	15(83.3%)
Sex	Male	9 (30.0%)	3 (25.0%)	3(16.7%)
	Range	80-120	86-243	98-272
FBS mg/dl	Mean ± SD	95.6 ± 13.8	(125.4 ± 47.7)	(123.3 ± 40.3)
	Range	90-150	110-242	120-250
PP.BS mg/dl	Mean ± SD	119.6 ± 20.6	(152.9 ± 32.3)	(155.1 ± 27.9)
	Range	3-5.5	4.5-7.0	5.1-7.2
Hba1C %	Mean ± SD	4.22 ± 0.79	(5.7 ± 0.7)	(5.8 ± 0.6)
Microalbumin	Range		16.5-29.0	185-310
mg/24h	Mean ± SD	---------	(23.97 ± 2.9)	(253.8 ± 33.8)

FBS: Fasting blood sugar, PP; BS = postprandial blood sugar; SD = standard deviation.

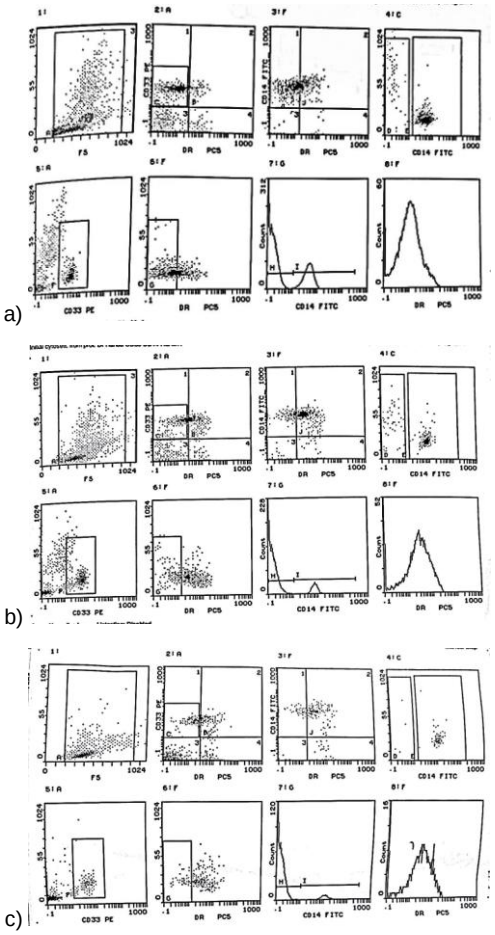

Figure 1: a) Flowcytometry analysis of the percentage of CD33+ HLA-DR- MDSCS in T1D patients with diabetic nephropathy; b) Flowcytometry analysis of the percentage of CD33+ HLA-DR- MDSCS in T1D patients without diabetic nephropathy; c) Flowcytometry analysis of the percentage of CD33+ HLA-DR- MDSCS in healthy control group

Increased frequency of $CD33^+$ HLA - DR^- MDSCs in the peripheral blood of T1D patients

The frequency of total MDSCs was increased in the peripheral blood of type 1 diabetes patients compared to the control group with a highly significant difference (Figure 2), (Table 2).

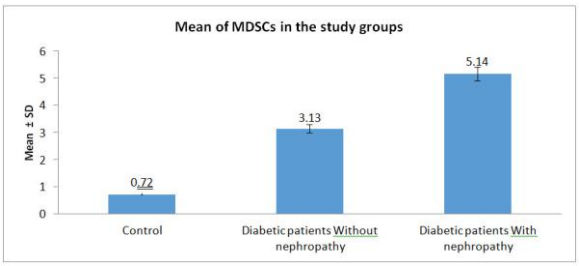

Figure 2: Mean of MDSCs in the study groups

Then, we subdivided the group of diabetic patients according to nephropathy into two groups: Diabetic patients without nephropathy (n = 12), and Diabetic patients with nephropathy (n = 18), and we compared the frequency of total MDSCs among

the two groups and the control group. We found that the frequency of total MDSCs in a diabetic patient with nephropathy was increased than diabetic patients without nephropathy with a highly significant difference (P - value < 0.001) (Figure 2), (Table 2).

Table 2: The frequency of MDSCs in both groups of T1D and the control group. Percentage of the two subpopulations of MDSCs CD14$^+$ and CD14$^-$ in both patients group compared to the control group

Descriptive parameters		Control N = 30	Diabetic patients Without nephropathy N = 12	Diabetic patients With nephropathy N = 18	Total Diabetic Patients
MDSCs	Range	0.3-1.01	2.4-3.8	3.0-10.3	2.4-10.3
	Mean ± SD	0.72 ± 0.24	(3.13 ± 0.5)	(5.14 ± 2.3)	(4.4 ± 2.07)
CD14 Positive	Range	98.9-99.7	96.0-97.5	87.5-99.5	87.5-99.5
	Mean ± SD	99.3 ± 0.3	(96.9 ± 0.6)	(95.4 ± 3.8)	(96.5 ± 3.02)
CD14 Negative	Range	0.1-1.1	2.5-4.0	0.52-12.5	0.52-12.5
	Mean ± SD	0.62 ± 0.33	(3.09 ± 0.56)	(4.6 ± 3.8)	(3.98 ± 3.0)

The total population of CD33$^+$ HLA–DR$^-$ MDSCs obtained from peripheral blood of T1D patients were further divided into two subpopulations of cells expressing CD14$^+$ and CD14$^-$. We compared the two subsets in the two groups of diabetic patients in relation to the control group. Then, we compared the percentage of these 2 MDSCs subsets in the two groups of diabetic patients (Table 2).

Analysis of the previous results of MDSC subsets (CD14$^+$ and CD14$^-$) demonstrated that all healthy individuals in the control group, diabetic patients with nephropathy and diabetic patients without nephropathy had higher numbers of CD14$^+$ MDSC and lower number of CD14$^-$ MDSCs.

The percentage of CD14$^+$ subset tended to decrease in the T1D patients compared to the control group even though their absolute number increased due to the increase of total MDSCs. On the contrary, Percentage of CD14$^-$ subset has increased in T1D patients than the control group.

Among the diabetes patients, the percentage of CD14$^+$ subset was less in diabetic patients with nephropathy than diabetic without nephropathy. On the contrary, the percentage of CD14$^-$ was increased in diabetic patients with nephropathy than diabetic without nephropathy (Figure 3), (Table 2).

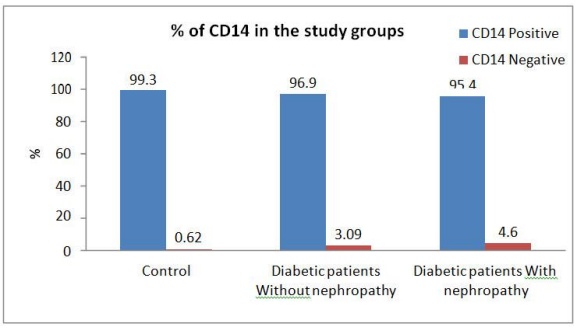

Figure 3: Comparison of CD14$^+$ and CD14$^-$ subsets among the three tested groups

In Brief, comparing diabetic patients with and without nephropathy to the control group demonstrated a highly significant difference in the frequency of total MDSCs and a significant difference in the proportion of CD14$^+$ and CD14$^-$ subset of MDSCs among the three groups.

Correlation between CD33$^+$ HLA - DR$^-$ MDSCs and HbA1C

We examined the possible correlations between CD33$^+$ HLA - DR$^-$ MDSCs and HbA1C in T1D patients. As shown in Figure 4, CD33$^+$ HLA - DR$^-$ MDSCs were positively correlated with levels of HbA1C (r = 0.702, P < 0.001) (Figure 4).

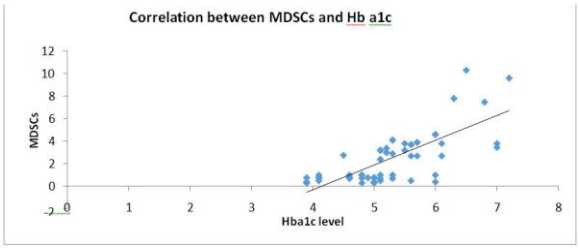

Figure 4: Positive correlation (direct proportion) between MDSCs and Hba1c (r = 0.702; P < 0.001)

Correlation of CD33+ HLA - DR$^-$ MDSCs, microalbumin

We examined the possible correlations of CD33$^+$ HLA - DR$^-$ MDSCs, levels of microalbumin in T1D patients. As shown in Figure 5, CD33$^+$ HLA-DR$^-$ MDSCs were positively correlated with levels of microalbumin (r = 0.814, P < 0.001) (Figure 5).

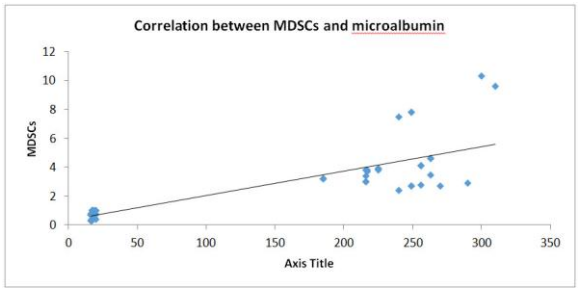

Figure 5: Positive correlation (direct proportion) between MDSCs and microalbumin (r = 0.814; P < 0.001)

Discussion

MDSCs are a heterogeneous population of cells that may inhibit the immune response, which makes them important goals for the treatment of autoimmune diseases [21]. Some studies focused on the importance of MDSCs, and they suggested

the possibility of their use in the prevention and treatment of T1D due to their potential suppressor function [22]. On the contrary, MDSCs was found at different stages of differentiation, accumulating in various pathological conditions like inflammation and autoimmune diseases [21]. These findings appear to be ambiguous. In our work, we studied the frequency of MDSCs in T1D patients.

According to *Kracht et al.* destruction of Beta-islets of the pancreas and development of autoimmune T1D and the antitumour immunity may share some common pathways and may differ in others [23]. Environmental factors have an important role in the aetiology of T1D as well as the antitumor immune response. The subsequent inflammation induces massive destruction of the target cells. Both beta cells and tumour cells respond to inflammatory signals by releasing proinflammatory cytokines such as (IL-1b), chemokines (CCL2, CXCL10) [24][25], and by producing neoantigens that are recognised by a tumour - specific or autoreactive T cells [26][27]. Overexpression of human leukocyte antigen (HLA) is an early indicator of islet distress, seemingly before insulitis in the case of T1D [28]. While tumours can evade immune detection by downregulation of HLA class I or direct inhibition of lymphocytes [29]. The hyperexpression of HLA class I by beta – cells together with the production of chemo-attractants, results in amplification of the immune response [30]. This, in conjunction with signals produced by infiltrating immune cells (CD8 and CD4 T cells), that lead to a microenvironment comparable to antitumor response. The microenvironment of progressive tumours contains numerous cells that promote tumour growth. Among these cells, we can find MDSCs that prevent cytotoxic T cell activity [31]. In case of T1D, Whitfield - Larry *et al.,* reported an increased frequency of MDSCs in T1D patients in contrast to the islet of diabetic Non- Obese Diabetic (NOD) mice that showed fewer MDSCs suggesting an underlying defect in immune suppression [22]. Even though studies of MDSCs in autoimmunity in mice are frequent, only a few data about MDSCs in human autoimmune diseases are available.

Our work reveals a highly significant increase in the frequency of $CD33^+$ $HLA\text{-}DR^-$ MDSCs in the peripheral blood of T1D patients. The underlying mechanism of this increase, however, is not well known. In a previous study, *Haile et al.* had described an increased frequency of peripheral MDSCs in Crohn's disease and ulcerative colitis [32]. A strong association reported in their study was that most of their patients followed an immunosuppression regimen. In our study, none of the T1D patients was on immunosuppressive treatment, yet our results similarly show an increased frequency of peripheral MDSCs. Therefore, we suggest that the high frequency of peripheral MDSCs might be a general finding in autoimmune diseases and not a result of the use of immunosuppressive drugs.

A possible explanation of MDSCs increase in T1D raised when a previous cancer study suggested that some factors produced by cancer cells and inflammatory cells may have a role in the increased frequency of MDSCs [33][34]. These factors included three cytokines known to be important in the pathogenesis of T1D which are IFN - gamma, IL-1beta, and IL-6 [35][36]. So, increased frequency of peripheral MDSCs in T1D patients is likely a result of the elevation of these cytokines. Some recent studies reported a relation between end-stage renal disease (ESRD) and expansion of MDSCs [37], so we investigated the effect of renal complication of T1D known as diabetic nephropathy on the frequency of MDSCs and whether MDSCs would significantly differ in T1D without nephropathy than in T1D with diabetic nephropathy. We found a highly significant increase of MDSCS in diabetic patients with nephropathy than in those without nephropathy.

Human MDSCs can be identified as $CD33^+$ $HLA\text{-}DR^-$, and further divided into 2 subsets: granulocytic $CD14^-$ and monocytic $CD14^+$ MDSCs [38][39]. In our study, we analysed the peripheral blood of T1D patients with and without diabetic nephropathy for the presence of 2 recently described MDSCs subpopulations (G-MDSCs and M-MDSCs). Our data demonstrate that MDSCs show highly significant increase in the peripheral blood of patients with T1D compared to healthy controls. M-MDSC phenotype ($CD14^+$ $CD33^+$ $HLA\text{–}DR^-$) were the most abundant MDSCs subpopulation in the blood of healthy control group, T1D with and without nephropathy. To our knowledge, this is the first study that compares the difference in the percentage of MDSCs subsets ($CD14^+$ and $CD14^-$) in T1D and stating the decrease in the percentage of $CD14^+$ and the increase in $CD14^-$ in T1D compared to the control group.

In conclusion, this study reports an increase in MDSCs in the peripheral blood of T1D patients versus healthy control with a predominance of the $CD14^+$ MDSCs subset. Considering the immunosuppressive effect of MDSCs, we realise that continuous monitoring of the composition of these cells in T1D patients may be highly rewarding. Further future studies are needed to test the immune suppression function of MDSCs in T1D and their potential use as targets to inhibit the immune response in autoimmune disease.

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Clinical Science

Immunological Evaluation in Patients with Familial Mediterranean fever

Shams Kholoussi[1], Naglaa Kholoussi[1], Moushira Erfan Zaki[2], Hala T. El-Bassyouni[3], Hala Elnady[4], Botros Morcos[1], Asem Abo–Shanab[1*]

[1]National Research Centre - Immunogenetics Department, Cairo, Egypt; [2]National Research Centre - Biological Anthropology Department, Cairo, Egypt; [3]National Research Centre - Clinical Genetics Department, Cairo, Egypt; [4]National Research Centre - Child Health, Cairo, Egypt

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***Correspondence:** Abo – Shanab. National Research Centre - Immunogenetics Department, Cairo, Egypt. E-mail: asem_nrc@yahoo.com

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Abstract

OBJECTIVE: This study aimed to investigate T & B lymphocyte subsets and Natural Killer (NK) cells patterns in children with FMF versus normal control subjects, to estimate the immunoglobulins IgG, IgM, and IgA levels, and to scrutinize the possible use of Neutrophil / Lymphocyte ratio (NLR) as a marker for subclinical inflammation in FMF patients.

PATIENTS AND METHODS: A group of 42 patients with FMF attending the Genetics Clinic at National Research Centre were included in this study. They were 13 males and 19 females; their age ranged from 2 to 17 years old. Normal healthy subjects within the same age and sex range were included as a control group. Complete blood picture was done for all cases, and neutrophil/ lymphocyte ratio was calculated. Flow cytometer analysis was done for CD3, CD4, CD8, CD19 and CD16 using monoclonal antibodies. Immunoglobulins IgG, IgA and IgM were estimated in serum using nephelometry.

RESULTS: Positive consanguinity was present in 20 patients (47.6%). Abdominal pain was the most common manifestation followed by fever, arthritis, and red rash. CD3, CD4 and CD8 were statistically increased in patients group as compared to normal control group, while CD16 was statistically decreased.

CONCLUSION: The study suggests that quantitative measurement of CD expressions of CD3, CD4 and CD8 as well as NLR might be used as valuable markers for subclinical inflammation in FMF.

Introduction

Familial Mediterranean fever (FMF) is the most common systemic autoinflammatory disorder worldwide [1]. It is an autosomal recessive disease [2] that affects mainly the Mediterranean population [3], is characterised by recurrent self - limited fever and aseptic serosal inflammation, causing abdominal, thoracic and joints pain [4]. Children with FMF are more prone to growth retardation [5], chronic normocytic normochromic anaemia [6], and splenomegaly [7].

Malicious activation of many inflammatory pathways occurs during the attacks of FMF, in which T, B and Natural killer cells play a major role [8][9]. The interaction of these cells with endothelial cells and several inflammatory and immune mediators produced by these cells leads to the formation of atherosclerotic plaques followed by its destabilisation and vessel rupture [10].

In between attacks, inflammation continues subclinically with the associated processes of endothelial dysfunction, increased atherosclerotic changes and platelets activation. The persistent inflammation in FMF may cause endothelial dysfunction, atherothrombosis and systemic amyloidosis [11]. The Neutrophil / Lymphocyte ratio (N/L ratio) showed significant, rapid, and serial changes as immune system response to different conditions like surgical stress, systemic inflammation or severe infections [12], and can be used as a marker for predicting amyloidosis development [13].

The most distressing complication in FMF is renal amyloidosis, leading to nephrotic syndrome and chronic kidney failure. A macromolecular complex, called inflammasome, plays a major role in the activation of IL - 1 and thus induction of inflammation and when inflammasome activity is abnormally stimulated through a mutation, it may be involved in the pathogenesis of FMF [14][15][16]. Natural killer (NK) cells are cytotoxic lymphocytes that participate in innate immunity. In addition to their cytotoxic response, these cells produce cytokines to assist the adaptive immune response [17][18].

The aim of this study was to investigate T and B lymphocytes and Natural Killer (NK) cells patterns through flow - cytometric analysis of CD3, CD4, CD8, CD16 and CD19 expression, to estimate the immunoglobulin levels IgG, IgM and IgA in FMF patients as compared to normal control subjects, and also to scrutinize the possible use of NLR as a marker for subclinical inflammation in patients with FMF.

Patients and Methods

Forty - two patients with FMF attending the Clinical Genetics Clinic at National Research Centre were included in this study. They were following up patients during the year 2015. They were 13 males and 19 females; their age ranged from 2 to 17 years old. Twenty normal healthy subjects within the same age and sex range were included as a control group.

All subjects were subjected to full clinical examination and history taking. Written consents were taken from the children parents', and the study was approved by Medical Ethics Committee of NRC (13/146). Complete blood picture was done for all cases, and neutrophil/lymphocyte ratio was calculated. Flow - cytometer analysis was done for CD3, CD4, CD8, CD19 and CD16 using monoclonal antibodies [19]. Immunoglobulins IgG, IgA, Ig M was estimated in serum using nephelometry [20].

Statistical analysis was performed using SPSS program version 13. Quantitative variables were presented as a mean and standard deviation. The unpaired t-test was used to evaluate differences between the two groups of continuous variables. Two-tailed $P < 0.05$ was considered statistically significant.

Results

The study group consisted of 23 males (54.8%) and 19 females (45.2%), all were diagnosed as FMF and during the attack; while the control group were normal subjects matching age and sex. The age range in the FMF study group was 2 - 17 years and 2 - 14 years in the control group respectively (Table 1). This study included 42 patients, with the clinical characteristics of FMF. Positive consanguinity was present in 20 patients (47.6 %).

Abdominal pain was the most common manifestation which usually occurs in repeated attacks and stays from 2 hours to several days followed by fever, arthritis, and erysipelas-like rashes. All the patients were treated with colchicine after being identified by molecular diagnosis.

Table 1: Age and sex of patients and control group

	Patients n = 42	Control n = 20
Age range (years)	2-17	2-14
Sex (male/female)	23/19	11/9

CD3, CD4, and CD8 were statistically increased in patients' group as compared to normal control group; p-value were 0.001, 0.002, 0.004, respectively (Table 2). While CD16 has statistically decreased, the p-value was 0.007 as compared the control group.

Table 2: Cellular expression of CD3, CD4, CD8, CD19 and CD16 in FMF patients as compared to control group

	Patients n = 42	Control n = 20	p - value
CD3 %	51.91 ± 15.19	36.31 ± 10.41	0.001*
CD19 %	18.29 ± 9.07	19.78 ± 5.38	0.81
CD16 %	12.81 ± 5.38	19.06 ± 6.9	0.007*
CD4 %	33.74 ± 6.46	22.88 ± 6.81	0.002*
CD8 %	19.37 ± 5.98	12.2 ± 4.15	0.004*

*statistical significant $p < 0.05$.

Immunoglobulins IgA, IgM, IgG and also TLC and N/L ratio did not show the significant statistical difference between the patients and control groups (Table 3).

Table 3: Immunoglobulins IgA, IgM, IgG, TLC and N/L ratio in FMF patients and control group

parameters	Patients n = 42	Control n = 20	p - value
TLC ($\times 10^6$ /mm^3)	7.54 ± 2.39	9.16 ± 2.23	0.13
N/L ratio (NLR)	1.23 ± 0.62	0.8 ± 0.16	0.10
IgA (g/L)	1.35 ± 0.7	1.31 ± 1.06	0.89
IgM (g/L)	1.26 ± 0.45	1.41 ± 0.60	0.54
IgG (g/L)	10.67 ± 3.8	9.62 ± 3.45	0.49

*statistical significant $p < 0.05$

Discussion

Autoinflammatory diseases originate from inappropriate activation of antigen-independent inflammatory mechanisms [21]. FMF is characterised by fever and serositis which flare up as paroxysmal attacks, with intervening asymptomatic periods. The attacks of fever and diffuse abdominal pain in FMF are characterised by subclinical inflammation and associated endothelial dysfunction [11]. We aimed to

investigate a possible immune regulation imbalance in familial Mediterranean fever (FMF) by measuring levels of peripheral blood lymphocyte subsets using flow - cytometry. In this study, we found the increased percentage of T cells CD3, CD4 and CD8 as compared to the control group. This was in agreement with Musabak et al., [22], who stated that CD3, CD4 and CD8 were increased in FMF patients than in the control group indicating that the T cell system is abnormally activated in patients with FMF. Rimar et al., [23] suggested that regulatory T cells play a role in cutting short FMF acute attacks. This may explain the fact that inflammation in FMF is self - limiting and also the absence of autoantibodies, antigen-specific T cells and tissue damage [24].

NK cells mediate cytotoxicity that is regulated by their inhibitory and activating surface receptors. An increase in the activation of NK cells may result in proliferation and change the immune response [25]. In this study, CD16+ NK cells were decreased in FMF patients group as compared to the control group in contrary to what was reported that NK cell numbers were significantly increased in FMF patients as compared to controls. In autoinflammatory diseases, dysregulation is believed to occur in the innate immune system without primary involvement of T and B lymphocytes. Nevertheless, autoinflammation involves crosstalk between the innate immune system – neutrophils, macrophages, and NK cells – with the adaptive immune system [26].

To investigate the B - cells, CD19 showed no significant difference between patients and control groups. As regards to serum immunoglobulins IgA, IgM and IgG estimated in this study, there was no statistically significant difference in patients when compared to the control group. In another genetic disease, results obtained showed a significant difference between patients and controls, in which the results of the control group is nearly the same [27]. However, Livneh et al., [28] compared the immunoglobulin levels in patients with FMF about patients with hyperimmunoglobulinemia D syndrome (HIDS) and not about healthy control subjects, and that the patients with HIDS showed the significantly higher prevalence of elevated immunoglobulins levels than patients with FMF.

The N/L ratio is a reliable marker for evaluating and monitoring the systemic inflammatory response, and prognosis of clinical outcome of inflammatory diseases [29].

No statistically significant difference was detected in TLC nor NLR in FMF patients as compared to the control group while Özer et al., [30] and Duksal et al., [29], found TLC and N/L ratio were significantly higher in children with FMF than in healthy individuals. Celikbilek et al., [31], reported that the N/L ratio was higher in active FMF than in FMF in remission as well as in control subjects.

In conclusion, dysfunction of the innate immune system is the central matter in FMF as a self - reactive autoinflammatory disease.

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Utility of Tissue Inhibitor Metalloproteinase-1 and Osteopontin as Prospective Biomarkers of Early Cardiovascular Complications in Type 2 Diabetes

Doaa Samir Salah El-Din*, Ahmed Ibrahim Amin, Ahmed Osman Egiza

Chemistry, Faculty of Science, Cairo University, Giza 12613, Egypt

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***Correspondence:** Doaa Samir Salah El-Din. Chemistry, Faculty of Science, Cairo University, Giza 12613, Egypt. E-mail: sdoaa@sci.cu.edu.eg

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Abstract

AIM: This work investigated associations between tissue inhibitor metalloproteinase-1 and diabetic cardiovascular diseases in type 2 diabetic patients; also it investigated the role of osteopontin in the diagnosis of type 2 cardiovascular diabetes complications.

SUBJECTS AND METHODS: These were examined on eighty subjects, divided into three groups as follows: twenty volunteer healthy control subjects, thirty type 2 diabetes mellitus (DM) patients, and thirty cardiovascular, diabetic patients. Full clinical measurements were carried out, and the expression level of tissue inhibitor metalloproteinase-1 in blood samples was analysed by real-time PCR, using gene-specific primer pairs. Also osteopontin concentrations had been measured by the enzyme-linked immunosorbent assay. Data were tested statistically by parametric tests.

RESULTS: The concentrations of osteopontin and the expression levels of tissue inhibitor metalloproteinase-1 were significantly increased in diabetic and cardiovascular diabetic groups compared to control group also they were significantly increased in the cardiovascular diabetic group compared to the diabetic group.

CONCLUSION: Tissue inhibitor metalloproteinase-1 and osteopontin concentrations were significantly increased in diabetic patients with cardiovascular complications than other groups.

Introduction

The expanding commonness of type 2 diabetes mellitus (T2DM) calls for creating corresponding screening systems for early recognisable proof of high-risk people for the disease or its complexities [1]. The condition is comorbid with macrovascular disorders, for example, coronary artery disease, peripheral arterial disease, and stroke. Diabetes mellitus is a major risk cause for cardiovascular disease (CVD) which is the main cause of death among the diabetic patients. Diabetes mellitus intensifies atherosclerosis and heart failure mechanisms. Regrettably, these mechanisms are not sufficiently modulated by therapeutic strategies focusing only on the glycemic control with currently available drugs or approaches [2], underscoring the requirement for forceful CVD risk factor management.

The diagnosis and screening of diabetes mellitus have been changed since the earlier scientific statement, with the incorporation of glycated haemoglobin (HbA1c) in the diagnostic gauges of type II diabetes of at least 6.5% [3]. Specifically, HbA1c was used as a preferable standardised assay over glucose, a better indicator for overall glycemic exposure, and as less subject to pre-analytic instability, prandial status, biological variability, and intense stress [4].

While insulin and glucose are the most entrenched biomarkers, progresses in the “omics” technologies have recognised the extent of molecules including genetic variants, small metabolites, RNA transcripts, and proteins that could serve as indicators of diabetes risk [5].

Circulating markers of extracellular matrix turnover, such as matrix metalloproteinases (MMP) and their tissue inhibitors (TIMP), are essential to the vascular alternations of atheromatous vascular disease. TIMP-1 was considered as an endogenous MMP inhibitor that might include in the vascular matrix

fibrosis [6]. Also, it has a role in diastolic dysfunction and left ventricular hypertrophy by diminishing cardiac collagen type I turnover and expanding cardiac stiffness and mass [7].

In diabetes, TIMP-1 and MMP-9 levels are significantly increased [8], also in this condition, peripheral and central artery stiffness is increased [9]. Amelioration in the metabolic control leads to decreasing in TIMP-1 levels, and this raises TIMP-1 probability as a marker of vascular composition in diabetes mellitus [10], a potential purpose of mediation.

Phosphoprotein osteopontin (OPN), also called secreted phosphoprotein 1 (SPP1), urinary stone protein, early T lymphocyte activation 1, and bone sialoprotein is found in various cell types [11]. In prevalent patients, a significant association was observed between atherosclerotic plaque development and plasma OPN levels, independently of conventional risk factors [12]. Moreover, high glucose and advanced glycation end product may lead to upregulation of OPN expression in the vascular wall of diabetic animal models and diabetic patients [13].

In this study, the hypothesis that there may be a relationship between elevated both OPN levels and TIMP-1 expression levels and the presence of CVD in type 2 diabetic patients were tested.

Subjects and Methods

Patients' data

T of 80 subjects was recruited for that study and divided into three groups. Twenty volunteer healthy control subjects (G1), thirty type 2 diabetes mellitus (DM) patients (G2); disease duration 5 ± 3 years, and thirty type 2 diabetic patients with cardiovascular (coronary heart) diseases (G3); disease duration 3 ± 1 years. Type II diabetic patients were those consecutively referred during routine medical care visits from an outpatient diabetes clinic (National Institute of Diabetes in Egypt). If patients had any known systemic diseases other than diabetes that can influence the needed results such as epidemiological diseases, chronic periodontitis, nephropathy, retinopathy, neuropathy, gallbladder, liver, and lung diseases, they were excluded in this study. Participation in this study is completely voluntary. Patients decide to participate, can stop participating at any time and may decide not to answer any specific question. Participants' decision not to continue participating will not influence the nature of their relationship with a researcher or with a staff of this study either now or in the future. If a patient withdraws from the study, all associated data collected will be immediately destroyed wherever possible.

Laboratory markers of diabetes mellitus

Blood samples were collected from all fasting participants in this study, Lipemic and hemolyzed samples were excluded, samples were analysed for HbA1c by cation - exchange resin method [14]. Total cholesterol (TC) and triglycerides (TG) in serum were measured by colourimetric enzymatic method [15]. Also, high-density lipoprotein cholesterol (HDL cholesterol) was measured [16], and low-density lipoprotein cholesterol (LDL cholesterol) was evaluated by Friedewald formula [17]. Moreover, heart function biomarkers such as creatine phosphokinase-MB (CK-MB), creatine phosphokinase (CK), and lactate dehydrogenase (LDH) were measured by enzymatic method [18].

Tissue inhibitor of metalloproteinases-1 (TIMP-1) analysis TIMP-1 expression levels were determined using Real Time PCR (qPCR) which performed with SYBR Green (5X HOT FIREPol®EvaGreen® qPCR Mix Plus) for tenfold diluted cDNA samples in a triplet set using specific primers pairs which synthesized in HVD Life Sciences co., Vienna, Austria; forward primer sequence (CTGGCTTCTGGCATCCTGTTG) and reverse primer sequence (GTCTGGTTGACTTCTGGTGTCC) (NG_012533.1, NM_003254.2) against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference gene; forward primer sequence (CAGCCTCAAGATCATCAGCAATG) and reverse primer sequence (CAGTCTTCTGGGTGGCAGTGA) (NG_007073.2, Variant1: NM_002046.5, Variant 2: NM_001256799.2, Variant 3: NM_001289745.1, Variant 4: NM_001289746.1). Blood RNA was extracted by EZ-10 column blood RNA purification kit (cat.# BS82313) according to the manufacturer's instructions, and it was eluted from the membrane in the presence of RNase - free water. First cDNA strand was synthesised immediately after RNA extraction. Reaction components were mixed as follows: 5 µg RNA, 0.5 µl (10 µmol) Oligo dT and 0.5 µl (10 µmol) random primer, then the volume was adjusted to 12.5 µl using diethyl pyrocarbonate (DEPC) treated water. The reaction tubes were mixed well and incubated 5 min at 60^{o}C to remove the RNA secondary structure, then chilled on ice. Finally, the following components were added to the reaction mixture: 2 µl (10 mM) dNTPs, 4 µl (5 X) Moloney murine leukaemia virus (MMLV) buffer, 0.5 µl ribolock (40 u/µl) and 1 µl reverse transcriptase enzyme (MMLV-RTase) (20 u/µl). As a termination step, the final reaction mixture (20 µl) was spun down and incubated at room temperature for 10 min, then at 42^{o}C for 1hour followed by 10 min at 70^{o}C. cDNA samples were stored at - 20^{o}C for the amplification steps which performed on Bio-Rad CFX ManagerTM software, version 3.1. The qPCR was performed according to the following program: Initial denaturation and polymerase activation step for 10 min at 95°C, denaturation step at 95°C for 30 sec, annealing step for 30 sec at 57°C for GAPDH and TIMP-1 and finally, the elongation step for 45 sec at 72°C. The denaturation,

the annealing and the elongation steps were performed at 50 cycles.

Osteopontin (OPN) analysis

Osteopontin concentrations were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's protocol (BOSTER Biological Technology Co., Inc., USA; cat.# EK0482). All samples were analysed in duplicate. Horseradish peroxidase was conjugated with the secondary antibody, and tetramethyl benzidine (TMB) was used as a substrate. The absorbance was measured at 450 nm. Osteopontin concentration was expressed as pg/ml.

Statistical analysis

SPSS 17.0 software was used to execute the statistical analyses, and the results were represented by the mean ± SD. Student's t-test was used to make a comparison between the groups and correlations were assessed by Pearson's correlation test using $P < 0.05$ as considered to be significant.

Results

Clinical findings in the diabetic patients

Fasting blood sugar (FBS), glycosylated Hb (HbA1c%), Lipid profile; total cholesterol (TC), triglyceride (TG), low density lipoprotein cholesterol (LDL-C), LDL-C/HDL-C ratio, cardiac biomarkers; creatine phosphokinase (CK), creatine phosphokinase-MB isoenzyme (CK-MB), and lactate dehydrogenase (LDH) activities were significantly higher in the cardiovascular diabetic groups than that in the control and the diabetic group, while high-density lipoprotein cholesterol (HDL-C) concentrations were significantly lower in the cardiovascular, diabetic groups than that in the control and the diabetic group as clarified in Table 1.

Table 1: Some clinical data in control and different tested groups

	Normal range	Control group	Diabetic Group	Cardiovascular diabetic group
FBS (mg/dl)	60-110	84.0 ± 8.9**	268.6 ± 94.2**	347.0 ± 66.9
HbA1c (%)	< 6.5	4.9 ± 0.7**	9.1 ± 2.0**	10.3 ± 1.1
TC (mg/dl)	< 200	105.8 ± 9.1**	157.9 ± 28.9**	262.7 ± 19.8
TG (mg/dl)	35-140	75.6 ± 8.7**	123 ± 22.8**	387.8 ± 71.9
HDL-C (mg/dl)	>55	59.9 ± 4.6**	52.9 ± 6.8**	22.9 ± 4.7
LDL-C (mg/dl)	< 150	30.6 ± 11.0**	80.4 ± 30.1**	162.2 ± 21.6
LDL-C/HDL-C	< 2	0.5**	1.6**	7.4
CK (U/L)	Up to 120	54.5 ± 10.2**	42.5 ± 7.8**	128.1 ± 23.7
CK-MB (U/L)	0-24	21.9 ± 3.9*	18.5 ± 3.8**	25.2 ± 3.7
LDH (U/L)	160-320	207.3 ± 12.5**	214.6 ± 23.8**	620.3 ± 101.9

Mean ± SD, vs. cardiovascular diabetic group. *P ≤ 0.05; **P ≤ 0.001.

TIMP-1 expression findings in the diabetic patients

The resulted data of the normalized expression ratio of TIMP-1 to GAPDH as a reference gene was represented in Table 2, and Fig. 1, A. Also individuals' data were statistically analyzed from the point of view as ± S.D and S.E. Then the t-value results were checked on student's t-test to detect the significance level (p-value).

Table 2: Statistical analysis of TIMP-1 expression levels and osteopontin concentrations in control and different tested groups

			Control group	Diabetic group	Cardiovascular diabetic group
Statistical analysis of TIMP-1	Avg. dC_T		7.03	2.27	1.39
	ddC_T		0.0	-4.77	-5.65
	FC than control group		1.0	27.20	50.07
	FC than cardiovascular diabetic group		0.12	0.54	1.0
	±S.D		0.75	0.9	0.72
	S.E		0.17	0.16	0.13
	F-value (ANOVA)			327.0	
	T-value	G1	-	20.3**	26.4**
		G2	-	-	4.2**
Statistical analysis of osteopontin	OPN conc. range		6000-8500	9000-14700	9550-22000
	OPN conc. mean		7140	10765	14421
	±S.D		619.8	1588.3	3411.6
	S.E		138.6	290.0	622.9
	F-value ANOVA			59.6	
	T-value	G1	-	9.7**	9.4**
		G2	-	-	5.3**

**P ≤ 0.001.

The present study showed that; the expression level of TIMP-1 gene significantly increased by 27.2 and 50.07 folds to reference gene; GAPDH, in diabetic and cardiovascular diabetic patients respectively than normal volunteers, so it might be considered as diagnostic marker for DM, and its expression level significantly increased by 1.84 folds in cardiovascular, diabetic patients than the diabetic patients, so it might be considered as diagnostic marker for detection of cardiovascular complications in type 2 diabetes.

Osteopontin (OPN) concentration findings in the diabetic patients

The individuals' data of osteopontin (OPN) concentration of different tested groups was statistically analysed in Table 2 and evaluated from the point of view as ± S.D and S.E. The result of the t-value is then checked on student's t-test to find out the significance level (p-value).

From the table, it appeared that highly significant difference ($p \leq 0.001$) was found in diabetic group (G2) (10765 ± 1588.3 pg/ml), and cardiovascular, diabetic group (G3) (14421 ± 3411.6 pg/ml) as compared with the control group (G1) (7140 ± 619.8 pg/ml), also highly significant difference was appeared in G3 as compared with G2 (Fig. 1, B).

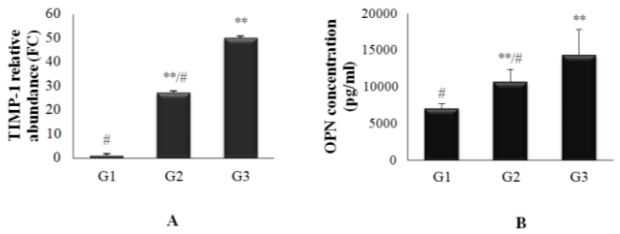

*Figure 1: (A) TIMP-1 relative expression level (FC ± S.D). (B) Osteopontin concentrations (Mean ± S.D) in control and different tested groups. **Highly significant different with the control group G1 (p ≤ 0.001), # Highly significant different with cardiovascular, diabetic group G4*

Table 3 showed highly significant positive correlation observed between osteopontin with some variables; FBS, HbA1c, cholesterol, triglyceride, LDL-C, CK, and LDH, and between TIMP-1 and HDL-C, while there was low significant positive correlation between osteopontin and CK-MB concentrations.

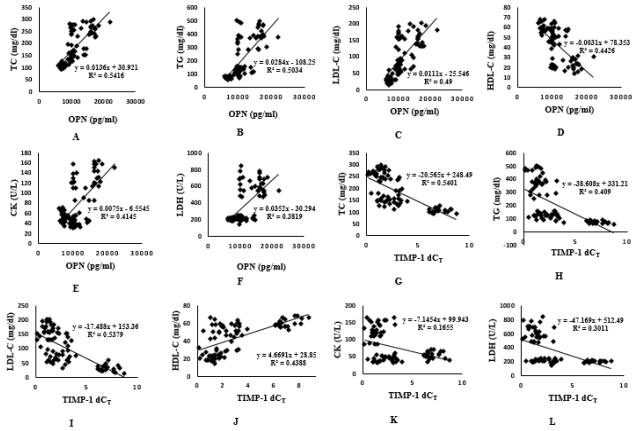

Figure 2: Correlation between osteopontin concentrations and TIMP-1 with some variable

Also there was highly significant negative correlation between osteopontin and HDL-C, also between TIMP-1 and some variables; FBS, HbA1c, cholesterol, triglyceride, LDL-C, CK, and LDH, while there was no correlation between TIMP-1 and CK-MB concentrations. (Fig. 2, A-L).

Table 3: Correlation between osteopontin concentrations and TIMP-1 with other variables

Osteopontin			TIMP-1	
Variable	Pearson Correlation (r)		Pearson Correlation (r)	Sig. (2-tailed)
FBS	0.605	H.S**	-0.740	H.S**
HbA1c	0.656	H.S**	-0.777	H.S**
Cholesterol	0.736	H.S**	-0.735	H.S**
Triglyceride	0.709	H.S**	-0.640	H.S**
HDL – cholesterol	- 0.666	H.S**	0.663	H.S**
LDL- cholesterol	0.701	H.S**	-0.734	H.S**
CK	0.644	H.S**	-0.407	H.S**
CK-MB	0.257	L.S*	-0.074	N.S
LDH	0.618	H.S**	-0.549	H.S**

N.S. P > 0.05; *P ≤ 0.05; **P ≤ 0.001.

Discussion

Diabetes mellitus is a complex disease affecting nearly all tissues and organs, with 158 metabolic ramifications extending far beyond impaired glucose metabolism. Biomarkers may reflect the presence and/or severity of hyperglycemia and the vascular complications of diabetes [19].

TIMP-1 is a diagnostic marker for diabetes, in this study, the expression level of TIMP-1 was significantly increased in diabetic and cardiovascular diabetic patients than normal volunteers, also increased significantly in cardiovascular, diabetic patients than other diabetic groups, higher levels of circulating TIMP-1 was connected with the higher risk of diabetes and cardiovascular demise. This finding was consistent with Usmanova [20], furthermore, Lee and his colleagues who found that plasma TIMP-1 concentration was significantly raised in type II diabetic patients [21]. Also in this study, the TIMP-1 expression level was significantly higher in diabetic patients with cardiovascular diseases (CVD) than in those without cardiovascular diseases, these were in agreement with Papazafiropoulou and Tentolouris results [22]. TIMP-1 is an endogenous MMP inhibitor that might be involved in vascular matrix fibrosis [6] and has a role in left ventricular hypertrophy and diastolic dysfunction by reducing cardiac collagen type I turnover, thereby increasing cardiac mass and stiffness [6][23]. Increased Central and peripheral artery stiffness [9] occurs with diabetes [24], and higher TIMP-1 circulating levels complete the hypothesis that altered TIMP-1 activities can be related to arterial stiffness.

Another biomarker studied in that work, was osteopontin (OPN) which is a phosphoprotein found in different types of cells [11]. In this study, Levels of serum OPN increased in all diabetic groups significantly in comparing with the control group, the expression of OPN was highly induced by glucose [25], this agreed with Takemoto and his colleagues [26] who reported an increase of serum OPN in diabetic patients, suggesting that it may be involved in the accelerated atherosclerosis-related to diabetes. Also in this study, its concentration increased in a cardiovascular, diabetic group significantly in comparing with the diabetic group. OPN is a pleiotropic cytokine that is a common and relevant component of many acute and chronic vascular or endothelial responses to injury characterised by inflammation and/or fibrosis, including atherosclerosis, arterial neointimal hyperplasia, and aortic stenosis [27], as well as the vascular damage, accompanied diabetes [28]. OPN has been acting as the main factor in the atherosclerosis development [29][30]. Plasma OPN concentration is elevated in the essential hypertension, coronary artery disease (CAD), and restenosis [31][32][33]. In the cardiovascular system, OPN is one of the main regulators of the vascular disease and the chronic inflammation [34]. In other aspects of vascular diseases, Yan and his

colleagues [35] found that plasma OPN level, parallels with the severity of nephropathy and CAD in diabetes, suggesting that an elevated plasma OPN level can be used as indicator for the diabetic vasculopathy screening.

This study revealed that, the expression levels of TIMP-1 and osteopontin concentrations had highly significant differences between diabetic and cardiovascular diabetic patients in Egypt which appeared in Table 2, so they could be used as molecular biomarkers in the diagnosis of cardiovascular complications in type 2 diabetic patients to predict potential progression and aid in case management.

In the future, we can be hopeful that new blood-based biomarkers will facilitate the discovery, prohibition, and treatment of diabetes and its complications much sooner than overt disease develops.

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Clinical Science

Dynamic MRI Evaluation of the Gastric Fundus and Splenic Circulation to Assess the Gastric Breves Dissection during Laparoscopic Nissen Fundoplication

Gökhan Akkurt[1*], Önder Sürgit[1], Hakan Ataş[1], Mustafa Alimoğullari[2]

[1]Keçiören Training Hospital Ankara, Ankara, Turkey; [2]General Surgery Ankara, Ankara, Turkey

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***Correspondence:** Gökhan Akkurt. Keçiören Training Hospital Ankara, Ankara, Turkey. E-mail: drakkurt06@gmail.com

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Abstract

AIM: We aimed to evaluate the possible effects of dissecting gastric breves (GB) during the Laparoscopic Nissen Fundoplication (LNF) on the gastric fundus and splenic circulation using dynamic Magnetic Resonance Imaging (MRI).

METHODS: In total 14 patients with gastroesophageal reflux disease (GERD) that was diagnosed with esophagogastroduodenoscopy and 24 - hour PH monitorization and undergoing LNF surgery were included. All patients underwent LNF surgery between October 2006 and March 2010. All patients were evaluated regarding gastric fundus and splenic circulation one week before and 15 days after the surgery with dynamic MRI. Alteration of the signal intensity before and after surgery was used to assess gastric fundus and splenic circulation.

RESULTS: We detected a significant decrease in DeMeester score before and after surgery ($p < 0.001$). There were no statistical differences between preoperative and postoperative dynamic MRI measurements of the spleen, anterior wall measurements, posterior wall measurements in different MRI phases (Bonferroni corrected $p > 0.01$). Postoperative measurements of anterior and posterior gastric wall measurements were comparable (Bonferroni corrected $p > 0.0033$).

CONCLUSIONS: We did not detect any significant differences in the abovementioned tissues regarding perfusion.

Introduction

Gastroesophageal reflux (GER) is a condition that is characterised by a retrograde movement of the gastric content into the oesophagus. Retrosternal burn at least two days of the week affecting life quality or mucosal loss (erosion or ulcer) in endoscopy is considered as “Gastroesophageal Reflux Disease” (GERD). Defective lower oesophagal sphincter (LES), transient lower oesophagal sphincter relaxations (TLESR), delayed oesophagal clearance, acid hypersecretion are implicated in the etiopathogenesis of the GERD [1][2]. GERD may cause clinical findings including pyrosis, acid regurgitation, chest pain, dysphagia, and odynophagia [3][4]. In some of the GERD cases, columnar epithelium with intestinal metaplasia may develop in the oesophagal mucosa, which is called as Barrett’s o esophagus [5]. It is known that this condition is a risk factor for oesophagal adenocarcinoma [4][6].

Even though acid might be controlled with medical treatment, non - acid reflux persists because defective LES remains untreated [7]. Contemporarily, surgical treatments are commonly used, as they are cheaper and more effective than medical treatment [8]. Among those surgical methods, Laparoscopic Nissen Fundoplication (LNF) is a standard gold treatment with less than 1% mortality and complication risk [9].

We aimed to evaluate the effects of transaction gastric breves (GB) during the LNF on

the gastric fundus and splenic circulation. Our hypothesis in the current study is to explore whether gastric breves in LNF is effective on gastric fundus and splenic circulation using dynamic magnetic resonance imaging (MRI).

Material and Methods

We included 14 patients with symptoms of gastroesophageal reflux disease (GERD), endoscopically identified hiatal hernia, esophagitis and Demeester score above 14.72 in 24 - hour Ph monitoring were included in the study. Esophagitis is evaluated using Los Angeles classification [10]. A 24 - hour pH monitorization was used. Flexible pH catheters had their reference pH levels, and the distance between two censors of the catheters was 15 cm. These catheters were calibrated in solutions with a pH of 1 and seven before usage. The data were assessed with a pH analysis program (MMS, the Netherlands) and Johnson and DeMeester scores were obtained. Patients with DM, HT, a history of pulmonary embolism, DVT or coronary artery disease and who had previously undergone gastrointestinal system or cardiac operation or received chemotherapy & radiotherapy were not included in the study. All patients underwent LNF surgery by the same operator (Surgit) using the standard technique between October 2006 - March 2010. All patients were evaluated regarding gastric fundus and splenic circulation one week before and 15 days after the surgery with dynamic MRI. Alteration of the signal intensity before and after surgery was used to assess gastric fundus and splenic circulation.

MR Imaging

A dynamic upper abdominal MRI was performed on a 1.5 T MR system (Intera Achieva 1.5 T; Philips Medical Systems, Best, The Netherlands) using a four channel SENSE body coil. A 15 - mL bolus of contrast material (gadodiamid [Omniscan, GE Healthcare Ireland]) and 20 mL of saline were delivered at 2 mL/second into an antecubital vein using an infusion pump injector (Medrad, Spectris Solaris; Indianola, Pa) and 5 sequential axial three - dimensional image datasets were acquired at precontrast, postcontrast 30 (Phase 1), 60 (Phase 2), 100 (Phase 3), and 130 (Phase 4) seconds using a T1 High Resolution Interpolated Volume Examination (THRIVE) perfusion sequence. (TR 4 msec, TE 1.9 msec, flip angle 10°, slice thickness 4 mm, field of view 320 - 380 mm). After transferring the images to Philips Extended Workspace, regions of interest were located over anterior, posterior gastric fundus wall and spleen parenchyma to generate signal intensity - time curves. One measurement was performed from spleen parenchyma in each pre - and postoperative stages (Figure 1, 3).

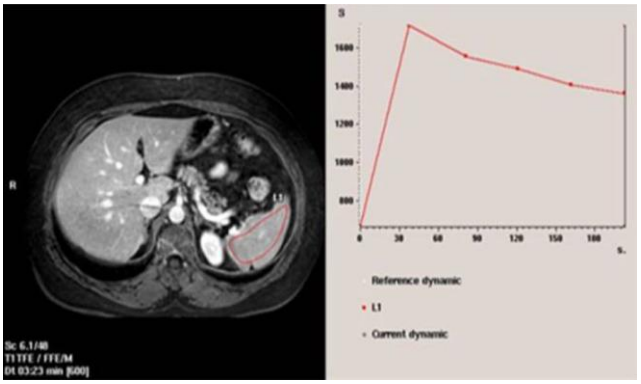

Figure 1: Preoperative measurements of the spleen with Dynamic MRG

A single measurement was performed from gastric fundus wall in preoperative stage (Figure 2), and two measurements were done from the anterior and posterior wall in postoperative stage (Figure 4). The signal intensities were calculated as arbitrary units (a).

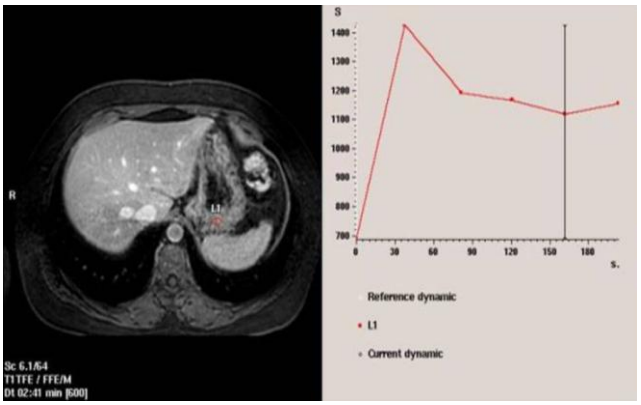

Figure 2: Preoperative measurements of the stomach with Dynamic MRG Image

Surgical Technique

Patients were placed in a Trendelenburg position following general anaesthesia. The operating surgeon stood between the patients' legs while the assistant operating the camera was on the patient's right and the second assistant holding the liver refractor was on the left side of the patient.

Following the positioning of the patient in 20 degrees reverse - Trendelenburg position, a 10mm incision was made approximately 3 cm above the umbilicus, and CO_2 insufflation into the abdomen was performed with a Veress needle. A 10 mm - port was introduced through the incision when the abdominal pressure reaches 14 mgHg. Then, two 5 mm trocars from both subcostal regions (ports that surgeon worked), a 10 mm trocar from the right side of the abdomen for the liver refractor and another 10mm trocar from the left side of the abdomen were placed.

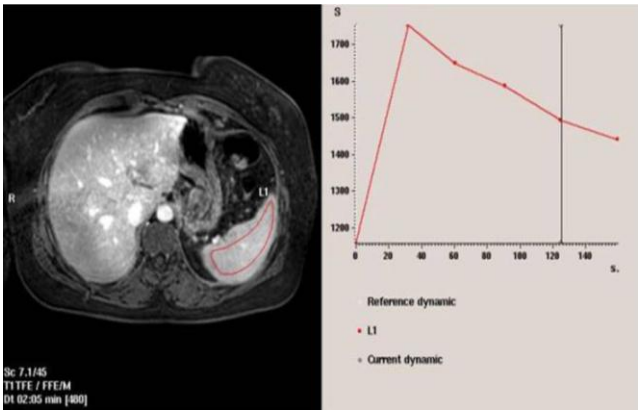

Figure 3: Postoperative measurements of the spleen with Dynamic MRG

First, the gastrohepatic ligament was opened, and the hiatus was dissected. External margins of the oesophagus, interior margins of both crura and nervus vagus were identified. Both oesophagus and nervus vagus were taken into the Goldfinger. Then, a blunt dissection in an avascular area was performed to identify crura completely. A window in which the fundus can easily pass through is opened.

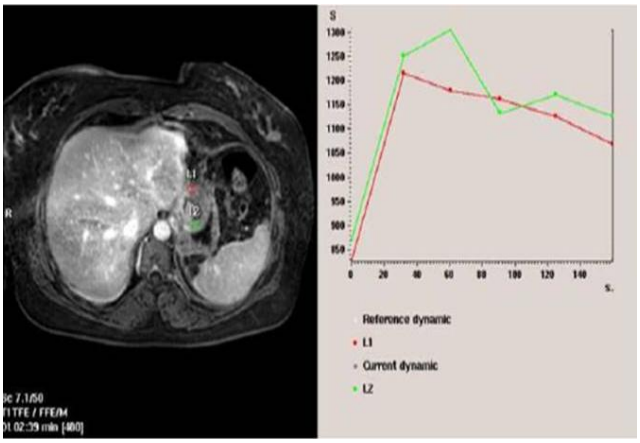

Figure 4: Postoperative measurements of anterior and posterior walls of the stomach with Dynamic MRG

The oesophagus was taken into the abdomen approximately 3 – 4 cm. After finalising hiatal dissection, Goldfinger was taken out, and GBs were transected using 5 mm - LigaSure®. The fundus is released including the left crus. Then the Goldfinger is inserted through the window that was previously opened. Crura were brought closer to each other using a 2 - 3 non - absorbable sutures and a 3 x 2 cm sized polypropylene graft (GalliniS.r.I, Mirandola, Italy) were laid out on it. The crura are fixed with a tacker. A suspending suture is placed on the fundus, and the suture is fixed to the Goldfinger. Then, it is moved through from the posterior to the anterior of the window. Thus, a 360 - degree wrap is completed. This position is fixed with three sutures with the first one passing through the oesophagal wall. This way, a 2 – 3 cm "floppy" loose fundoplication was completed. The fundal part was fixed to the graft with one suture.

The Patients started began oral nutritional intake the next day. They were advised to be on a pureed food diet for one month. On post - operation day 15, a dynamic MRI was performed to evaluate gastric fundal and splenic circulation. On post - operation month 3, they were evaluated with an upper GIS endoscopy and a 24 - hour pH monitorization.

Table 1: Demographic characteristics

Variables	n=14
Age	48.9±8.6
Sex	
Men	8 (%57.1)
Women	6 (%42.9)
Comorbid disorders	2 (%14.3)

Statistical analyses

We used the SPSS for Windows®version 11.5 to analyse the data. A Shapiro Wilk test was used to check the normality of the continuous variables. Descriptive statistics were presented as median (minimum-maximum). We used Wilcoxon Sign test with a Bonferroni correction to test the differences between pre - and post - operation phase 1, 2, 3, four dynamic MRI measurements. For the splenic measurements, $p < 0.010$ and for gastric measurements, $p < 0.0033$ were considered statistically significant according to the Bonferroni correction.

Results

Preoperative median DeMeester score was 75.8 (minimum: 19.2 - maximum: 211.4) and it receded to 3.6 after surgery (minimum: 0.4 - maximum: 6.6). This decrease was statistically significant ($p < 0.001$) (Table 2).

Table 2: Pre - and post - operative DeMeester Scores

Time	DeMeester Score
Pre-operative	75.8 (19.2-211.4)
Post-operative	3.6 (0.4-6.6)
p-value	< 0.001

Median pre - and postoperative splenic measurements in phase 1, 2, 3, and 4 were statistically not different (Table 3).

Table 3: Pre - and postoperative Dynamic MRI measurements in each phase of the spleen

Phases	Pre-op (a)	Post-op (a)	the
Phase I	1962,5 (1174-2697)	2273 (1172-2874)	0.245
Phase II	2098 (1279-2836)	2105 (1153-3117)	0.975
Phase III	2032 (1241-2912)	2013 (1131-2894)	0.975
Phase IV	1957 (1196-2753)	1954,5 (1076-2594)	0.975

a Results were considered significant according to the Bonferroni corrected p<0.01.

Median pre - and postoperative gastric measurements in phase 1, 2, 3, and 4 were statistically not different (Table 4).

Table 4: Pre - and postoperative Dynamic MRI measurements in each phase of the stomach

Phases	Pre-op (a)	Post - op Ön (a)	the
Phase I	1582 (1126-2486)	1558.5 (663-2085)	0.363
Phase II	1791 (964-2545)	1756 (876-2351)	0.530
Phase III	1744,5 (888-2719)	1612 (996-2704)	0.272
Phase IV	1649 (794-2273)	1530 (942-2346)	0.300

Median preoperative gastric and postoperative gastric posterior wall measurements in phase 1, 2, 3, and 4 were statistically not different (Table 5).

Table 5: Preoperative gastric and postoperative posterior gastric wall Dynamic MRI measurements in each phase

Phases	Pre-op (a)	Post - op Arka (a)	the
Phase I	1582 (1126-2486)	1927.5 (1129-2529)	0.363
Phase II	1791 (964-2545)	1966.5 (1312-2903)	0.875
Phase III	1744,5 (888-2719)	1831 (1161-2286)	0.826
Phase IV	1649 (794-2273)	1761 (1094-2357)	0.975

[a] Results were considered significant according to the Bonferroni corrected $p < 0.0033$.

Postoperative anterior and posterior gastric wall measurements in phase 1, 2, 3, and four were significantly not different in Bonferroni corrected analysis (Table 6).

Table 6: Postoperative anterior and posterior gastric wall Dynamic MRI measurements in each phase

Phases	Post - op Anterior (a)	Post - op Posterior (a)	the
Phase I	1558.5 (663-2085)	1927.5 (1129-2529)	0.019
Phase II	1756 (876-2351)	1966.5 (1312-2903)	0.026
Phase III	1612 (996-2704)	1831 (1161-2286)	0.055
Phase IV	1530 (942-2346)	1761 (1094-2357)	0.008

[a] Results were considered significant according to the Bonferroni corrected $p < 0.0033$.

Discussion

Many studies have shown that ischemia and necrosis in the gastric fundus, splenic ischemia and infarcts may occur following LNF. The prevalence of these complications is reported in several studies [11][12]. In the current study, we measured gastric fundus ischemia and spleen circulation with dynamic MRI before and after LNF and evaluated the effects of these measures on postoperative complications and the choice of laparoscopic antireflux technique. It is important to consider that in our study all patients were operated by the same surgeon with the same technique and evaluated by the same observer. Unlike previous studies, Dynamic MRI was used for the first time to evaluate gastric fundus and splenic circulation in patients who underwent LNF, which was performed dissecting the GB. The development of novel MRI may provide a new opportunity for evaluating abdominal pathologies [13].

Laparoscopic Nissen fundoplication was performed by the same surgeon without any complications. In our study, preoperative median DeMeester score was 75.8 (minimum: 19.2 - maximum: 211.4), and postoperative median DeMeester score was 3.6 (minimum: 0.4 - maximum: 6.6), and there was a significant difference between pre - and postoperative DeMeester score ($p < 0.001$).Gastric necrosis following Nissen Fundoplication was first described in an 11 year - old girl who presented with gastric dilatation and was operated at the emergency room two years after Nissen Fundoplication surgery. Gastric necrosis was related to delayed gastric emptying after removing gastronomy tube and compartment syndrome [14]. Gastric necrosis and perforation after Nissen Fundoplication were described first by Patuto N. in adults. Obstruction related to small intestine adhesion 14 years after the surgery and gastric dilatation related with tight fundus wrap were thought to be responsible [15]. Experimental studies have shown that tight fundoplication wraps may cause gastric dilatation and infarcts due to the vomiting difficulties. The most dramatic outcome of the gastric dilatation is gastric necrosis, which is a life-threatening condition with 73% mortality [16][17]. Animal studies reported that the ligation of the four main arteries and 80% ligation of the small arteries do not result in gastric necrosis [15]. Disturbed intramural venous circulation due to increased intragastric pressure (over 20 to 30 mmHg) may cause gastric ischemia and rupture [16].

Kenedy T. et al. performed Nissen fundoplication and proximal gastric vagotomy to 33 patients with chronic duodenal ulcer and hiatal hernia. They detected necrosis of the lesser gastric curvature in three patients within the first week after the operation. This was related to gastric dilatation, which occurred due to swallowed air a couple of days after the operation, and subsequently distension and pressure on the lesser curvature vessels [11].

Rudolf Nissen in 1956 suggested forming a less tight gastric fundus wrap during the operation dissecting the GB to prevent extreme narrowing at the bottom of the oesophagus and postoperative dysphagia. However, dissection of GB during fundal mobilisation is still being debated. Some believe that it is necessary to dissect these vessels to have a less tight wrap and to prevent postoperative dysphagia [18]. Others, on the other hand, do not find it necessary to have a loose wrap. Moreover, they suggested that a larger dissection might increase the risk of bleeding during the operation, gastric fundus and splenic infarcts and abscess formation [19].

In a study, 99 patients with chronic GER who had an antireflux procedure with or without preserving the GB were followed for ten years. There was no statistical difference between the groups regarding heartburn, gas - bloating syndrome and burping. Life quality was evaluated with Psychological General Well - Being (PGWB) index and both groups revealed similar results [20]. The surgeon should decide to dissect the GBs during the operation. In some publications, it was shown that dissecting the GBs result in similar postoperative outcomes as in LNF without GB dissection and it was

concluded that dissecting the GBs is a safe procedure [12].

A hiatal hernia and bloating related to gas is seen more commonly in patients that underwent LNF with GB dissection. Surgeons developed the Nissen - Rosetti technique in which these vessels were preserved. Even though postoperative dysphagia was reported less in LNF with dissection of GBs when compared to the LNF without GB dissection, randomised, controlled studies are needed to confirm these findings [21].

Luostarinen ME, et al. suggested that GB dissection during LNF increases the risk of a hiatal hernia [22]. It was thought that dissecting GBs might cause loosening of the gastric fundus. Thus, the hernia may occur more easily, and hernia recurrence is increased [21]. In the last five prospective randomised studies, the beneficial effect of dissecting GB was not proven. However, many surgeons dissect GB during LNF in their clinical practice [23].

LNF with or without GB dissection, however, has been shown to be beneficial in relieving GER symptoms [24]. Wyman et al. proposed that dissecting GBs during LNF might damage the afferent vagal nerves that stimulate gastric tension receptor in the fundus responsible for burping reflex [25].

Markar S.R. et al. reviewed literatures between January 1950 - October 2009 for patients that LARP with and without GB dissections and compared them in terms of primary outcomes including need of reoperation, postoperative dysphagia, and postoperative GER secondary outcomes including duration of operation, duration of hospitalization, presence of postoperative complications, postoperative gas - bloating syndrome, postoperative resting state lower esophageal sphincter pressure, and postoperative DeMeester scores that is calculated using 24 - hour pH monitoring. There were no significant differences and heterogeneity between groups for the need of reoperation, postoperative GER, duration of hospitalisation; postoperative DeMeester score and postoperative resting state lower oesophagal sphincter pressure. However, duration of operation was significantly longer in patients who received LNF with GB dissection. There were no significant differences and heterogeneity for postoperative complications between the groups. They did not detect any significant differences in gas - bloating syndrome, but a difference in heterogeneity was detected [12].

In our study, we showed that dissecting GBs during LNF does not affect gastric fundal circulation as evaluated with dynamic MRI.

Splenic bleeding and infarcts were also described in previous publications [18]. Splenic infarctions have been reported commonly in the laparoscopic surgery and prevalence was generally < 1% [18][26].

An occlusion or injury in the peripheral splenic artery that has limited collateral circulation may generate ischemia or infarct areas in the splenic parenchyma [19]. Cardiac emboli or hypercoagulability are considered to be an underlying factor for occlusion [27].

In total, 30% of the patients with splenic infarcts are asymptomatic, and they may develop splenic rupture or abscess. Rarely, the whole spleen may have infarct [28]. In the short term, haemorrhage can be seen, but in the long term, it is healed by fibrosis [27]. In our patient group, none of the patients had postoperative splenic ischemia or infarct.

Damaging or ligating risk of the splenic vessels is high in LNF and laparoscopic sleeve gastrectomy (LSG) with GB dissection as they are located in the posterior field of the surgical area. In that case, splenic ischemia or infarct might occur. Splenic infarct has been reported in some case reports [18][25], and in a study of 1600 patients, it was seen in < 1% of the patients [26]. Stamou KM et al. reported splenic infarcts as 4.1% in 287 patients who had laparoscopic sleeve gastrectomy. Splenic infarcts are recognised by observing the colour alterations in the spleen during the operation or with USG and BT postoperatively [19][29].

Similarly, we believe that ischemia and infarcts following LNF occur due to the dissection or ligation of the splenic artery and its branches after dissection of the GB. In the current study, no statistical differences between pre - and postoperative measurements of gastric fundus and splenic circulation with dynamic MRI were detected.

To the best of our knowledge, effects of the dissection of GBs during LNF on gastric and splenic perfusion have not been tested with dynamic MRI. We did not find any significant difference regarding perfusion between pre - and post - operative measurements. However, small sample size is a limitation in the current study. More studies with bigger sample sizes are needed. In the light of our current findings, we believe that LNF with GB dissection has no effect on the gastric and splenic perfusion and it is a relatively safe technique for GERD.

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Clinical Science

Comparative Clinical Efficacy between Electrodesiccation with Curettage and Application of 80% Phenol Solution in Treatment of Common Warts

Dina Arwina Dalimunthe*, Remenda Siregar, Chairiyah Tanjung

Department of Dermatology and Venereology, Faculty of Medicine, Sumatera Utara University, Medan, Indonesia

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Keywords: Common warts; Electrodesiccation; Phenol; Efficacy; Treatment outcome

***Correspondence:** Dina Arwina Dalimunthe. Department of Dermatology and Venereology, Faculty of Medicine, Sumatera Utara University, Indonesia. E-mail: dinadalimunthe15@gmail.com

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Abstract

BACKGROUND: Common warts are skin diseases caused by human papillomavirus. Several treatment modalities available for common warts, two of them are electrodesiccation with curettage and application of 80% phenol solution.

AIM: This study aims to compare clinical efficacy between these two modalities.

MATERIAL AND METHODS: Open clinical trial was conducted at Dr Pirngadi General Hospital Medan and H. Adam Malik General Hospital Medan from February to June 2013 on 17 patients with multiple common warts. Both treatments began and applied simultaneously on the same day on each patient.

RESULTS: Cure rate was higher in electrodesiccation with curettage (76.5%, 100%) compared to the application of 80% phenol solution (11.8%, 64.7%) on three weeks and six weeks of follow up. Statistical analysis showed a significant difference of common warts cure rate between electrodesiccation with curettage and application of 80% phenol solution after three weeks ($p < 0.001$) and six weeks ($p = 0.018$) of treatment.

CONCLUSION: As a conclusion, electrodesiccation with curettage has higher cure rate than the application of 80% phenol solution on the treatment of common warts. Further study is needed to find out the best concentration and time interval for application of phenol solution to improve its clinical efficacy as an alternative treatment of choice for common warts.

Introduction

Human papillomavirus (HPV) infection is very common, as most people will experience infection during their lifetime [1]. The most common manifestation of HPV infection is common warts [2]. Common warts may appear at any age [3].

Treatment of common warts aims to cure the patient's physical and psychological discomfort and to prevent the spread of infection [4]. Treatment should present no hazard to the patient and side effect should be minimal [5]. Many treatment modalities are available for the treatment of common warts, such as topical, systemic, and surgery [6].

One of surgical treatment is electrodesiccation with curettage which is the most common treatment for common warts in Medan. Some of the patients feel discomfort with this treatment because of the trauma of pain from the injection of local anaesthesia.

Topical treatment could be an alternative to avoid that discomfort, and the tools and the procedure are simpler than surgical treatment. There are several topical treatments for common warts, like salicylic acid, lactic acid and anthralin. Gibbs & Harvey (2009) and Gibbs, Harvey, Sterling & Stark (2002) show there is no the best topical treatment for common warts [7][8].

Phenol is formerly obtained from coal tar and has been using in our daily life. In low concentration, phenol can be used as an antiseptic and antimicrobial agent, while in high concentration phenol can act as acoustic agent [9][10]. Banihashemi, Pezeshkpoor, Yasdanpanah & Family (2008) found that treat common warts with 80% phenol has no significant different compared to cryosurgery [11].

The objective of this study is to compare clinical efficacy between electrodesiccation with curettage with the application of 80% phenol solution in the treatment of common warts.

Methods

Patients

This study was conducted after receiving approval from Ethical Committee of Sumatera Utara University. The open clinical trial was done at Dr Pirngadi General Hospital Medan and H. Adam Malik General Hospital Medan from February to June 2013. The sample was 17 patients with multiple common warts, older than eight years, not pregnant, not lactating, not using a cardiac pacemaker, did not have keloid history, and agreed to participate in this study. First of all, the patient must sign the informed consent. After that one of common warts of the patients was treated with electrodesiccation with curettage and another one was treated with application of 80% phenol solution.

Procedure of treatment

The treatment procedure of electrodesiccation with curettage is:

a. The patient is lying on the bed

b. Disinfection of common warts and its surrounding with povidone iodine

c. Injection of 2% lidocaine with adrenalin by infiltration procedure around the lesion, except for lesion in acral region which was done without adrenalin

d. Wait for 10 - 15 minutes

e. Electrodesiccation was done from the centre to the edge of lesion

f. Use curette to take lesion until its base

g. After clean apply gentamycin ointment

The treatment procedure of application of 80% phenol solution are:

a. Patient sit is lying on the bed

b. Application of Vaseline around the lesion using toothpick

c. Application of 80% phenol solution using a cotton bud to the lesion until the colour changes into white

d. This procedure is done once a week until lesion dismiss, maximum in 6 weeks

Follow up

Follow up is used to see clinical improvement and if there is any complication. For electrodesiccation with curettage the first follow up was done two days after the treatment, then weekly until the wound heals or maximum six weeks. Meanwhile, the 80% phenol solution treatment was followed up every week until the lesion dismisses or maximum six weeks. At the last visit, the patient was asked about the two methods of treatment for their common warts, which one they prefer and why.

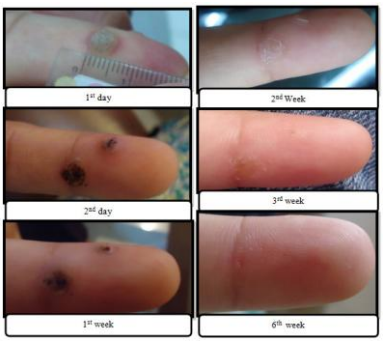

Figure 1: Treatment with electrodesiccation and curettage

Statistical analysis

Chi-square test and Fisher exact test with a level of significance 0.05 were utilised to test the significance of the difference of cure rate between electrodesiccation with curettage and application of 80% phenol solution.

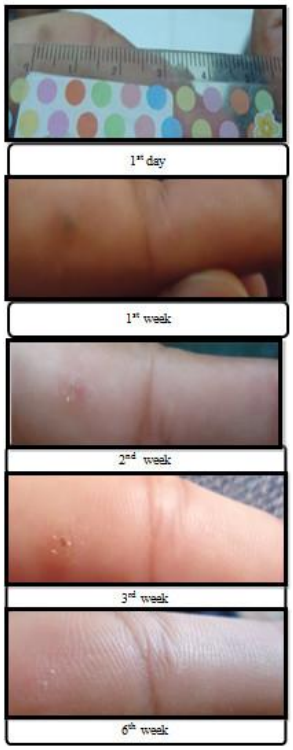

Figure 2: Treatment with 80% phenol solution

Results and Discussion

Characteristic of patient

In this study, common warts were found more on male (58.8%) than female (41.2%). It caused by low awareness of hygiene in men, and usually, they are physically more active than women, making them vulnerable to have trauma on stratum corneum.[12] Moreover, women are more concern and aware of their common warts and treated them with over – the - counter medication. In contrast, men are more unaware of their common warts because they do not cause any discomfort [13]. Al - Mutairi & Al Khalaf (2012) in Kuwait found similar result that common warts found more frequent in male (58,7%) than female (41,3%) while Bruggink et al. (2012) in Leiden found prevalence of patient with common warts more on female (58.9%) than male (41.1%) [12][14].

Table 1: Characteristic of patient

Characteristic	N	%
Sex		
Male	10	58.8
Female	7	41.2
Age (years)		
9-13	9	52.9
14-18	1	5.9
19-23	5	29.4
24-28	-	-
29-33	2	11.8

The most frequent was patient with age 9-13 years (52.9%), while the most frequent in Bruggink et al. study (2012) in Leiden was 4 - 11 years (43.5%) [14], and according to Kilkenny, Merlin, Young & Marksl (1998) in Australia the most frequent was 4 - 12 Tahun (59.0%) [15]. The incidence of common warts in this age group may be related to school attendance and exposure from peer group [16]. Transmission of HPV could be indirect via fomites [12].

Location of common warts

Common warts were found on finger hand, hand, foot, knee, elbow and ankle, the most frequent was finger (64.7%). The hands, especially fingers, are most in contact with the surroundings which increase their possibility of trauma and become the entry point of HPV infection than in other parts of the body.

Table 2: Location of common warts

Location	N	%
Finger	22	64.7
Hand	4	11.8
Foot	3	8.8
Knee	3	8.8
Elbow	1	2.9
Ankle	1	2.9

A study by Al - Muairi & Al Khalaf (2012) in Kuwait showed that most common warts are found on the hands [12]. In Bruggink et al study (2012) warts were found most frequent on hand (58.1%) [14], according to Kilkenny et al (1998) the most location of warts was upper limb (84.2%) [15], Theng, Goh, Chong, Chan & Giam (2004) in Singapore reported that hand was the most location of warts (39.1%) [17].

Relation of method of treatment and curing at the end of the third week

On follow up at the end of the third week, it could be seen that common warts that underwent electrodesiccation with curettage treatment had higher cure rate (76.5%) than warts treated with application of 80% phenol solution (11.8%). The difference was statistically significant ($p < 0.001$), which means there was different clinical efficacy between electrodesiccation with curettage and application of 80% phenol solution.

Table 3: Curing at the end of the third week based on method of treatment

Curing	Method of treatment				p
	Electrodesiccation with curettage		Apply of 80% phenol solution		
	n	%	n	%	
Cured	13	76.5	2	11.8	
Not yet cured	4	23.5	15	88.2	< 0.001
Total	17	100,0	17	100,0	

Compare to Ginting (1988) in Medan that reported the percentage of healing at the end of the third week on 39 patients whom treatment with electrodesiccation with curettage was 95%, result of this study was lower [18]. The result of this study was similar to Banihashemi et al. (2008) in Iran that reported the percentage of healing at the end of the third week on 23 patients who were treated with application of 80% phenol solution was 13% [11].

Although all patients who were treated with electrodesiccation with curettage were cured, there was hypopigmentation complication in 3 patients (17.7%). Al - Muairi & Al Khalaf (2012) in Kuwait, revealed that the side effect of electrodesiccation is erythema, blisters and hyperpigmentation [12]. On the other hand, there was no any complication on patients who were treated with 80% phenol solution.

Table 4: Curing at the end of the sixth week based on method of treatment

Curing	Method of treatment				p
	Electrodesiccation with curettage		Apply to 80% phenol solution		
	n	%	n	%	
Cured	17	100.0	11	64.7	
Not yet cured	0	0.0	6	35.3e	0.018
Total	17	100.0	17	100.0	

Perception of patient to method of treatment

At the last visit, patients were asked how their perception of the two methods of treatment, which one they prefer and what was their reason (table 5). Most patients prefer electrodesiccation over curettage because although it is more painful, the common

warts were cleared in one session, resulting in fewer visits to doctors. Some others choose to have less painful procedure despite longer duration of treatment.

Table 5: Preference of patient to method of treatment

Preference to method of treatment	n	%	Reason
Electrodesiccation with curettage	9	53.0	Practical (6) Faster cure and not repeatedly (2), Faster cure (1) Afraid of electrodesiccation because of the tools and injection (3)
Apply to 80% phenol solution	4	23.5	On electrodesiccation, patient must take care the wound not to wet for two days which not match with her works as a midwife (1)
Electrodesiccation with curettage as same as applying to 80% phenol solution	4	23.5	Not worry about applying to phenol solution repeatedly and not afraid with electrodesiccation (3) It depends on which of method of treatment available (1)
Total	17	100.0	

In conclusion, there was clinical efficacy difference between electrodesiccation with curettage and application of 80% phenol solution on the treatment of common warts, where electrodesiccation with curettage has a better result. Although electrodesiccation with curettage has higher cure rate than the application of 80% phenol solution, for patients, who feel discomfort or afraid to electrodesiccation, especially children, application of 80% phenol can be the appropriate choice.

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Clinical Science

The Kapandji Technique of Closed Reduction Using Sommer - Pins in the Treatment of Completely Dislocated Fractures of the Distal Radius in Children

Marjan Kamiloski*, Lazar Todorovik, Shaban Memeti, Lazo Jovcevski, Slavica Shuperliska, Zlatko Aleksovski

University Clinic of Paediatric Surgery, Faculty of Medicine, Ss Cyril and Methodius University of Skopje, Skopje, Republic of Macedonia

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Keywords: Kapandji; Fractures of the distal radius; Pediatric; Fractures; Surgical treatment

***Correspondence:** Marjan Kamiloski. University Clinic of Paediatric Surgery, Faculty of Medicine, Ss Cyril and Methodius University of Skopje, Skopje, Republic of Macedonia. E-mail: mkamiloski@yahoo.com

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Abstract

BACKGROUND: The fractures of the distal radius are one of the most frequent cases in the pediatric population. The primary way of treating these fractures is conservative, with manual reduction and cast immobilisation. In patients where reduction and retention of the fracture cannot be achieved, a surgical approach is the treatment of choice.

AIM: To evaluate the benefits of using the minimally invasive surgical treatment of closed reduction using Sommer - pins in the treatment of the fractures of the distal radius in the pediatric population with the method of the Kapandji technique.

MATERIAL AND METHODS: In this study, we used cases treated from 2012 to 2017, of 48 completely dislocated fractures of the distal radius in patients ages 6 -14 yrs., where the use of non-surgical treatment proved ineffective. In the surgical treatment, we used one or two Sommer - pins to achieve a correct reduction and fixation.

RESULTS: The post-op immobilisation lasted 4 - 7 weeks with an underarm cast. The patients were closely followed in the period of 6 months following the intervention. The anatomic reduction was easily achieved with this type of technique in every case. In the post-op period, there was no significant loss of reduction and another surgical procedure was not needed in any of the cases.

CONCLUSION: With the use of the closed reduction Kapandji technique, an easy and good anatomical reduction is achieved with good post-op results in the treatment of completely dislocated fractures in the distal radius in children.

Introduction

The distal radius is the most frequent location of fractures in the pediatric population - about 20 - 30% of all fractures belong to this group [1]. The main principle in the treatment of fractures in the pediatric population is to use a non - invasive, conservative approach to the treatment of the fractures of the distal radius. In the cases where we have a non - optimal reposition of the fragments of the fracture, surgical or semi-conservative techniques are used to treat these fractures.

The achievement of a good reposition of the fracture can prove difficult in the completely dislocated pediatric fractures of the distal radius (PDR) using a closed reduction technique which consists of manually manipulating the fracture. It has been shown that the traction is especially non – efficient [2], when there is an intact ulna or a “greenstick” ulnar fracture [3], because of which completion of the periosteal “greenstick” ulnar fracture is suggested [4]. Several authors have identified the factors of risk for dislocation of the fractures of the distal radius which can be categorised in two categories: primary and secondary causes[5][6][7][8]. The **primary cause** includes: ages of more than 9 yrs., a complete dislocation to begin with, a translation of the fracture of more than 50%, an angulation of more than 20^0, an oblique fracture line, comminuting, a bayonet fracture with dorsal positioning accompanied with ipsilateral distal ulnar fractures. The **secondary causes** include the inability to achieve a primary perfect reduction, a suboptimal technique of cast immobilization with a cast index larger than 0.8,

additional reduction maneuvers and reduction in sedation or using a hematoma block, instead of full anaesthesia.

The intrafocal technique of Kapandji is a well - established method of reduction and fixation of the distal radial fractures in the adult population, and the authors of this study have used it during their practice in this institution for many years. Because of its simplicity, and effectiveness, the authors of this study have used the same technique for the treatment of the completely dislocated fractures of the distal radius in the pediatric population [9].

Our study aims to evaluate the benefits of using the minimally invasive surgical treatment of closed reduction using Sommer - pins in the treatment of the fractures of the distal radius in the pediatric population with the method of the Kapandji technique.

Material and Methods

This is a prospective study of the treatment of 48 pediatric patients, presented with a completely dislocated, closed fracture of the distal radius with or without an accompanying ulnar fracture, after a non - optimal reposition of the fragments at the University Clinic of pediatric surgery in Skopje, in the period from 2012 - 2017. All of the 48 patients included in the study were treated surgically. The surgical method used was the Kapandji technique of closed reduction using Sommer - pins. In every patient, a pre-op and post-op AP and lateral X - rays were made. An informed agreement for the surgical procedure (The Kapandji technique) was given by the parents/guardians in every case included in the study.

Surgical technique

After adequate anaesthesia, the arm was positioned on the radio - translucent table (Figure 1, 2).

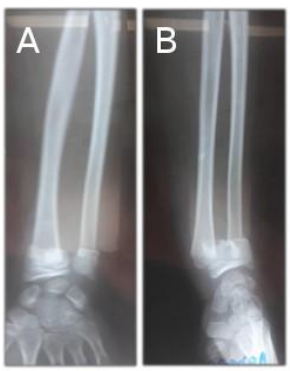

Figure 1: The preoperative anteroposterior (A) and lateral X-rays of a completely dislocated fracture of the distal radius in a child (B)

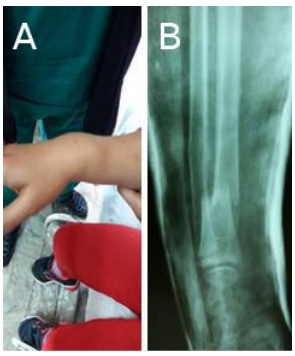

Figure 2: Photo of the presentation of the fracture (A) and an initial X-ray of the fracture (B)

To achieve a full radial length, gentle traction and contra-traction were used. In all of the cases, the distal fragment was posteriorly moved. The Sommer pin was manually introduced at the site of the fractured form the posterior aspect (Figure 3). A 2.2 mm Sommer pin was used in the smaller children, while a 2.8 mm Sommer pin was used in the larger patients.

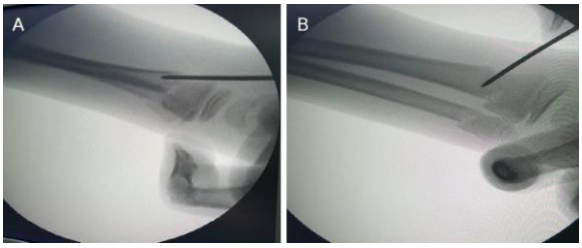

Figure 3: (A) Lateral and (B) AP presentation after the introduction of the Sommer – pin

The preoperative anteroposterior (A) and lateral X - rays of a completely dislocated fracture of the distal radius in a child (B).

The Sommer-pin was introduced trough the distal fragment in the site of the fracture towards the proximal fragment. The posterior cortex of the proximal fragment was elevated posteriorly with a manual borer for pinning reducing the posterior cortex of the distal fragment (Figure 4).

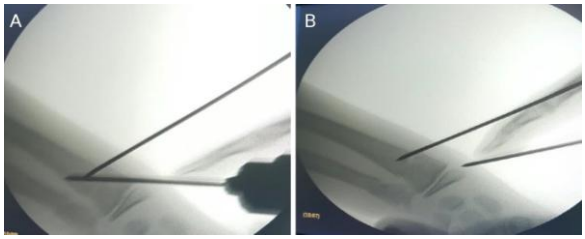

Figure 4: An x-ray in which the reduction of the proximal (A) and distal fragment (B) of the fracture was made using Sommer - pins

An x-ray in which the reduction of the proximal (A) and distal fragment (B) of the fracture was made using Sommer – pins.

After the posterior cortexes of the fragments have been aligned, the Sommer - pin was forced obliquely across the fracture so it could reach the anterior cortex of the proximal fragment. After this, a drill was placed on the Sommer - pin which later is introduced across the anterior cortex, stabilising the fracture. The underarm was in full pronation and then checked on an AP and lateral x-ray (Figure 5). If the fracture was reduced, as was in most of the cases, the extra - focal Sommer - pin has passed through the lateral side or from the distal lateral to the proximal medial side of the radial styloid processes, or has passed above the physical line, or form proximal lateral position to distal medial.

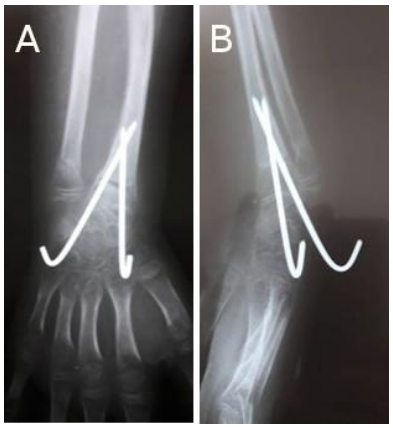

Figure 5: An X-ray of the anteroposterior (A) and lateral (B) presentation after the fixation with the Sommer - pins

An X-ray of the anteroposterior (A) and lateral (B) presentation after the fixation with the Sommer - pins.

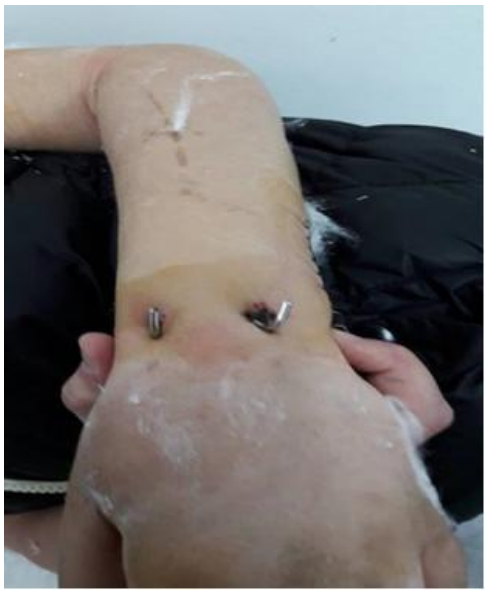

Figure 6: A clinical presentation of the introduction site of the Sommer - pins through the skin

If there was a residual lateral translation/dislocation of the distal fragment in the AP presentation, another intra - focal Sommer - pin was introduced trough the lateral site of the fracture (Figure 6). If the child was admitted during the night shift, it was released from the hospital the next day. A control x-ray in lateral and AP presentation were obtained before releasing the child. The first control X-ray was done on the first post-op day, then control X - rays were obtained according to the age of the child.

After 3 to 6 weeks, control X-rays were obtained. After the results were optimal, the extraction of the Sommer pins was done as an outpatient procedure. We used a compressive bandage, and the mobilisation of the wrist began after the extraction of the Sommer pins. The patients were followed at least three months post-op.

Results

In this study are presented our starting results of 48 patients with completely dislocated fractures of the distal radius treated with the minimally invasive surgical approach of the Kapandji technique.

The age of the patients was 6 - 14 yrs., out of which 26 were male, 22 female. According to the mechanism of injury – 14 patients were injured after falling from a height, while most were sports injuries, or injury during play – 34 patients (Table 1). The average age was 9.8 yrs. The average time of the procedure of reduction and fixation with the Sommer - pins was 19 minutes. The combination of intra and extra - focal Sommer - pins were used and adjoined ulnar fractures were observed in 21 patient. The results were based on the function of the wrist (Figure 7). The normal pronation/supination was defined as 90/0/90 degrees (A), the normal flexion/extension as 30/0/30 degrees (B), while the normal radial flexion as 20/0 degrees and the normal ulnar flexion as 0/30 degrees (C). The control group consisted of children with a healthy non - injured wrist.

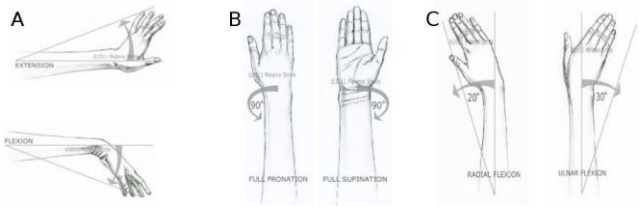

Figure 7: Function of the wrist

The evaluation of the results was done six months after the intervention, and in our study, we used the “Mayo wrist score” system for evaluation of the function of the wrist with analysis of several parameters: mobility, the strength of grip, level of satisfaction and pain (Figure 8).

Category	Score	Findings
Pain (25 points)	25	No pain
	20	Mild pain with vigorous activities
	20	Pain only with weather changes
	15	Moderate pain with vigorous activities
	10	Mild pain with activities of daily living
	5	Moderate pain with activities of daily living
	0	Pain at rest
Satisfaction (25 points)	25	Very satisfied
	20	Moderately satisfied
	10	No satisfied, but working
	0	No satisfied, unable to work
Range of motion (25 points)	25	100% percentage of normal
	20	75~99% percentage of normal
	10	50~74% percentage of normal
	5	25~49% percentage of normal
	0	0~24% percentage of normal
Grip strength (25 points)	25	100% percentage of normal
	15	75~99% percentage of normal
	10	50~74% percentage of normal
	5	25~49% percentage of normal
	0	0~24% percentage of normal

Final result (total points)	
90~100	Excellent
80~89	Good
65~79	Fair
<65	Poor

Figure 8: "Mayo wrist score."

With the method of lifting by Kapandji, an anatomical or almost anatomical reduction of the fracture was achieved in every case. Open reduction was not used in any of the cases. The post-op period in each of the 48 patients, went without any complication.

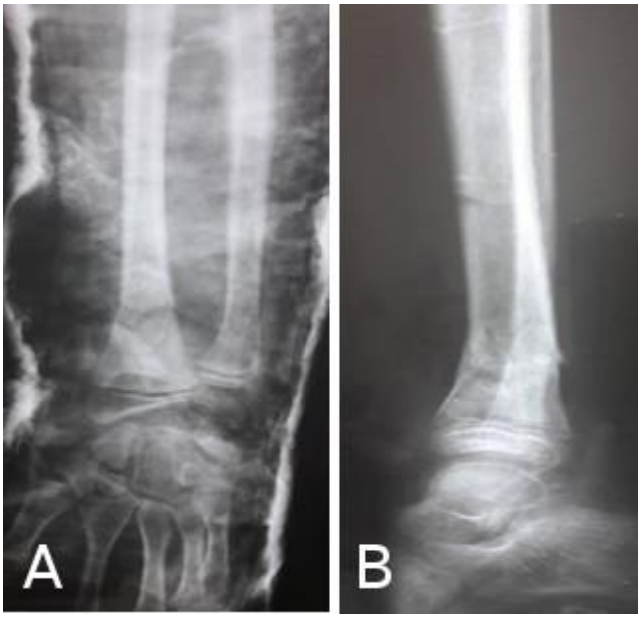

Figure 9: A postoperative anteroposterior (A) and lateral X-ray (B), showing the healed fracture of the distal radius

The X-ray signs of consolidation were seen after four weeks, and the treatment of the fractures was fully completed three months after the injury, with the extraction of the pins after 8 - 18 weeks. In each patient, a full clinical and radiologic healing was obtained with a normal range of motion of the wrist. On the final X-ray, just before the extraction of the Sommer pin, there has been no recorded angular translation or angulations.

All the fractures have healed fully (Figure 9). The cosmetic effect was excellent after the cast was removed in all the cases. All of the patients achieved full flexion and extension of the wrist (Figure 11).

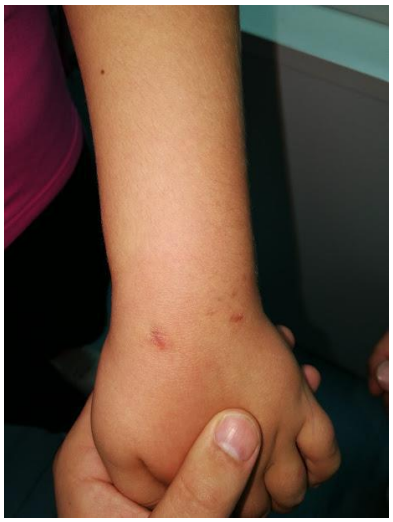

Figure 10: A good cosmetic effect

The average length of time needed to achieve a full range of motion after the initial immobilisation was four weeks (range 2 - 5 weeks). There was no loss of reduction or re-manipulation. No complications with the pins or the cast were noted.

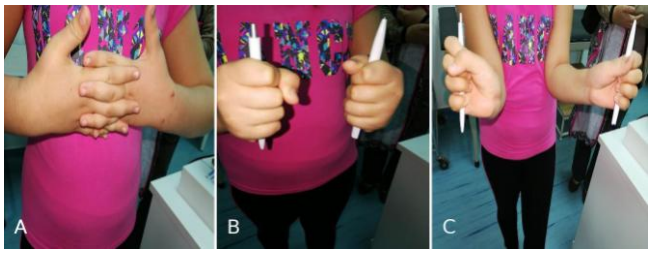

Figure 11: Good flexion and extension of the wrist

Discussion

There are three methods for the treatment of the completely dislocated fractures of the distal radius in children: a careful manual repositions without optimal reduction of the fracture and cast immobilisation without the use of anaesthesia, a closed reduction and cast immobilisation with the use of anaesthesia, and closed reduction and pinning with the use of anaesthesia.

The closed reduction is usually reserved for cases of late treatment or loss of initial reduction with complete angulation. The completely dislocated fractures of the distal radius are with the risk of new reposition and reduction after the initially closed manipulation and cast immobilisation. Despite the overall good functional and radiological results, on the long run, in most of the non - reduced fractures of the distal radius, the loss of reduction is considered a real problem.

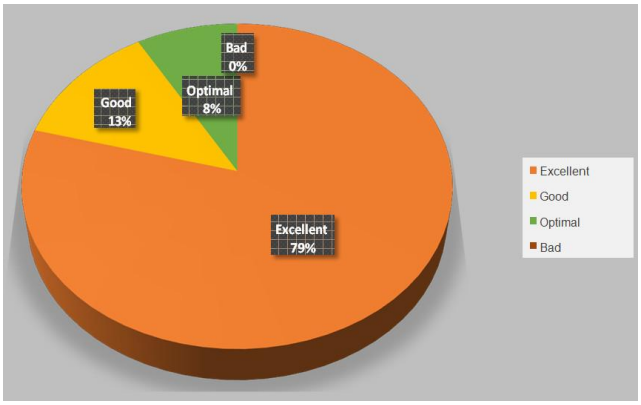

Figure 12: Display results according to the "Mayo wrist score" scoring system for evaluation

Some factors that have to be considered before electing the optimal method of treatment are the age of the child, the seriousness of the initial angulation or an angulation after a secondary displacement which might be accepted for a given patient, the length of time needed for the remodeling and another intervention if needed, and weather the second intervention can give the same result as the primary intervention, as well as the full length of treatment.

Table 1: Statistical analysis of clinical data

Age	Sex	Associated fractures	Mechanism of injury	Pin extraction in weeks	Functional results according to the Mayo wrist score
10	M	Ulna	Fall from bike	10	Excellent
10	F		Fall from rollers	11	Excellent
11	F	Ulna	Fall from sling	15	Excellent
9	F		Fall from sling	14	Excellent
12	M		Fall during football	15	Excellent
10	M		Fall from bike	13	Excellent
9	F		Fall from bike	9	Excellent
10	M	Ulna	Fall from trampoline	10	Good
10	M	Ulna	Fall from ladder	13	Excellent
7	F		Fall while skating	12	Excellent
8	F		Fall from bike	18	Optimal
10	M		Fall from trampoline	14	Good
12	M	Ulna	Fall from ladder	9	Excellent
11	F		Fall while skating	16	Excellent
13	F		Fall during football	11	Excellent
8	F		Fall from bike	17	Excellent
11	M	Ulna	Fall from trampoline	11	Optimal
9	M	Ulna	Fall from ladder	13	Excellent
12	M	Ulna	Fall while skating	8	Good
10	F	Ulna	Fall from trampoline	10	Excellent
11	F		Fall from ladder	15	Excellent
13	F		Fall while skating	14	Excellent
9	M		Fall from trampoline	9	Excellent
12	F	Ulna	Fall from trampoline	18	Excellent
11	M	Ulna	Fall from ladder	14	Excellent
10	F		Fall while skating	13	Excellent
9	M		Fall during football	10	Good
11	F		Fall from bike	15	Excellent
14	F	Ulna	Fall from ladder	18	Excellent
8	M	Ulna	Fall from trampoline	14	Excellent
10	M		Fall from ladder	12	Excellent
6	F	Ulna	Fall while skating	15	Excellent
8	M	Ulna	Fall during football	18	Good
9	M		Fall during football	17	Excellent
13	F		Fall during football	12	Optimal
7	F	Ulna	Fall from bike	11	Excellent
9	M		Fall while skating	18	Excellent
9	M		Fall during football	16	Excellent
9	F		Fall from ladder	16	Excellent
10	M	Ulna	Fall from sling	12	Excellent
11	M		Fall from rollers	10	Excellent
6	M		Fall from ladder	13	Optimal
7	F		Fall from trampoline	18	Excellent
14	F	Ulna	Fall while skating	18	Excellent
12	M	Ulna	Fall from bike	16	Good
9	M	Ulna	Fall from trampoline	15	Excellent
9	M		Fall while skating	14	Excellent
7	M	Ulna	Fall from ladder	14	Excellent

Even though the achievement of an optimal closed reduction with any used technique is the unified first step in the treatment, the other thing that must be highlighted is the use of the technique which allows the best post-op reduction that will last during the period of healing. Well-Placed cast immobilisation on three points and the percutaneous application of the Sommer pins are two options that are proven to keep a good stabile reduction of the fracture while healing. Although a perfectly modelled cast is required, that might not be possible because of inappropriate and sufficient application of gauze, a too quick or maybe even a prolonged manipulation of the cast itself, swelling of the soft tissues, or the use of suboptimal anaesthesia. An above average swelling can be present in the initial exam of the fracture, because of trauma under high velocity, an accompanying ulnar fracture primary immobilisation that was never achieved right after the injury. The swelling can increase after later forced manipulations with the fracture, especially in cases of prolonged presentation. The reduction of the swelling several days after the initial cast immobilisation is placed can result in a concomitant dislocation of the initially achieved reduction.

In one prospective randomised trial of over 100 fractures of the distal radius in adults treated with the application of Sommer pins, Sthorm et al., [10] have found the functional and radiographic results of the Kapandji method used and proved that they are much better than the conventional (Willenegger) technique. The conventional extra - focal Sommer - pins in completely dislocated distal radial fractures can be introduced only after achievement of a satisfying reduction.

The authors of this study had done only two post-op X-ray exams, one right after surgery, the other when the Sommer pins were extracted. After extraction, they have conducted only two controls, the first after 2 - 3 weeks, the second after 4 - 6 weeks after extraction. During the first control exam, the authors were satisfied with the cosmetic effect. On the second exam, the majority of patients had already achieved full range of motion of the wrist, which was an indicator of full recovery. Using the Kapandji technique which is a method of closed fracture reduction, the force used is applied in full effect without traumatising the soft tissues surrounding the fracture. Many orthopaedic surgeons agree that the majority of distal radius fractures could be treated with minimal or no cosmetic and functional abnormalities, especially in the pediatric population.

According to our results, in the treatment of the dislocated fractures of the distal radius which are by the results by other relevant authors, we can safely recommend the minimally invasive Kapandji technique as a method of choice when treating this type of fractures.

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Clinical Science

Prevalence of Coxitis and its Correlation with Inflammatory Activity in Rheumatoid Arthritis

Ismet H. Bajraktari[1], Blerim Krasniqi[2], Sylejman Rexhepi[1], Sadi Bexheti[3], Elton Bahtiri[4*], Halit Bajraktari[5], Besim Luri[1]

[1]Clinic of Rheumatology, University Clinical Center of Kosovo, Prishtina, Kosovo; [2]University of Medical Sciences "Rezonanca", Prishtina, Kosovo; [3]Institute of Anatomy, Faculty of Medicine, University of Prishtina, Prishtina, Kosovo; [4]Department of Pharmacology, Faculty of Medicine, University of Prishtina, Prishtina, Kosovo; [5]Emergency Center University Clinical Center of Kosova, Prishtina, Kosovo

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Keywords: Rheumatoid Arthritis; Coxitis; CRP; ESR; Anti CCP; DAS - 28

***Correspondence:** Elton Bahtiri. Department of Pharmacology, Faculty of Medicine, University of Prishtina, Prishtina, Kosovo. E-mail: elton.bahtiri@uni-pr.edu

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Abstract

BACKGROUND: Rheumatoid arthritis (RA) is an autoimmune inflammatory disease characterised by intra-articular and extra-articular manifestations but very rarely with coxitis.

AIM: This study aimed to investigate the prevalence of coxitis, clinical changes, and its correlation with the parameters of inflammatory activity.

METHODS: A cohort of 951 patients diagnosed with ACR/EULAR (American College of Rheumatology/European League against Rheumatism) 2010 criteria was enrolled in this prospective, observational and analytic research study. The CBC (Complete Blood Count), ESR (Erythrocyte sedimentation rate), CRP(C - reactive protein), Anti CCP (Antibodies to cyclic citrullinated peptides), X-ray examination of palms and pelvis, and the activity of the disease as measured by DAS - 28 (28 - joint disease activity score) were carried out in all subjects. Independent samples t-test was used to compare the group's characteristics, whereas Pearson correlation test was used to analyse the correlation between study variables.

RESULTS: Of the total number of the subjects, 730 (76.8 %) were females, whereas 221 (23.2%) were males. The average age was 51.3, y/o while the most of them were between 40 - 49 y/o (32.6%). The prevalence of coxitis was 14.2%, mostly found in males (19.46%). The echosonografic prevalence of changes was 21.45%, while the radiological changes were 16.3%; in both cases, the changes were more expressed in males. The analysis showed that inflammatory parameters were significantly higher in patients with coxitis.

CONCLUSION: Coxitis has high economic cost because it ends up with a mandatory need for a total hip joint prosthesis. Thus the results of this study can serve to plan and initiate early preventive measures.

Introduction

Rheumatoid arthritis (AR) is an autoimmune chronic inflammatory disease which is characterised by small and large joint polyarthritis which over the time can lead to invalidity. It is spread all over the world, and it can affect all races, both genders and all age groups, and it has an incidence rate that ranges from 0.5 - 1% of the total population [1]. The onset of disease may be slow (the more frequent form) or rarely fast [2]. The disease has articular and extra-articular systemic manifestations [3]. The disease is not related to vocation, social status, nationality, religion or level of education [4].

Coxitis starts with restrictive pain in the hip joint which radiates to the knee. The pain is expressed more in the external and internal rotation, and those movements are hard to execute. Anteflexion and retroflexion among those patients are limited, and therefore their steps are short and slow. Movement restrictions are also found in adduction and abduction. The examination tests for hip joint are positive [5].

In the terminal phase of the disease, the patients have severe pain and large limitations as they can move only with the help of a second person or

with crutches. High activity of the disease as measured by DAS – 28 is also an important factor for hip affection [6]. The damage is caused not by the only disease itself, but also from the use of the glucocorticoids which can cause the osteonecrosis of femur head [7].

Due to migration or displacement of the femoral head from the process of inflammatory synovitis, the acetabular protrusion is presented and the dislocation of the femur head that can be measured with delta angle [8]. Progression of damage may be faster in time and can be measured [9]. Osteoporotic fractures can be presented in this pathology, and they can pose a threat to patient's life [10]. Diagnosing and monitoring the changes in coxal articulation with ultrasound during rheumatoid arthritis is an excellent and irreplaceable clinical method [11]. On average 3 - 5 years from the onset of coxitis, it is necessary to perform the prostheses implantation by an orthopedist (arthroplasty joint).

The main purpose of this study was to investigate the prevalence (clinical, echosonografical and radiological) of coxitis in RA. Also, we aimed to investigate its correlation with the inflammatory activity parameters. The specific objectives of the study were to investigate the prevalence of coxitis based on gender and age, to investigate the results of inflammatory parameters in patients with and without coxitis, and if there are any changes in clinical manifestations and radiologic findings according to gender in patients with RA.

Material and Methods

In this prospective study were included 951 patients that were treated in Rheumatology Clinic (inpatients and outpatients) during period January 2012 – December 2016 through the descriptive, investigative and analytic method. Patients are diagnosed with RA according to ACR - EULAR 2010 criteria.

Every patient is examined for complete blood count, ERS, CRP, RF, Anti CCP, pelvic X-ray (Philips Bucky Digital Diagnose apparatus), coxo-femoral articulation echo sonography (Sonoscape S 40), and when these methods were not clinically definitive for diagnose, we moved on to MRI of coxofemoral articulation (GE Signa HDe 1.5T MRI) or CT scan (Siemens Biograph 6 PET/CT).

Touching of coxofemoral joint is marked as positive to: pain in external and internal rotation, anteflexion and retroflexion, adduction and abduction, limitation of these movements, synovitis (echo of art. coxae), impossibility of walking and sitting, as well as radiological changes: erosion, narrowing of articular space, protrusion, subluxation and other changes. Also, the activity of disease is measured with DAS - 28.

In the research are not included patients with the degenerative disease, periarticular rheumatism, infection coxitis, palindrome rheumatism, and those with congenital or acquired pathology of the hip joint. All patients were informed and agreed to be part of our manuscript. The research is approved by the local ethical committee.

Statistics

Statistical processing was performed with SPSS 20.0, 2:03 SigmaStat, SigmaPlot 2000 and Excel 2010. From the statistical analysis we drew a descriptive analysis, and from statistical parameters, we have determined the structure index, arithmetic average, standard deviation, standard error, and the confidence interval with reliability 95% (95% CI). The data are presented in tables and graphs.

A t-test of arithmetic averages was used for parametric data with a normal distribution of variables, while the Mann - Whitney Rank Sum Test was used for variables with non-normal distribution. Pearson's correlation test was used to test the correlation between study variables.

Results

In our research, we have analysed 951 patients with duration of illness ranging 1 to 18.2 years, with the average of morbidity of 4.85 years.

The majority of patients belonged to the group age 40 - 49 (32.6%) and 50 - 59 years old (24.5%), who altogether made up nearly 2/3 of all patients, and the least number of patients were of group age 70 - 79 years old (8.6%) (Table 1).

Table 1: Demographic data, distribution of group age by gender and average age by gender

	Females		Males		Total	
	Nr.	(%)	Nr.	(%)	Nr.	(%)
Frequency*	730	76.8	221	23.2	951	100.0
Group age						
30-39	120	16.4	31	14.0	151	15.9
40-49	237	32.5	73	33.0	310	32.6
50-59	189	25.9	44	19.9	233	24.5
60-69	122	16.7	53	24.0	175	18.4
70-79	62	8.5	20	9.0	82	8.6
Age, MEAN (SD) years **	51.0	(11.4)	52.4	(11.7)	51.3	(11.5)

* Chi-test (F vs. M) = 8.047, df =4, (P = 0.09); ** Mann-Whitney Rank Sum Test.

The prevalence of coxitis was 14.2% in the study population, mostly found in males (19.46%), with the prevalence of echosonografic of changes of 21.45%, and of radiological changes of 16.3%; in both cases, the changes were more expressed in males (Table 2).

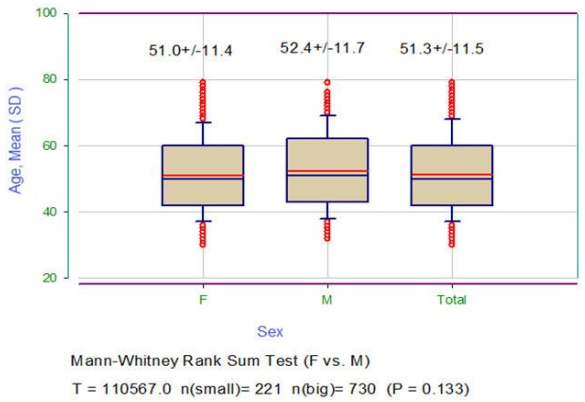

Figure 1: Patients age by gender

The patients with coxitis had a significantly higher level of all parameters of inflammatory activity (p<0.001) compared to those without coxitis (Table 3).

Table 2: Coxitis prevalence, its radiologic, echosonographic, topographic changes and localization

Prevalence of clinical, radiological and echosonographic changes		Female (n=730) Nr. (%)	Male (n=221) Nr. (%)	Total (n=951) Nr. (%)
Coxitis prevalence by gender		92 12.6	43 19.4	135 14.2
Prevalence of echosonographic changes in art. coxae		140 19.18	64 28.96	204 21.45
Prevalence of radiological changes in art. coxae.		92 12.6	44 19.9	136 14.3
Localization of coxitis	right	58 7.94	18 8.14	76 7.99
	left	42 5.75	17 7.7	59 6.20
Topographic-radiologic changes in art.coxae measured by delta angel	Cranio - lateral subluxacion	56 7.67	22 9.95	78 8.20
	Acetabular protrusio	42 5.75	16 7.24	58 6.1

The results of Pearson's correlation analysis between the study variables are shown in Table 4, where it can be seen that all parameters of inflammatory activity are positively correlated with the clinical manifestation of the coxitis, the radiological changes as well as the mode of onset of the disease.

Table 3: Laboratory characteristics of patients with rheumatoid arthritis according to presence or absence of coxitis (Independent samples compared with t-test)

	Performed analysis	All patient	Patient with Coxitis	Patient w/o Coxitis	Value of P
CRP (mg/dl)	N = 943	26.07 ± 22.00	48.18 ± 25.20 (n =134)	22.41 ± 15.15 (n=809)	< 0.001
SE (mm/h)	N = 951	45.77 ± 36.00	79.38 ± 36.76 (n=135)	40.20 ± 25.28 (n=816)	< 0.001
Anti-CCP (U/dml)	N = 575	137.83 ± 84.00	275.25 ± 171.75 (n=97)	109.94 ± 123.18 (n=478)	< 0.001
Nr.(%) of patient positive in RF and WR	N = 951	N = 734 (77.2%)	N = 123 (91.1%)	N = 611 (74.9%)	< 0.001
Nr.(%)of patient on fast on set	N = 951	N = 227 (23.8%)	N = 102 (75.6%)	N = 125 (15.3%)	< 0.001

Discussion

Coxitis in RA is understudied, even when its presence causes a highly functional disability which can fast lead to disability. Surprisingly, even the few publications currently available are clinical case reports; surfing on Pub Med, we could not find any research on the prevalence of coxitis in RA.

Table 4: Correlation of Pearson analysis between laboratory parameters, clinical and radiologic manifestation and the onset of disease in patients with RA (r)

	Clinical manifestation of coxitis	Radiological changes in art.coxae	Mode of beginning of the disease
CRP (mg/dl)	0.469**	0.317**	0.702**
SE (mm/h)	0.150**	0.326**	0.688**
Anti-CCP (U/dml)	0.424**	0.261**	0.606**
RF or WR positive	0.135**	0.698**	0.113**

*p<0.05; **p: 0.01; CRP Protein C reactive; SE (ESR) erythrosedimentation rate; Anti CCP - Anti cyclic citrulinated peptide, RF rheumatoid factor: WR Waler Rose.

This clinical condition of patient – is crucial for dynamic function, has not received the proper attention of scientific research yet due to a "trap" caused from rarely touching data of hip joint and difficulties in the examination (in the past) of this joint compared to other joints. The results of our research prove that coxitis has not such low prevalence, thus should be clinically evaluated to maintain the motoric function of movement in patients. This finding is more expressed in men than in women and is more frequent in old group ages (Senile Rheumatoid Arthritis), compared to other group ages, and there is an important statistical significant difference between the high inflammatory parameters in patients with and those without coxitis. There is also a positive correlation between laboratory parameters, clinical and radiological manifestations and the quick start of the disease.

When calculating the prevalence and incidence of RA which is high (affects about 1% of world population) appears that coxitis is a big socio-medical problem. There are some studies that have compliance with the findings of our research in every element. Coxitis and its clinical features have been researched by authors Bourqui M, Gerster JC in 20 patients and they have noticed that more than half of patients with this pathology within a short time must undergo surgery because functional impediments while walking were significant [12]. Author Pučar studied the prognosis of coxitis in 81 patients with Rheumatoid Arthritis, by analysing the opening angle of the acetabulum with X - rays, and he found that there is a statistically significant increase in millimetres of this angle [13].

Nagao Y et co. investigated radiographic methods for measuring the angle of inclination in the acetabulum and noticed that the reduction of this angle is a precursor of injuries of movements in art coxae [14]. We have explored some scientific researchers by patients treated with total hip joint Arthroplasty. Since 1990 many studies are developed for surgical treatment correction of large joints damaged by RA and now this procedure is part of patient's treatment [15]. With this achievement, the global orthopaedic community has increased the quality of patient's life significantly with rheumatoid

arthritis, and these methods are constantly improving [16].

Australian authors have investigated the reasons for the deployment of the femoral head of total hip joint arthroplasty, and rheumatoid arthritis has been the second cause of their clinical findings [17].

However, before undergoing surgical treatment, patients with RA should undergo a thorough clinical assessment because these methods despite the great advances have their complications that must be taken into consideration [18-19].

Finally, it is a great fortune for patients with rheumatoid arthritis that after a total hip joint arthroplasty they have rare complications compared to those with rheumatoid arthritis [20].

In conclusion, rheumatoid arthritis is a destructive inflammatory disease of joints, including the hip joint. Prevalence of coxitis is higher in males with a higher prevalence in older age groups. Prevalence of echosonographic changes is higher than the radiologic ones, whereas the prevalence of clinical changes is the lowest. Craniolateral subluxation is more frequent than an acetabular protrusion. There is an important statistical significance between inflammatory parameters, fast onset of RA and development of coxitis. As coxitis has a high impact on the health care system education of the patients about the disease activity plays a key role in the prevention of coxitis and its consequences.

Results from the research for the prevalence of coxitis can serve as important data for calculating some patients in need for Arthroplastic hip joints. These data can serve as a planning tool for the Ministry of Health as well as for planning the needs for arthroplastic hip joints in orthopaedics and traumatology clinics in the Balkan region and beyond because the incidence and prevalence of rheumatoid arthritis in the region are assumed approximately the same.

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Clinical Science

Prevalence of Depression Symptoms in Diabetes Mellitus

Salih Eker*

Family Medicine, Adnan Menderes Street, Health Street 195, 54100 Adapazarı / Sakarya, Turkey

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***Correspondence:** Salih Eker. Şirinevler Family Medicine, Adnan Menderes Street, Health Street 195, 54100 Adapazarı/Sakarya, Turkey. E-mail: salihekerdr@hotmail.com

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Abstract

BACKGROUND: Diabetes mellitus (DM) is one of the most prevalent diseases all over the world. Prevalence of DM in Turkey is 13.7%. Depression is another condition which has a high prevalence. All over the world, an estimated 300 million people of all ages suffer from depression. The relevance between depression and DM is a well - known condition.

AIM: We aimed in this study to find out the prevalence of depression symptoms for DM in an attempt to better manage the disease.

METHODS: We preferred the Beck Depression Index (BDI) to evaluate the depression symptoms.

RESULTS: The number of patients introduced the study were 171 (101 (59.1%) female). As a results of BDI 67 (39.2%) patients evaluated as normal [29 (28.7%) female], 54 (31.6%) had poor symptoms [35 (34.7%) female], 46 (26.9%) had moderate symptoms [34 (33.7%) female] and lastly only 4(2.3%) had strong symptoms 3 (3.0%) female]. So 50 (29.2%) of patients had median plus strong symptoms. There were statistically significant association between HbA1c stages and depression (P = 0.018).

CONCLUSION: Being a patient with DM is a strong indicator that the patient may have a depressive disorder. So the physician who takes care patients with DM should be alert about depression, and the simplest way to accomplish is BDI.

Introduction

Diabetes mellitus (DM) is one of the most prevalent diseases all over the world and has devastating complications for patients and economic burden for nations [1]. Prevalence of DM in Turkey has increased from 7.2% [2] to 13.7% [3] in 8 years.

Depression is also a condition with high prevalence. All over the world, an estimated 300 million people of all ages suffer from depression [4]. Also, the World Health Organization (WHO; 2010) declared that major depression carries the heaviest burden of disability among mental and behavioural disorders (3.7 % of all U.S. disability-adjusted life years; and 8.3% of all U.S. years lived with disability) [5].

A study conducted in Istanbul determined that 40.1% of all participants have moderate to severe depressive symptoms [6]. Furthermore, it is observed to be prevalent in the city that the study is conducted.

The correlation between depression and DM is a well - known condition which is investigated by many researchers [7]. Prevalence of depression in patients with DM may vary according to the type of DM, gender and conditions they live in. While the DM is a serious and important condition, depression must be recognised to manage better and improve the condition to prevent devastating outcomes well known for DM.

This study aims to reveal the prevalence of depressive symptoms among patients with diabetes in our region. This way, we can rightly presume and

diagnose depression in patients with DM and manage it more precisely to improve outcome.

Methods

We selected patients randomly who came for their routine Diabetes control as outpatients. After evaluation of biochemical parameters along with patient's condition and informing the patient about the test, the Beck Depression Index form was given to the patient to fill out either at the clinic or to take home. Data of 171 diabetic patients were collected, recorded and evaluated on SPSS 20 statistical program.

The study was conducted in a cross-sectional manner to find out if a correlation exists between depression symptoms and DM. Parameters used were HbA1c values along with independent parameters like age, gender and educational background. The study was carried out in Sakarya Research and Training Hospital in Turkey from December 2016 to January 2017. All the patients had diabetes. The exclusion criteria for patients were a diagnosis of depression and using any antidepressant drug.

The sample population consisted of 171 patients with diabetes. Within this population, 96 typed 2 using the oral antidiabetic drug (OAD), 61 were using OAD plus insulin, 5 patients were using insulin only, 2 were prediabetic, and 7 were type 1. We used the Beck Depression Index (BDI) to evaluate depression symptoms.

BDI was developed by Aron T. Beck. In BDI there are 21 questions and 4 answers to choose, and each question is graded from 0 - 3. The total points are calculated and assigned ranging 0-9 is normal, 10 - 16 poor, 16 - 30 modest and over 30 is strong [14][15].

Before using the BDI, the conducting physician has been trained by psychologists. The BDI questionnaire forms were given to patients at the time of regular visit and had been collected for review. All the forms were recorded together with other parameters on SPSS 20 statistical program and then evaluated. A p-value ≤ 0.05 was accepted as statistically significant. Parameters such as gender, age, marital status, and educational background, the age of patient's diabetes, diabetes therapy, HbA1c and other biochemical parameters that were collected in 3-month intervals were recorded. These were then reviewed to determine the relationship between these parameters and depression symptoms.

HbA1c is the strongest indicator for DM status and is used to find out the control level of the disease and to direct therapy [13]. HbA1c is analysed with the PremierHb9210^{TM} HbA1c analyser. We stratified HbA1c in five stages as 5.0 - 6.5, 6.5 - 7.0, 7.0 - 8.0, 8.0 - 9.5 and over 9.5 [12] and we calculate a mean value of A1c for each patient collected in years to determine the relevant stage of A1c.

The study protocol was approved by ethics committee of Sakarya University. Approval was obtained on July 31 of 2016 with the number of 71522473/050.01.04/18. Before giving the questionnaire forms, all patients were informed about the test, and their approvals were taken.

Results

There were 171 patients. The majority of patients were female (n = 101; 59.1%).The mean age was 59.11yrs with a min 30yrs, and a max 79yrs and the majority of patients were under 65 years old (77.2%). fifteen (8.8%) patients were illiterate, 100 (58.5%) elementary school, 18 (10.5%) middle school, 28 (17.0%) high school and 9 (5.3%) had college degrees. The marital status of patients were 2 (1.2%) single, 157 (91.8%) married, 2 (1.2%) divorced and 10 (5.8%) widowed. Most of the patients were housewives (94; 55%), and the rest were retired (26.9%), and 28 patients had other jobs, and only 3 were jobless. Smoking status of patients was 96 (56.1%) never smoked, 34 (19.9%) currently smoking and 41 (24%) had smoked before. Alcohol status of the patients was; 160 (93.6%) were alcohol naïve, 8 (4.7%) had rarely used, and 3 (1.8%) had previously used. Mean duration of diabetes was 13.47 years (ranging from newly diagnosed to 36 years). Data were collected over a mean period of 7. 68 years.

DM treatment profile of patients were Oral Antidiabetic Drug (OAD) 97 (56.7%), insulin 12 (7%) and OAD + insulin 62 (36.3%). Most of the patients had concurrent diseases especially hypertension [118 (69%)] and dyslipidemia [141 (82.5 %)]. Further 16 (9.4%) patients had ischemic heart disease (IHD), and 16 (9.4%) hypothyroid, and also 1 had allergic asthma, 1 colitis, 1 hepatitis carrier, 1 chronic obstructive pulmonary disease, 1 ischemic heart disease + asthma bronchial, 1 larynx carcinoma (CA), 1 has had tuberculosis, 1 premature menopause, 1 prostate CA, 2 rheumatoid arthritis and 1 ulcerative colitis.

As mentioned before we tried a new method of staging HbA1c (We stratified HbA1c in five stages as 5.0 - 6.5, 6.5 - 7.0, 7.0 - 8.0, 8.0 - 9.5 and over 9.5) to reveal the association between HbA1c and depression. The relevance is depicted in the table. Also as shown in Figure 1, there was a statistically significant association between HbA1c stages and depression p = 0.018.

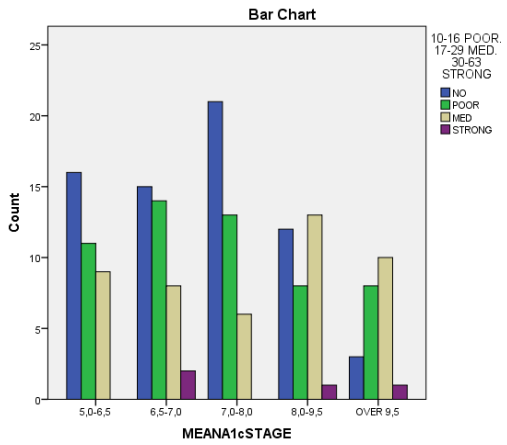

Figure 1: Relation between depression and mean A1c stage

As a results of Beck Depression Index 67 (39.2%) patients appreciated as normal [29 (28.7%) female and 38 (54.3%) male], 54 (31.6%) had poor symptoms [35 (34.7%) female and 19 (27.1%) male], 46 (26.9%) had moderate symptoms [34 (33.7%) female and 12%) male] and lastly only 4 (2.3%) had strong symptoms [3 (3.0%) female and 1 (1.4%) male]. Therefore, 50 (29.2%) of patients had shown median plus strong symptom (Table 1).

Table 1: Correlation between depression symptoms and gender, education, DM age and HbA1c levels

		Normal	Poor Symptoms	Med Symptoms	Strong Symptoms	P
Subjects	Total N=171	67(39.18%)	54(31.57%)	46(26.90%)	4(2.33%)	
Gender	Female n=101 (59.06%)	29(28.7%)	35(34.7%)	34(33.66%)	3(2.97%)	0.007
	Male n=70 (40.93%)	38(54.3%)	19(27.1%)	12(17.1%)	1(1.4%)	
Education	No Education n=15 (8.77%)	4(26.7%)	6(40.0%)	4(26.7%)	1(6.7%)	0.459
	Pre n=100 (58.47%)	40(40%)	25(25%)	32(32%)	3(3.0%)	
	Med n=18 (10.52%)	6(33.3%)	7(38.9%)	5(27.8%)	0(0.0%)	
	High n=29 (16.95%)	14(48.3%)	11(37.9%)	4(13.8%)	0(0.0%)	
	University n=9 (5.26%)	3(33.3%)	5(55.6%)	1(11.1%)	0(0.0%)	
DM Age	0-5 Years n=16 (9.4%)	7(43.75%)	6(37.5%)	3(18.75%)	0(0%)	0.863
	6-10 Years n=47 (27,4%)	21(44.68%)	14(29.78%)	11(23.40%)	1(2.12%)	
	11 Years And Over n=108 (63.2%)	39(33.33%)	40(34.18%)	35(29.91%)	3(2.56%)	
HbA1c Levels	%5,0-6,5 n=36 (21.05%)	16(44.4%)	11(30.6%)	9(25.0%)	0(0.0%)	0.018
	%6.5-7.0 n=39 (22.80%)	15(38.5%)	14(35.9%)	8(20.5%)	2(5.1%)	
	%7.0-8.0 n=40 (23.39%)	21(52.5%)	13(32.5%)	6(15.0%)	0(0.0%)	
	%8,0-9.5 n=34 (19.88%)	12(35.3%)	8(23.5%)	13(38.2%)	1(2.9%)	
	%9,5> n=22 (12.86%)	3(13.6%)	8(36.4%)	10(45.5%)	1(4.5%)	

While the relationship between DM type and depression symptoms was not statistically significant in a general mean (P = 0.892) statistically significant differences between patients with type 2 OAD (25% moderate plus strong symptoms) and patients with type 2 OAD plus Insulin (32.8 % moderate plus strong symptoms) was noted (Table 2).

Table 2: Correlation between depression symptoms and DM type, HT and dyslipidemia

		Normal	Poor Symptoms	Med Symptoms	Strong Symptoms	P
Prediabetes	n=2 (1.16%)	1(1.5%)C↓ (50%)R→	0(0.0%) (0.00%)	1(2.2%) (50%)	2(1.2%) (0.00%)	0.879
Type 1 Diabetes	n=7 (4.09%)	1(1.5%) (14.2%)	2(3.7%) (28.57%)	4(8.7%) (57.1%)	0(0.0%) (0.00%)	
Type 2 DM (OAD)	n=96 (56.14%)	39(58.2%) (40.62%)	33(61.1%) (34.37%)	22(47.8%) (22.91%)	2(50.0%) (2.08%)	
Type 2 DM (Insulin)	n=5 (2.92%)	2(3.0%) (40.00%)	2(3.7%) (40%)	1(2.2%) (20%)	0(0.00%) (0.00%)	
Tip 2 DM OAD&Insulin	n=61 (35.67%)	24(35.8%) (39.34%)	17(31.5%) (27.86%)	18(39.1%) (29.50%)	2(50.0%) (3.27%)	
Hypertension	Yes n=118 (69.00%)	49 (41.5%)	34 (28.8%)	32 (27.1%)	3 (2.5%)	0.675
	No n=53 (30.99%)	18 (34%)	20 (37.7%)	14 (26.4%)	1 (1.9%)	
Dyslipidemia	Yes n=141 (82.45%)	54 (38.3%)	45 (31.9%)	39 (27.7%)	3 (2.1%)	0.916
	No n=30 (17.54%)	13 (43.3%)	9 (30%)	7 (23.3%)	1 (3.3%)	

Although high school (13.8%) and college (11.1%) degree patients had less moderate depression symptoms, it should be kept in mind that most of the patients (n = 100) had elementary school degree. P = 0.59 (Table 1).

The association between dyslipidemia and hypertension is detailed in Table 2 respectively.

Discussion

This study investigated depression symptoms and its association with some parameters in patients with DM. This study revealed that 29.2 % of patients with DM have depression symptoms. It should be kept in mind that BDI results given here are not diagnostic but rather to give direction for further referral to a specialist. Most of the patients who have depression symptoms were female. Some studies found a high prevalence between depression and DM [8] and the results of our study complied with these results.

DM patients with depression are more prone to poor glycemic control in general. This vulnerability is yet to be explicitly understood. It is thought that depression is caused by changes of the neurotransmitters in the brain. These are dopamine, serotonin and norepinephrine. These affect mood and behaviour. Counterregulatory hormones such as catecholamines, glucocorticoids, glucagon and growth hormones are secreted during psychological stress [9]. Activation of counter-regulatory hormones counteract the insulin action and can worsen glucose excursion. Increasing glucose levels may complicate the control of diabetes. And poor glycemic control especially in DM with complications could worsen depression symptoms and could lessen the response to antidepressant therapy [10]. While the relationship between depression and DM is well known, only 31% of patients with DM and depression use antidepressant therapy [11].

In our study based on BDI, we found that 29.2% of patients with DM had depression symptoms with prevalence higher in females (36.7% moderate plus strong symptoms) when compared to males (18.5% moderate plus strong symptoms), p = 0.001.

In conclusion, the prevalence of depression symptoms in patients with diabetes in a region where Turkish people live was somewhat higher than reported for other countries. Being a diabetic especially a female and having a poor managed DM were strong indicators that the patient may have depressive disorder. Hence the physician who takes care of patients with DM should be alert to the possibility of depression and the simplest way to accomplish this is the Beck Depression Index.

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Clinical Science

Dynapenia and Sarcopenia as a Risk Factor for Disability in a Falls and Fractures Clinic in Older Persons

Angela-María Benjumea[1], Carmen-Lucía Curcio[1], Gustavo Duque[2,3], Fernando Gómez[1,3*]

[1]Research Group on Geriatrics and Gerontology, Faculty of Health Sciences, International Association of Gerontology and Geriatrics Collaborative Centre, University of Caldas, Manizales, Colombia; [2]Australian Institute for Musculoskeletal Science (AIMSS), Melbourne Medical School - Western Precinct, The University Of Melbourne & Western Health, Melbourne, Australia; [3]Ageing Bone Research Program, Sydney Medical School Nepean, The University of Sydney, Penrith, Australia

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***Correspondence:** Fernando Gómez. Research Group on Geriatrics and Gerontology, Faculty of Health Sciences, International Association of Gerontology and Geriatrics Collaborative Centre, University of Caldas, Manizales, Colombia. E-mail: gomez.montes@ucaldas.edu.co

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Abstract

BACKGROUND: The role of sarcopenia and dynapenia in disability in older persons from falls and bone health clinics remain unknown.

AIM: This study aims to compare the association of sarcopenia and dynapenia with physical and instrumental disability in a population of older persons attending a falls and fractures clinic.

METHODS: This is a cross-sectional study in Manizales, Andes Mountains, Colombia. A cohort of 534 subjects (mean age = 74, 75% female) Sarcopenia was measured according to the European Working Group on Sarcopenia in Older People (EWGSOP) including an index of skeletal mass, muscle strength, and gait speed. Dynapenia was defined as a handgrip force ≤ 30 kg for men and ≤ 20 kg for women.

RESULTS: Dynapenia and sarcopenia were present in 84.6% and 71.2% respectively. Both were more prevalent in older subjects and women than men. While sarcopenia was associated with body mass index and hypertension, dynapenia was associated with hypothyroidism and visual impairment. After controlling for all covariates, sarcopenia was associated with low IADL and mobility disability.

CONCLUSIONS: Sarcopenia was associated with mobility, ADL and IADL disability. Dynapenia was not associated with disability in this high - risk population. Systematic assessment of sarcopenia should be implemented in falls and fractures clinics to identify sarcopenia and develop interventions to prevent functional decline among elderly individuals.

Introduction

Sarcopenia has been defined as a loss of muscle mass and muscle strength related to ageing. [1][2]. However, there are divergences about the mechanisms, definitions and measurements of sarcopenia. The main point of discussion is the inclusion of muscle mass and strength in the same concept because the decline in muscle strength can be attributed to a combination of muscular and neural factors and not only to reduced muscle mass [3]. At the same time, recent data from longitudinal studies on ageing indicate that maintaining or gaining muscle mass does not prevent aging-related decline in muscle strength [4][5]. Dynapenia (Greek translation for the poverty of strength, power, or force) is the age-associated loss of muscle strength that is not caused by neurologic or muscular diseases [6]. Low muscle strength is well known to place older adults at an increased risk of mobility limitations and mortality [6]. Accordingly, the preservation of muscle strength and power with advancing age is of high clinical significance.

Since dynapenia is only partially explained by the reduction in muscle mass (sarcopenia), several authors insist that these two conditions need to be defined independently of one another [7]. However, The European Working Group on Sarcopenia in Older People (EWGSOP) recommendations for a diagnosis of sarcopenia, which is widely disseminated in clinical practice, includes not only the presence of low muscle

mass (LMM) but also low muscle strength (LMS) and/or low physical performance (LPP) [8]. Recent studies have highlighted the relationship between sarcopenia and falling [9][10]. Indeed, we previously identified a phenotype of osteosarcopenia in older individuals with a history of falling in a fall and Fractures Clinic in Australia [11]. However, the role of sarcopenia and dynapenia in disability in this high-risk population remains unexplored.

Also, few studies have been carried on in Latin America about sarcopenia o dynapenia as a predictor of disability [12][13].

Therefore, the present study aims to compare the association of disability with either EWGSOP - defined sarcopenia or dynapenia in a high - risk population of older fallers in Colombia.

Materials and Methods

Study population

This is cross-sectional study. The setting was the Falls, Dizziness, and Fractures Clinic at University of Caldas (Manizales, Andes Mountains, Colombia, South America). The participants were 534 subjects (mean age = 74, 75% female) assessed between January 2002 - 2014. To be included in the study, participants had to be at least 60 years old and have a complete data set. Participants were excluded based on severe medical conditions that may significantly affect their mobility or incomplete registration of data.

Eligibility criteria to attend the falls, dizziness and fractures clinic included ability to mobilize with a walker or cane(s), willingness to attend the clinic, and at least one of the following: multiple faller (more than two in the last year), single faller with established gait and/or balance problem (e.g., by Get Up and Go Test), unexplained fall with apparent complex medical cause(s), history of chronic dizziness (last 5 years and no earlier than 3 months) and/or history of self - reported symptomatic or asymptomatic fragility fracture(s). The University of Caldas Ethics Research Committee approved this study.

Dependent variables: disability

Respondents were asked if they had difficulty performing activities of daily living (ADL) using a Spanish version of the Barthel ADL scale [14]. If the respondents indicated difficulty or inability in performing one or more of the tasks, they were scored as having ADL disability. Despite its importance about functionality in elderly individuals, incontinence was not included in ADL because it does not necessarily imply physical limitation [15]. For the instrumental activities of daily living (IADL), respondents were asked if they were able to perform eight activities (using a telephone, shopping, preparing meals, performing light housework, taking medications, managing money, doing heavy housework and using transportation), using a modified Spanish version of the Lawton IADL scale [16]. If respondents indicated difficulty or inability in performing one or more of the tasks, they were scored as having IADL disability. A summary score for mobility, ADL and IADL variables was computed. The final disability variable was hierarchical, with three levels. A score of 0 indicated no mobility, ADL or IADL limitation; 1 indicated any IADL limitation or a mobility limitation, and 2 indicated IADL and ADL limitations [17].

Independent Variables

Muscle mass was estimated by appendicular skeletal muscle mass (ASM) using the Lee equation as follow [18]: ASM = (0.244 * body weight) + (7.8 * height) + (6.6 * gender) – (0.098 * age) + (race – 3.3). Body weight was measured in kilograms (kg), and height was measured in meters. This equation has been validated in older population from Latin America using dual-energy X-ray absorptiometry (DXA) as a gold standard with a high correlation between methods ($r = 0.86$ for men and $r = 0.90$ for women, respectively, $p < 0.05$) [19]. After estimating the values, we adjusted the ASM by height squared to create the skeletal muscle mass index (SMI).

Following the studies of Delmonico et al. [20] and Newman et al. [21], the cutoff for LMM used was based on the 20% lowest percentile of the population distribution, representing SMI of ≤ 6.37 kg/m^2 for women and ≤ 8.90 kg/m^2 for men. Muscle strength was assessed with handgrip strength in kg using a hand-held dynamometer (Takey hydraulic dynamometer, the Smedley Hand Dynamometer III). Grip size was adjustable so that each participant felt comfortable while squeezing the grip. The test was performed twice in the dominant limb with a 1 - minute rest between tests and the higher value of the two trials was used for scoring. Cutoff values of < 30 kg for men and < 20 kg for women were considered to represent Low Muscle Strength [8][22].

The Spanish validated the version of Physical performance was assessed with gait speed (in meters/second), determined using the walk test of the Short Physical Performance Battery assessing Lower Extremity Function. The faster of the two trials was used for analyses [23]. The cut - off point of ≤ 0.8 m/s was used to represent LPP [8][22].

Sarcopenia was defined using the EWGSOP criteria. Participants with LMM plus either LMS or LPP were considered as having sarcopenia [8]. Dynapenia was defined using the criteria of Laurentani et al. [22]: < 30 kg for men and < 20 kg for women.

Covariates

Demographic characteristics were age, gender, marital status and education. Education was measured as years of formal schooling completed and was analysed as a continuous variable. (range, 0 to 18). Health status variables included perceived health status, chronic conditions, visual and auditory impairment, depression and cognitive status. The presence of seven chronic conditions (osteoarthritis, hypertension, diabetes mellitus, stroke, chronic obstructive pulmonary disease, and hypothyroidism) was ascertained through self - report. Physical performance was assessed with gait speed (in meters/second), determined using the walk test of the Short Physical Performance Battery Assessing Lower Extremity Function [23]. The faster of the two trials was used for analyses. The cut - off point of ≤ 0.8 m/s was used to represent LPP [8]. Falls and hospitalisations in the previous 12 months were assessed. Sensory impairments were assessed by asking for troubles with vision and hearing (yes or no). Cognition was assessed by the Mini-Mental State Examination; participants with a score of less than 20 were considered to be cognitively impaired [24][25]. An abbreviated (score 0 to 15) Spanish-validated Geriatric Depression Scale (GDS - S) was used to assess the presence of depressive symptoms [26]; respondents with a score of 6 or more on GDS - S are considered likely to be depressed. Body mass index (BMI) was computed by dividing weight in kilograms by height in square meters (kg/m^2).

Statistical analyses

The characteristics of the participants were described by means and standard deviations (SD) or frequencies and percentages according to the type of variable (continuous or categorical, respectively). The chi-square test was used to test qualitative data, while analysis of variance (ANOVA) was used to evaluate continuous data. Statistical differences between groups were determined. To identify the factors associated with disability, variables were selected based on the strength of the associations, higher prevalence (10% or more), clinical relevance, and low potential for collinearity. We calculated OR and 95% confidence intervals (CI). A three-step procedure was developed. First, univariate logistic regression analyses were used to describe the unadjusted effect of sarcopenia and dynapenia and covariates, in the second step, multivariate linear regression models were created to adjust by potential confounder covariates. Based on previous results, we proceeded with multivariate analysis using multiple multinomial logistic regressions, which estimates the prevalence odds ratios (OR). Model 1 includes sarcopenia as an independent variable and model 2 includes dynapenia. Statistical analyses were performed using SPSS for Windows version 22.0.

Results

The mean age ± standard deviation of the participants was 74.4 ± 8.2 years; 75.5% were female, 41.1% were married and the mean years of scholarly were 6.2 ± 4.4. The most prevalent medical conditions were hypertension (65.9%), hypothyroidism (21.9%) and diabetes (15.5%). Sarcopenia and dynapenia were present in 84.6% and 71.2% of the participants, respectively. Table 1 shows the baseline characteristics of the total sample and by sarcopenia and dynapenia status. Participants with sarcopenia and dynapenia were significantly more likely to be older. Those with dynapenia were more female and reported more hypothyroidism, falls and visual impairments, while those with sarcopenia had lower BMI and reported more hypertension. Visual and auditory impairments were reported by 80.4 % and 49.3% of the participants, respectively.

Table 1: Characteristics of the total sample and by sarcopenia and dynapenia status at baseline in clinic of fractures, falls and dizziness

Characteristic	Total sample N = 534	No Sarcopenia N = 154 (15.4)	Yes Sarcopenia N = 380 (84.6)	Yes Dynapenia N = 452 (71.2)	No Dynapenia N = 82 (28.8)
Sociodemographics					
Age, mean (DE)	74.4 (8.2)	74 (7.9)*	76 (8.3)*	75.9 (8.1) **	72.6 (8.6) **
80 or more years (%)	173 (32.4)	42 (27.3)	131 (34.5)	156 (34.5)	17 (20.7)
Women (%)	403 (75.5)	117 (76)	286 (75.3)	364 (80.5)**	39 (47.6)**
Schooling (years) (Mean DE)	6.24 (4.4)	5.9 (4.2)	6.4 (4.5)	6.4 (4.4)*	5.5 (4.4)*
Marital status (single) (%)	77 (14.4)	15 (9.7)	62 (16.3)	72 (15.9)	5 (6.1)
Self perceived health Bad /very bad (%)	73 (13.8)	22 (14.2)	51 (13.6)	61 (13.7)	12 (14.6)
Health conditions					
Hipertension (yes)	344 (65.9)	114 (75)**	230 (62.2)**	294 (66.5)	50 (62.5)
Cancer	26 (5)	8 (5.3)	18 (4.9)	23 (5.2)	3 (3.8)
Stroke	43 (8.4)	18 (12.1)	25 (6.9)	39 (9)	4 (5)
Diabetes (yes)	81 (15.5)	31 (20.3)	50 (13.5)	73 (16.4)	8 (10)
Osteoartritis (yes)	33 (6.3)	8 (5.3)	25 (6.7)	29 (6.5)	4 (5)
Hipothiroidism	115 (21.9)	42 (27.5)	73 (19.7)	108 (24.3) **	7 (8.8) **
COPD	55 (10.7)	13 (8.7)	42 (11.4)	48 (11)	7 (8.8)
Falls in last year (yes)	309 (60.4)	216 (59.5)	93 (62.4)	261 (72.7)*	38 (47.5)*
Hospitalization last year (yes)	289 (54.1)	80 (51.9)	209 (55)	246 (54.4)	43 (52.4)
Vision impairment (yes)	426 (80.4)	122 (79.7)	304 (80.6)	370 (82.4) **	56 (69.1) **
Hearing impairment (yes)	255 (49.3)	76 (50.7)	179 (48.8)	207 (47.4)*	48 (60)*
BMI: mean (DE)	26 (7.1)	30.3 (6.7) **	24.2 (6.4) **	25.6 (7.06)	28 (6.9)
Cognitive status MMSE < 20 (%)	66 (12.4)	14 (9.1)	52 (13.7)	59 (13.1)	7 (8.5)
Depressive symptoms GDS > 5 (n %)	233 (43.4)	76 (49.4)	156 (41.1)	205 (45.4)	27 (32.9)
Physical performance (SPPB) Gait Speed (M/seg). mean (DE)	0,80 (0.99)	0.91 (1.15)**	0.54(0.17)**	0.73 (0.62)*	1.18 (2.03)*

* P<0.05; **P<0.01.

In the bivariate analysis, there were strong associations between dynapenia and age (OR = 1.06, 95% CI = 1.01 - 1.11, p = 0.01), sex (OR = 1.16, 95% CI=1.16 - 1.31, P < 0.01), hypothirodism (OR = 6.09, 95% CI = 1.56 – 23.7, p < 0.01) and visual impairment (OR = 2.21 (1.03 - 4.75, p < 0.01). While significant associations were noted between sarcopenia and age (OR = 1.04, 95% CI = 1.0 - 1.08, p < 0.01), sex (OR = 1.37, 95% CI = 1.00 – 2.51, p = 0.29) and BMI (OR = 1.02, 95% CI = 1.0 – 1.09, p < 0.01).

Table 2 presents the weighted multinomial regression analysis for disability. In model 1, the following were risk factors for incidence in mobility or IADL disability: age and sarcopenia; some falls had a marginal statistical relationship. The risk factors for

ADL and IADL disability were: age, female and sarcopenia. In model 2 the same risk factors were found. However, dynapenia was not associated with disability.

Table 2: Weighted multinomial regression analysis for disability

	Mobility or IADL Sarcopenia model N = 259 OR (95%)	ADL and IADL Sarcopenia model n= 144 OR (95%)	Mobility or IADL Dynapenia model n= 332 OR (95%)	ADL and IADL Dynapenia model n= 192 OR (95%)
Age	1.09 (1.05–1.11)	1.12 (1.08 – 1.16)	1.08 (1.05-1.12)	1.11 (1.08 – 1.15)
Sex (female)	1.50 (0.87 – 2.58)	2.29 (1.32 – 3.96)	1.43 (0.81 – 2.52)	1.97 (1.11 – 3.49)
Hypertension	0.67 (0.40 – 1.12)	0.78 (0.48 – 1.27)	0.63 (0.38 – 1.04)	0.71 (0.43 – 1.15)
Hypothyroidism	1.52 (0.83– 2.80)	1.15 (0.65 – 2.03)	1.45 (0.79 – 2.66)	1.13 (0.64 – 1.98)
Falls (number)	1.01 (0.93- 1.11)	1.06 (0.98 – 1.14)	1.02 (0.94 – 1.11)	1.06 (0.98 – 1.15)
Visual impairment	1.24 (0.69 – 2.23)	0.70 (0.30 – 1.65)	1.34 (0.74 – 2.42)	1.29 (0.71 – 2.33)
Sarcopenia	2.03 (1.16 - 3.53)	2.03 (1.18 – 3.50)	-	-
Dynapenia	-	-	0.87 (0.45-1.67)	0.52 (0.26 – 1.06)

Discussion

Sarcopenia, according to the EWGSOP, was associated with low mobility and disability. Our data correspond with those previous reports [12][27] by showing an association between LMM, LMS and LPP and IADL, mobility and ADL disability. In addition, studies in different settings as community-dwelling older people [28][29][30][31][32][33][34][35][36], acute care [37][38][39] and nursing homes [40][41] showed a significant association between sarcopenia and functional limitation and disability.

Our results also correspond with other studies in Latin America, which have demonstrated that sarcopenia is a risk factor for disability in the elderly. In a four year prospective study in 478 individuals from SABE study, founded after controlling for all covariates, Aleixandre et al., reported that sarcopenia was associated with mobility or IADL disability [12]. Similar to our results, dynapenia was not associated with disability. In another cross-sectional study in 90 hospitalised women in Mexico City, Velasquez - Alba et al., reported that sarcopenia was associated with difficulties in mobility, particular difficulties in climbing stairs [42].

To the best of our knowledge, this is the first paper testing the role of sarcopenia in the clinical measurements performed at falls and fractures clinics. The prevalence of sarcopenia is difficult to establish. The percentage of sarcopenia (84.6%) found in our study is higher than reported for older people living in the community (1-29%), for those living in long-term care institutions (14-33% and up to 68% in men), and for those in acute hospital care 10% [43]. In another study about of sarcopenia in geriatric outpatient clinics, a prevalence of sarcopenia was higher in women (22.9%) than in men (12.7%) [44]. Indeed, this prevalence can differ depending on the characteristics of the studied population and the cut off applied for measuring [45]. Our sample had a mean walk speed of 0.8 mt/sec and cut off point for EWGSOP criteria by sarcopenia case finding [8]. Another possible reason is the eligibility criteria of the sample, with complex medical problems, chronic dizziness and symptomatic or asymptomatic fragility fracture(s). Furthermore, muscle mass reflects ethnicity and lifestyle characteristics [32]. As a consequence, more data are required to determine standardised cut - off values for sarcopenia in falls and fracture clinics. However, the findings here and elsewhere [27][28][29] support the view that intervention strategies designed to preserve skeletal muscle mass should be initiated in all older people attending falls and fracture clinics.

Sarcopenia as a risk factor for falls in elderly individuals remains controversial. In this study, we could not find an association between sarcopenia (based on muscle mass) and fall number in the last year. There are conflicting reports regarding the relationship of sarcopenia with fall. Our findings are in agreement with the results of several cross-sectional surveys on sarcopenia [27][28], but other study using EWGSOP criteria examined 260 individuals aged 80 years or older in Italy found that sarcopenic individuals had a high risk of fall incidents compared with non-sarcopenic individuals [46] Another study in Japan with the same criteria examined 1160 individuals aged 65 years or older revealed that sarcopenia was significantly associated with a history of fall [9].

This study has some limitations. First, the use of the use of the regression equation to estimate muscle mass may overestimate the prevalence of sarcopenia. The availability of DXA as “gold standard” for measuring sarcopenia is limited in coffee grower zones in Colombian Andes Mountains. However, this equation has been used previously in assessing Latin American populations [18][19]. Second, the study design was cross-sectional, and the results do not establish cause-effect relationships between sarcopenia and disability and falls. Theoretically, certain types of morbidity (e.g., lung disease, stroke, and uncontrolled noninsulin - dependent diabetes mellitus) could produce sarcopenia that would result in functional impairment and disability. It is also possible that disability could lead to sarcopenia by limiting physical activity and subsequently predisposing people to some chronic diseases. Also, none of the chronic diseases was significantly associated with sarcopenia, except hypertension. Third, we used handgrip strength and usual walking speed cutoff values that were based on values derived from other reference population. Future studies are required to determine the optimal muscle strength and physical performance cutoff values for defining sarcopenia on this population.

This study has several strengths. First, it was conducted on a large sample of patients comes from falls, dizziness and fractures clinic in Latin America. Second, this study is the first to analyse sarcopenia using the EWGSOP criteria at a falls, dizziness and fracture clinic and to compare this method with dynapenia as a risk factor for mobility, IADL, disability and ADL disability.

In conclusion, sarcopenia is a risk factor for developing IADL, mobility and ADL disabilities in older people. The diagnostic criteria from (EWGSOP) using normative data should be implemented at falls and fractures clinics to identify sarcopenia and develop early interventions to prevent functional decline among this population of high-risk elderly individuals.

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Survey about the Extubation Practice among Anaesthesiologists in Kosovo

Nehat Baftiu*, Islam Krasniqi, Kastriot Haxhirexha, Rudin Domi

Clinic for Anaesthesiology and Intensive Care, University Clinical Centre of Kosovo, Prishtina, Kosovo

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***Correspondence:** Nehat Baftiu. Clinic for Anesthesiology and Intensive Care, University Clinical Center of Kosovo, Kosovo. E-mail: nehatbaftiu@hotmail.com

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Abstract

BACKGROUND: Tracheal extubations may be performed before or after awakening from anaesthesia. The advantage of extubation during anaesthesia may avoid all the unpleasant effects of fully awake extubation such as severe hypertension and tachycardia, malignant dysrhythmias, myocardial ischemia laryngospasm, and cough induced high intraocular and intracranial pressure.

AIM: To show the current practice of performing extubations in Kosovo, as well as the advantage and disadvantage in performing this procedure in an awake patient or inpatient in light anaesthesia.

MATERIAL: This study is conducted at the Regional Hospitals and the University Clinical Center of Kosovo during the year 2015. A questionnaire is given to the anesthesiologists to collect information about the techniques used for extubation, timing and management of extubation.

RESULTS: Based on this survey results that 86% of an anesthesiologist (71) extubate the patients when they are completely awake, while 14% of them (12) prefer to extubate the patients under light anaesthesia. From all anesthesiologists involved in this study, forty of them reported problems during extubation. Complications were related to airway, and they are treated by oxygenation and jaw support, but in rare cases, reintubation were performed.

CONCLUSION: Complications during extubation remain important risk factor while extubation during light anaesthesia can minimise some of them.

Introduction

Perioperative airway management includes taking care of both intubation and extubation. Airway management and mechanical ventilation during anaesthesia is a crucial daily practice of every anesthesiologist. To ensure a successful anaesthesia process, the anesthesiologist must create a plan how to conduct anaesthesia and airway management. It is recently reported that extubation period can face to the anesthesiologist perhaps more sharp complications compared with endotracheal intubation [1].

No guidelines exist regarding the best extubation technique or timing, to prevent the post-extubation complications. There are many potential complications which may occur during extubation including post-extubation respiratory failure manifested by hypoxemia and/or hypercapnia, and cardiac disturbances including death. Minor complications are faced by the anesthesiologist as a laryngeal irritation (the incidence is reported up to 45%), sore throat and hoarseness (commonly in intubated patients for a long period or difficulty intubated trachea) occur in most intubated patients.

Difficult mask ventilation or difficult intubation can result in lips, mouth, or pharynx damage. It is reported even hypoglossal nerve damage, causing numb tongue 1 - 2 weeks after extubation [2]. The literature offers no significant convincing data on this topic.

The main aim of this study is to evaluate the extubation techniques related to the surgery procedures including extubation time, and patients are positioning as well. Secondly, the study tends to reveal all the complications after extubation, their incidence, and all treatment approaches.

Material and Methods

This study is conducted at the Regional Hospitals and the University Clinical Center of Kosovo during the year 2015. A questionnaire in Albanian language (native language) is given to the anesthesiologists who are working in Regional Hospitals in Kosovo and University Clinical Center of Kosovo. A total of 89 questionnaires were distributed to them, and they returned it after they completed the questionnaire. The modified questionnaire [3], was applied as a tick - box format with a dedicated space for further necessary explanations. The questionnaire included information about the gender, years of work as anesthesiologists, anaesthesia techniques used, and techniques used for extubation, the timing of extubation, management of extubation including patient position during extubation and if there were any complications during extubation in the last six months.

The last part dealt with the type of complications and management of these complications during extubation.

Results

A total 89 questionnaires were distributed, and 83 questionnaires were completed and returned (93% respond rate). The years of experience as anesthesiologist were included in this study. The mean value was 13 (range from 3 - 35 years). Ten of respondents (13%) were from Regional Hospitals of Kosovo and the others from University Clinical Center in the capital.

According to the data referred by anesthesiologists results that they extubate the patient either when is completely awake or under light anaesthesia 71 vs 12 (86% vs 14%). About 86% of respondents always use 100% of O_2 for 2 - 5 minutes before extubating, 36% of them occasionally use reversal agents, while aspiration during extubation is used by 89% of respondents, as summarised in Table 1.

Supine and head up position during extubation were used in elective surgery and at obese patients, but lateral left and lateral left with head down position were used significantly in emergency surgery patients.

Table 1: Airway management and extubation

	Always	Mostly	Occasionally	Never
100% oxygen for 2–5 min before extubation	86%	14%		
Reversal agents	22%	42%	36%	
Aspiration	64%	25%	11%	

The trachea is always extubated in the operation theatre, and all patients are transferred extubated to the recovery room breathing oxygen. From all respondents, 40 reported problems during extubation. Table 2 demonstrates the positions of patients in the moment of extubation.

Table 2: Position(s) at extubation

	Head up	Supine	Lateral left	Lateral left and head down
Elective	32	44	4	2
Emergency	36	14	26	12
Obese	52	18	18	0

We observed and memorised all the reported complications after the patients were extubated. As shown in Table 3, the most common complication after extubation is a cough, desaturation, laryngospasm, and airway obstruction.

Table 3: Incidence of complications after the extubation

Complications	Nr	%
Coughing	80	45%
Breath holding	10	6%
Airway obstruction	12	7%
Laryngospasm	10	6%
Desaturation	26	15%
Inadequate reversal	16	9%
Apnoea	8	5%
Vomiting	4	2%
Aspiration	2	1%
Haemodynamic instability	8	4%
Total	176	100%

Complications were treated with oxygen therapy, jaw support, but in some cases, the medicaments were used significantly to treat the complication. In fourteen patients reintubation is undertaken because of due to difficulties in maintaining adequate respiratory (Table 4).

Table 4: Treatment methods of the complications

Treatment of complications	n
Oxygen	96
Jaw support	74
Propofol	26
Midazolam	2
Reversal drugs	30
Suxamethonium	8
Re-intubation	14

Discussion

The extubation can be performed before or after the patient regains consciousness. The main benefits of extubation in deep anaesthesia include minimising several side effects of sympathetic stimulation as hypertension, tachycardia, laryngospasm, cough, and increased intraocular and intracranial pressure. This would be beneficial when avoidance of the hemodynamic and respiratory reflexes to extubation is advisable (e.g. following certain intracranial, ophthalmologic, or thoracic surgical procedures). The principal disadvantage of

tracheal extubation during deep anaesthesia is the increased risk of upper airway obstruction and inadequate airway protection rendering the patient prone to pulmonary aspiration [1]. During the extubation sequence, the anesthesiologist should be careful to provide oxygen to the patient [4]. Extubation has been performed when the patient is either fully 'awake' or deeply anaesthetised. During awake extubation, the patient can maintain the airway patency. Deep anaesthesia extubation reduces the incidence of coughing, and hemodynamic perturbations due to sympathetic stimulation, but can be associated with increased likelihood of upper airway obstruction [30][31][32]. This extubation approach is applied in patients whom airway management is easy, and the gastric aspiration risk is not evident.

For all participants, 86% of them always use 100% O_2 before extubation; these were answers if they use 100% O_2 before extubation of the patient, which is in line with recommendations for patient extubation. Before extubation, the anesthesiologist can often increase FiO_2 to maximise oxygenation in case of failed extubation [19]. Recently has been reported that higher FiO_2 can induce postoperative atelectasis, but controversies exist [20][21]. In order to avoid unnecessary post-extubation hypoxia, a higher FiO_2 can be recommended [22][23][24][25][26].

Anesthesiologists use reversal agents for reversal of neuromuscular block in most of the patient. In patients at high - risk for extubation failure, incomplete reversal of muscle relaxation will contribute to, or even be the primary cause of upper airway obstruction after extubation. Therefore clinically adequate return of neuromuscular function following administration of muscle relaxants is essential. This can only be assured by using quantitative relaxometry [47]. If such a technique is not available, muscle relaxants must be antagonised by appropriate doses of acetylcholine esterase inhibitors or sugammadex while ventilation needs to be continued for 3 - 4 h after the last dose of an intermediately long-acting muscle relaxant. According to our data results, 89% of anesthesiologists perform tracheal aspiration always during the extubation. Suction must be smooth and ideally performed under direct laryngoscopy [27][28]. Special care must be taken if there is bleeding or clots in the airway. The suction must be carefully performed or even avoided because of increased risk of the airway and tracheal tree obstruction by blood [29]. In elective operations, extubation was more frequently performed in the supine and head up positions. In emergency surgery, the head up and left lateral position was usually chosen. In obese patients, however, most anaesthetists preferred a head up position before tracheal extubation. There is no consensus on the suitable patient's position during extubation, but ahead - up (reverse Trendelenburg) or semi-recumbent position may be preferred. The anesthesiologist performed the obese patient's extubation in the head - up tilt because of benefits in airway monitoring and management. This position is accepted for the non - fasted patient [18, 48] as well. The trachea is always extubated in the operating room, and all patients are transferred extubated to the recovery room breathing oxygen. During extubation, the incidence of coughing was 45% of total complications, about 6% of patients were complicated with laryngospasm while desaturation represents 15% of all complications because is considered a major cause of upper airway obstruction. It may be provoked by sudden stimulation while the patient is in a light plane of anaesthesia, and by vocal cord irritation through secretions (e.g. saliva, blood, gastric content). Although suction of the oropharynx should routinely be performed, it must be performed while the patient is still deeply anaesthetised because any irritation of the vocal cords at a light plane of anaesthesia may provoke laryngospasm. Extubation during application of positive airway pressure is an additional means of removing secretions from around the vocal cords. This manoeuvre may, however, provoke coughing. If tracheal tube (TT) cuff pressure has been monitored and maintained at the recommended level, extubation without prior cuff deflation may be a further means of removing secretions from above the vocal cords during extubation [45]. If risk factors for the development of laryngospasm exist, extubation during deep anaesthesia should be considered. Patients being extubated during deep levels of anaesthesia are preferably placed in the lateral (and possibly slightly Trendelenburg) position to keep the vocal cords clear of secretions during emergence. Even that reversal agents were used in most of the patients, and most of the anesthesiologists reported that they extubate the patients always or mostly in awake or under light anaesthesia, 9% of complications where due to the inadequate reversal. It is important to highlight that in regional hospitals or University Clinical Center is not available equipment or tools for monitoring of the neuromuscular block and for neuromuscular block reversal. Oxygen is used mostly (n = 96) to treat the complications, then airway open techniques and reversal drugs including and propofol are drugs used mostly to treat the complications. Guidelines are designed to describe the actual practice on the topic and are evidence-based medicine helping the anesthesiologists to manage the life - threaten everyday issues [11][34][35][36][37][38][39][40][41]. None of the actually published guidelines addressed to the extubation approach [6][7][8][9][10][11]. The residency programs do not always deal with extubation which is, of course, one of the most important moments in anaesthesia. Extubation after anaesthesia differs from ICU extubation. Several events can occur after extubation [13][33][42][43], but outcomes are improved by planning, organization and communication [14][15][44].

Results of this survey show that complications during extubation remain important risk factor in the

management of anaesthesia, and in current practice in Kosovo" Usually the experience of the anesthesiologist is an important factor in avoiding complications of the extubations and not related if it is Regional Hospital or University Clinical Center.

Author contributions

Nehat Baftiu contributed to the original idea of the study, Islam Krasniqi collected data and analysed them, all authors contributed to the discussion section and manuscript preparation for submission.

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Evaluation of Fast Glycaemia in Hypertonic Population that Suffer from Diabetes: The Importance of Self-Monitoring of Glycemic Level and the Effects of Interactions, with the Aim of Reducing the Levels of Fast Glycaemia in These Patients

Edlira Lashi[1], Fatos Lashi[2], Klotilda Muca[2], Bora Ballta[3*], Suzana Kazazi[1]

[1]Primary Healthcare Clinic Nr. 1, Endocrinology, Tirana, Albania; [2]Primary Health Care Center, "Egian", General Medicine, Tirana, Albania; [3]Primary Healthcare Center, "Klinika Italiane S. Antonio", Tirana, Albania

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Keywords: HC; Self - monitoring; Glycemia; Hypertension; Glucometer

***Correspondence:** Bora Ballta. Primary Healthcare Center, "Klinika Italiane S. Antonio" – Tirana, Albania. E-mail: bora.ballta@gmail.com

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Abstract

AIM: Identification of glycemic level tendency rates in a hypertonic population that suffers from diabetes mellitus in Health Centre Nr. 1, Tirana, evaluation of self-monitoring and the effects of interactions, with the aim of reducing the levels of fast glycaemia in these patients.

MATERIAL AND METHODS: In the study participated 810 patients of Health Centre Nr 1 in Tirana that suffer from hypertension and diabetes mellitus type 1 and 2. The study was conducted through 10 months' period. The patients that owned glucometer passed through the process of calibration of the devices, the others that had no glucometer had been given one. All the patients had been instructed how to use the device properly. Informative and educative materials regarding hypertension and diabetes were given to them. A standardised table was used to collect all the data. Changes in therapy were done regarding the glycemic levels.

RESULTS: The most of the patient shown an important improvement in glycemic rates during ten months of study. From 810 patients, 617 of them shown an improvement of the glycemic level data (median = 24 mg/dl; IQR: 14 – 50 mg/dl), and the other 193 patients have shown no improvement (n = 11) or aggravation (n = 182). The data showed that the patients that had no improvement during the study have diabetes type one (40%), they that have shown improvement has diabetes type 2 (33%) The difference between 2 those groups were insignificant (p = 0.075). The data of glycemic levels shown a significant decreased of 19% of basal glycemic levels (128 ± 31 vs. 158 ± 55 mg/dl: $p < 0.05$) at the end of the study, and decreased of glycemic levels was visible especially after the first month of the study, in both groups male and females.

CONCLUSION: A total of 205 therapy changes like adding a new or two drugs or an increase of doses of the drugs, are done in some 181 patients that have diabetes, with a frequency of 1.1 changes in therapy per patient.

Introduction

Diabetes mellitus (DM) is a group of metabolic diseases characterised by chronic hyperglycemia resulting from defects in insulin secretion, insulin action, or both. Metabolic abnormalities in carbohydrates, lipids, and proteins result from the importance of insulin as an anabolic hormone. Low levels of insulin to achieve adequate response and/or insulin resistance of target tissues, mainly skeletal muscles, adipose tissue, and to a lesser extent, liver, at the level of insulin receptors, signal transduction system, and/or effector enzymes or genes are responsible for these metabolic abnormalities [1]. The severity of symptoms is due to the type and duration of diabetes mellitus.

Some of the diabetes patients are asymptomatic especially those with type 2 diabetes during the early years of the disease, others with marked hyperglycemia and with absolute insulin deficiency may suffer from polyuria, polydipsia, polyphagy, weight loss, and blurred vision. Uncontrolled diabetes may lead to stupor, coma and if not treated death, due to ketoacidosis or rare from nonketotic hyperosmolar syndrome [1][2][3].

Diabetes mellitus is an important health

condition for the ageing population; 26 % of patients over the age of 65 years have diabetes mellitus [1], and this number is expected to grow rapidly in the coming decades [3]. Older individuals with diabetes have higher rates of premature death, functional disability, and coexisting illnesses, such as hypertension, coronary heart disease, and stroke than those without diabetes. Older adults with diabetes mellitus also are at a greater risk than other older adults for several common geriatric syndromes, such as polypharmacy, cognitive impairment, urinary incontinence, injurious falls, and persistent pain. Screening for diabetes mellitus complications in older adults also should be individualised and periodically revisited, since the results of screening tests may impact therapeutic approaches and targets [4].

World Health Organization (WHO) predicts that in 2030 diabetes mellitus will be the 7th cause of death in worldwide [5]. A healthy diet, regular physical activity, normal weight and not smoking can prevent diabetes mellitus type 2 [6]. The prevalence of DM during the 1990-2006 is doubled and is in continuous increasing. Population ageing, urbanisation, increased caloric intake, decreased physical activity are some of the factors that lead to continuous increasing of DM rate in Albania [7]. In 2004 in Albania were 30,000 people with diabetes registered. In 2011 this number reached 55,000, and in 2013 were more than 65,000 patients with diabetes registered regarding ISKSH data [8]. In the world, 15 million people have diabetes mellitus and more than 59.8 million are in Europe region. In 2040 this number is predicted to be 71.1 million [9]. Diabetes mellitus is a chronic and complex disease that requires continuous medical care and a strategy to reduce the risk for this disease. Continuous medical education of the patients is very important in preventing acute complications and in the reduction of risks for long-term complications [10]. Self - monitoring of glycaemia is considered very important key in diabetes mellitus management.

The study aims to evaluate the collaboration between patient and doctor, self-monitoring of glycemia and the impact of these two actions in the decrease of glycemic levels. The study was based on the hypotheses that the treatment of diabetes is effective when the patient is active in monitoring his glycemic blood levels and collaborates with medical staff.

Patients and Methods

Patients

Health Center No 1 in Tirana has a total number of 82,500 patients. Twelve percent (12%) of them, 10,190 patients have chronic diseases, especially cardiovascular disease and diabetes mellitus. 89% of these chronic patients suffer from hypertension (9,120 patients), and 27% suffer from diabetes mellitus type 1 and type 2 (796 patients). In the study contributed 810 patients with hypertension and diabetes mellitus. The confidentiality and anonymity were secured for all the patients since the first clinical visit. Demographic data were collected retrospectively from the individual data registered in Health Center No 1 in Tirana database. Other data are collected prospectively during the time of the study. The criteria for patient selection are I: the patient must have been diagnosed with DM and hypertension from almost one year; II: the patient must be in continuous medical treatment for the last six months; and III: the patient must show a desire to be part of the study and collaborate with the doctor. All the data are registered in our database.

Methodology

Self - monitoring of levels of glycemia is considered as a very important part of diabetes mellitus management. In the study participated 810 patients of Health Center No 1 in Tirana that suffers from hypertension and diabetes mellitus type 1 and 2. The study was conducted through 10 months' period. The patients that owned glucometer passed through the process of calibration of the devices, the others that had no glucometer had been given one. Glucometers (one touch select) 270 pieces and strip tests were given to the patients. The patients were instructed regarding the exact monitoring of glycaemia (Table1), and instructions regarding diet were secured to them also (Table 2).

Table 1: Step – by - step patient instruction on blood glucose home monitoring

1	Wash hands with soap and warm water. Dry hands
2	Prepare the lancing device by inserting a fresh lancet. Lancets that are used more than once are not as sharp as a new lancet and can cause more pain and injury to the skin
3	Prepare the blood glucose meter and test strip
4	Use the lancing device to obtain a small drop of blood from your fingertip
5	If you have difficulty getting a good drop of blood from the fingertip, try rinsing your fingers with warm water, shaking the hand below the waist, or squeezing the fingertip
6	Apply the blood drop to the test strip in the blood glucose meter. The results will be displayed on the meter after several seconds
7	Dispose of the used lancet in a puncture-resistant sharps container

Regular physical activity is very important in the management of diabetes mellitus and hypertension, but also healthy balanced diet and medical therapy are necessary to control these diseases. The patients were suggested to maintain an active physical status by walking for 30 - 60 min per day.

Table 2: Diet instructions

1. Drink 6-8 glasses of water every day.
2. Eat five fruits and vegetables per day.
3. Eat whole grain bread or cereals.
4. Do not eat refined sugar foods, and with high fat.
5. Eat small portions.
6. Eat fish, white meat and drink milk with low fat.
7. Reduce salt intake, sugar, alcohol as much as possible.
8. Limit soft drinks and sweet foods.

Results

Demographics

More than half of the study patients were females (n = 435 vs. n = 375; p = 0.001; Table 3). Mean age at study initiation was 68 ± 10 years. Eight hundred ten patients had DM.

Table 3: Patient baseline demographics by gender

	Females (n = 435)	Males (n = 375)	*p*-value
Age	67 ± 9	68 ± 10	
Diabetes Mellitus (DM)			
Type I	160 (37%)	122 (33%)	0.133
Type II	275 (63%)	253 (67%)	0.235
Median time with DM	8 (4, 12)	8 (4, 11)	0.247
Baseline blood glucose			
Median (IQR)		145 (121-169)	0.762
Mean ± Std		158 ± 59	0.860

IQR-interquartile range (25th and 75th percentiles); Std-standard deviation.

There were significantly more patients with type II DM (65% [*n* = 528] type II DM *vs*. 35% [*n* = 282] type I DM; *p* < 0.001). The median time of patients with DM was 8 (interquartile range [IQR]: 4 - years. Mean blood glucose level at baseline was 158 ± 55 mg/dl and median 145 mg/dl (IQR: 125 - 170) (Table 4).

Table 4: Patient baseline demographics

	Total	*the*
Age	68 ± 10	
Gender		
Females	435 (54%)	
Males	375 (46%)	0.001*
Diabetes Mellitus (DM)		
Type I	282 (35%)	
Type II	538 (65%)	<0.001*
Median time with DM (years)	8 (4-11)	
Baseline blood glucose (mg/dl)		
Median (IQR)	145 (125-170)	
Mean ± Std	158 ± 55	

IQR-interquartile range (25th and 75th percentiles); Std-standard deviation.

The majority of the patients demonstrated improvement of DM after ten months of being in the study. Of the 810 patients, 617 patients demonstrated an improvement of their blood sugar (median = 24mg/dl; IQR: 14 – 50 mg/dl). The remaining 193 patients had no improvement (n = 11) or worsening of their DM (n = 182). There were more patients with type I DM that did not improve compared to patients with type 2 (40% vs 33%), although not significant (*p* = 0.075; Table 5).

Table 5: Patient baseline demographics by groups

DM	Group 1 (n = 617)	Group 2 (n = 193)	*p*-value
Age	67±9	68±11	0.26
Gender			
Female	333 (54%)	102 (53%)	0.80
Male	284 (46%)	91 (47%)	
Time/year with DM,	8 (4, 11)	8 (5, 12)	0.38
DM type I	204 (33%)	78 (40%)	0.075
DM type II	413 (67%)	115 (60%)	

Group 1 - showed improvement; group 2 - did not have any effect on improvement or worsening DM = diabetes mellitus.

We observed a steady decline in mean fasting glucose levels in all patients, females and males. This decline became statistically significant starting at one month after baseline readings. A 19.5% decline in blood glucose level was observed at the end of study follow - up (10 - months) when compared to baseline (128 ± 31 vs 158 ± 55 mg/dl; *p* < 0.05).

We also investigated whether the decline in blood glucose level was age-dependent we divided our study population into three groups (Table 6). The decline in blood glucose levels ranges from 18% (group 2) to 20% (group 1). Chi-square test showed that the decline was not statistically different between the age groups (*p* > 0.05).

Table 6: Age-dependent decline in blood glucose level

	BM	10-Months	Decline	BM	10-Months	Decline	BM	10-Month	Decline
DM	160 ± 60	128 ± 29	20%	156 ± 51	128 ± 35	18%	159 ± 56	128 ± 30	19.5%

DM = diabetes mellitus; BM=baseline measurements; Decline = represents the decline values in blood glucose level at the end of study follow-up (10-months) when compared with baseline measurements.

To confirm these results we ran a Cox regression analysis against gender and age, with age as a continuous variable (i.e. patients were not divided into age groups). This analysis also Cox regression analysis showed that this decline was independent of patient gender (i.e. the decline was found to be similar and significant in both males and females; *p* > 0.05).

A total of 205 interventions related to DM, were performed during the ten months of follow - up to 181 patients for a rate of 1.1 interventions/patient. Interventions included therapy changes.

Discussion

Self - monitoring of glycemia is the collection of detailed information of glycemic levels during the day, and with the aim of finding the appropriate drug doses for them, to improve diabetes mellitus and prevent complications [11].

Self-monitoring of glycemic levels can be improved with these steps:

I: finding the appropriate frequency for the tests.

II: education and the capability of the patients. III: capability and the education of medical staff.

IV: use of simple medical devices for glycemic measurements [12].

These steps help in limitation of overuse of glycemic tests at home, helping so in reducing the cost of the glycemic control [13]. The studies have concluded that the management of chronic diseases resulted in improvement of patient health and decreased of costs as result of the reduction. Our

study concluded that diabetes could be improved when doctor-patient relationship is well - established, and the patient is educated and instructed how to monitor his glycemia. But there were also some patients that regarding all the instructions and education have not shown any improvement in their health condition. Some of the factors that may have contributed in this result for the mentioned group may be incapability for following instructions, monitoring at home blood glycemic level, other existent diseases, eating not carefully regarding diets and having a sedentary lifestyle. To sum it up, our study has shown that it was a really clear improvement in our group study of the glycemic levels, not related to age or sex of the patients. This achievement it is believed to be as a result of continuous and carefully education of the patients for self-monitoring of glycaemia at home, and also different drug therapy changes that are specific for every patient. We also believe that the relationship doctor-patient is very important in the impact of the study result, making patent more comfortable to follow doctor's advice. We must admit that the time of the study was not long, and the possibility to follow the patients for a long time was not possible.

In conclusion, self - monitoring improve Diabetes Mellitus the final point, which may reduce Diabetes Mellitus complication, and the study has verified what we already know that relationship doctor-patient is important in diabetes mellitus improvement.

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Classification of Radiological Changes in Burst Fractures

Salim Şentürk[1], Ahmet Öğrenci[2*], Ahmet Gürhan Gürçay[3], Ahmet Atilla Abdioğlu[4], Onur Yaman[1], Ali Fahir Özer[1]

[1]Koc Universitesi Tip Fakultesi, Neurosurgery, Istanbul, Turkey; [2]Neurospinal Academy – Neurosurgery Kurtköy mah. Ankara Cad. 390/3, Pendik, Istanbul 34955, Turkey; [3]TC Saglik Bakanligi Ankara Ataturk Egitim ve Arastirma Hastanesi , Neurosurgery Ankara, Ankara, Turkey; [4]Trabzon Kanuni Egitim ve Arastirma Hastanesi, Neurosurgery, Trabzon, Turkey

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***Correspondence:** Ahmet Öğrenci, Neurospinal Academy – Neurosurgery Kurtköy mah. Ankara Cad. 390/3, Pendik, Istanbul 34955, Turkey. E-mail: drahmetogrenci@gmail.com

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Abstract

AIM: Burst fractures can occur with different radiological images after high energy. We aimed to simplify radiological staging of burst fractures.

METHODS: Eighty patients whom exposed spinal trauma and had burst fracture were evaluated concerning age, sex, fracture segment, neurological deficit, secondary organ injury and radiological changes that occurred.

RESULTS: We performed a new classification in burst fractures at radiological images.

CONCLUSIONS: According to this classification system, secondary organ injury and neurological deficit can be an indicator of energy exposure. If energy is high, the clinical status will be worse. Thus, we can get an idea about the likelihood of neurological deficit and secondary organ injuries. This classification has simplified the radiological staging of burst fractures and is a classification that gives a very accurate idea about the neurological condition.

Introduction

Sometimes neurological deficit is not observed in burst fractures while there may also be an evident neurological deficit and secondary organ injury and even death [1][2]. Several classifications have been developed for spine injuries. AO (Arbeitsgemeinschaft für Osteosynthese Fragen) divided thoracolumbar injuries into three groups from morphological and pathological aspects (A: Compression; B: Distraction; C: Axial Strain and rotational deformity). Each group was divided into subgroups according to the morphological injury and grade of instability. However, there is no information about the neurological deficit in this classification. Burst fractures take place in group A in AO classification [3]. Fractures passing through the pedicles were added to the classification, but interpedicular separation and bone fragments with the excess pediculolaminar junction (corner) (PLC) in the spinal canal were not included in the study of Magerl et al. [3]. Although several classifications were proposed, Thoracolumbar Injury Classification and Severity Score (TLICS) were introduced in 2005 [4].

This classification is based on the morphology of the injury, the status of the posterior longtidunal ligament (PLL) and neurologic examination [4]. The energy that is generated due to axial and flexional loading in burst fractures is transmitted to the corpus and forces the corpus, which leads to some changes.

Burst fractures are classified according to the pathomorphological changes based on their radiological appearance.

The aim of this study is to create a simpler radiological classification in the burst fractures and to present their relation to secondary injuries.

Materials and Methods

After the approval was obtained from the ethics board of our hospital, the tomographic images and medical charts of 80 patients who were diagnosed with burst fractures were examined.

The patients were evaluated concerning age, sex, fracture segment, neurological deficit, secondary organ injury and radiological changes that occurred.

The classification was made according to the changes on the tomographic images as an indicator of the energy that was exposed. Neurological status was classified according to the ASIA scoring system [5]. Secondary organ injury was assessed.

Secondary organ injury was evaluated in the light of the abdominal CT reports and abdominal USG reports. Rib fractures, lung contusions, haemothorax, pneumothorax, liver and spleen injuries were determined.

Radiological changes were assessed and classified according to the axial sections on CT.

Group 1: Fractures extend forward or laterally from the corpus. In general, a piece of the bone fragment may move to the spinal canal. The width of this spur usually depends on the distance between the radix of the pedicles (Because the pedicles are an obstacle before the bone fragment broken and detached from the corpus).

Different bone fragments can be protruded if the energy that is exposed also contains rotational motion in addition to the flexion and axial loading. PLL and spinal cord are the breaking points where some bone fragments stop moving and also leap or move backwards due to the effect of the moment's dynamism. Consequently, some bone fragments can be seen in front of the corpus or/and near the corpus.

Usually, one piece of bone fragment moves on to the spinal canal. The protruded bone fragment may get closer to the PLC. Interpedicular distance is constant.

Group 2: There may be bone fragments in front of the corpus or/and near the corpus. There are some bone fragments that come closer to the PLC, but they don't lead to the separation and splitting of the posterior components and don't move into the spinal canal. Interpedicular distance is constant.

Group 3: There can be fractures in front of the corpus and at the sides of the corpus. There are bone fragments in the spinal canal. There are fractures on the lamina and spinous process. Interpedicular distance is extended.

PLC is used as a reference point while classifying the burst fractures. If the bone fragments can't reach the PLC, it should be classified as Group 1. The fracture should be classified as Group 2 if it reaches the PLC and splits into pieces by crushing the corner. The fracture should be classified as Group 3 if it passes through the PLC and breaks the posterior components. The staging of burst fractures is shown in (Figure 1).

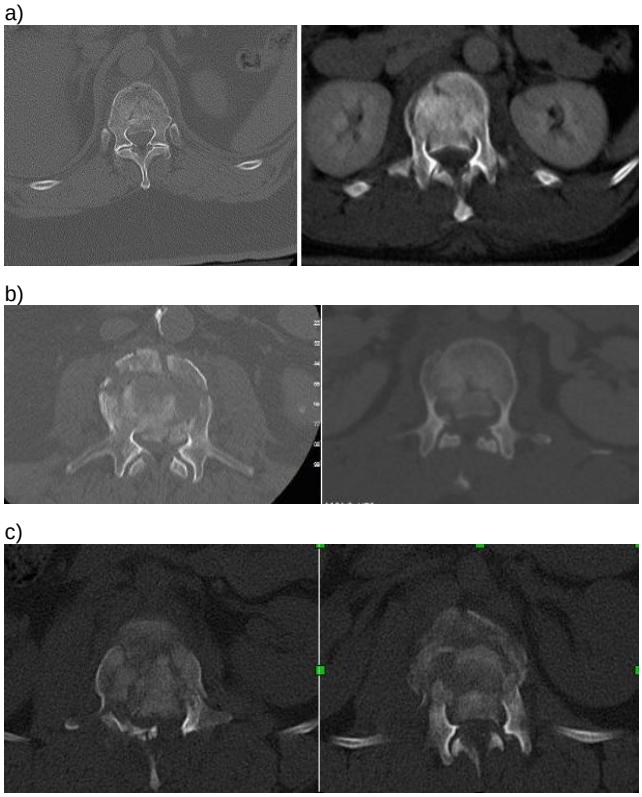

Figure 1: a) Grade 1) The bone fragments can't reach the PLC; b) Grade 2) The fracture reaches the PLC and splits into pieces by crushing the corner; c) Grade 3) The fracture passes through the PLC and breaks the posterior components

Results

There were 27 female and 53 male patients in the group diagnosed with burst fractures. The mean age of the patients was 49.3. The mean age of the male patients was 49.4 (16 - 84) while it was 49.2 (20 - 72) for the female patients.

Five of the cases were observed in the thoracic region (6.2%), 17 at T12 (21%), 2 at both T12 and L1 (2.5%), 35 at L1 (43.7%) and 21 at lumbar region (26.2%) (Table 1).

Table 1: Distribution of cases according to the region

Region	Number of Cases
Thoracic	5 (6.2%)
T12	17 (21%)
T12/L1	2 (2.5%)
L1	35 (43.7%)
Lumbar Region	21 (36.2%)

It was observed that burst fractures were grade 1 in 38% of the patients (22 male, 16 female), grade 2 in 24% (18 male, 6 female) and grade 3 in 18% (13 male, 5 female) of the patients (Table 2).

Table 2: Table shows the patients according to grades and also with neurological deficit rates and secondary organ injury rates

Grade	Female	Male	Total	Cases with neurological deficit	Percentage of neurological deficit	Cases with secondary organ injury	Percentage of secondary organ injury
1	16	22	38 (47.5%)	2	5.2%	2	5.2%
2	6	18	24 (30%)	13	54.1%	3	12.5%
3	5	13	18 (22.5%)	13	72.2%	8	44.4%
Total	27	53	80	28		13	

Neurological deficit was observed in 2 (2 / 38) of Grade 1 patients, in 13 (13/24) of Grade 2 patients, and in 13 (13/18) of Grade 3 patients (Table 2).

Two of Grade 1 patients were observed to have ASIA D neurological status; 12 of Grade 2 patients had ASIA D neurological status while 1 had ASIA C neurological status; 6 of Grade 3 patients had ASIA D neurological status, 4 had ASIA C neurological status, and 3 had ASIA A neurological status.

As regards secondary organ injury; rib fractures were observed in 2 of Grade 1 patients; 2 of Grade 2 patients had rib fractures while 1 had lung contusion. Rib fractures, lung, liver or spleen injuries were observed in 8 of Grade 3 patients (Table 2).

And also in our staging system, the proportion of dural injury was high in stage 2 and stage 3 patients. It was observed that if the grade of the fracture increased, CSF leak also increased.

Discussion

Tomographic changes in burst fractures may be observed in different ways. Burst fractures may occur in front and at the sides of the corpus, in the middle column; while depending on PLL injury, the bone fragment may flow to the spinal canal. A whole piece of bone may continue to progress along the canal by hitting the PLC. It may come back after hitting and can be divided into pieces there. Therefore, pediculolaminar corner is extremely important, because PLC may prevent the overflowing of the bone fragment. PLC can't cope with the high energy exposed by the trauma and does not resist anymore.[6] And thus bone fragments may lead to breakage and separation at pedicles. PLC will be broken, and the bone parts will not be able to bounce back there. So the bone fragments will continue to advance in the canal as a result. So the neurological condition will be worse because of the compromise in the spinal canal.And also in our data, the neurological deficit rate increases as the grade increases. The rate of neurological deficit in grade 3 patients is as high as 72%. At this point, we think that the PLC's resistance is an important point for a compromise that may occur in the spinal canal and for the neurological situation to be encountered.

Of course, it may not always be right to say that the severity of this neurological deficit correlates with the severity of trauma and radiological images. For example, the Grade 3 radiological appearance of the patients does not always necessarily mean that the deficit will be severe. There are patients in grade 3 group with ASIA D score while the patient is among the grade 2 patients with ASIA C score. But as it is seen in our study; in grade 3 patients, the neurological deficit rate is more than the others.

If the bone fragments cause breakage of PLC and extension of interpedicular distance, it may move further and may lead to fractures at laminas and separation of laminas [7].

It has been shown in many studies that separation of pedicles worsens the clinical picture in burst fractures [8][9][10][11]. The extension of the interpedicular distance is concordant with worse clinical status and worse radiological images.

Petersilge et al. reported that 9 of 12 patients whose interpedicular distance extended had at least 50% spinal canal compromise, and this group was found to have the worst clinical picture in their study [12].

The size of the bone fragment and the degree of energy that is exposed are highly associated.

These changes as an indicator of energy that is exposed can also give a hint for secondary organ injury and neurological deficit. Therefore, the size of the bone fragment in the spinal canal can indicate organ injury and neurological deficit that may occur [13][14][15][16][17]. The bone fragments in the canal were proportional to and neurological deficit secondary organ injury in our study.

In our study, rib fractures as secondary organ injury were observed in Grade 1 patients; lung contusions were also observed in one of Grade 2 patients. Severe secondary organ injuries were observed in Grade 3 patients. In a study on the condition of PLL and the size of bone fragment protruding to the spinal canal, Hu et al. concluded that the size of the bone fragment was associated with neurological deficit [18] In this study, the size of the bone fragments was statistically evaluated according to the axial width and height on the sagittal plane in CT. The results and their relations were calculated and observed.

In the study of Dai et al., the anterior and posterior side of the bone fragment was shown to be the most relevant parameter in ASIA scoring system, and it was also demonstrated that repositioning of the bone fragments provided a significant improvement only in that parameter [19]. Therefore, the fragments

in the canal should be repositioned and attempts should be made to decrease the grade of the burst fracture.

Some studies have reported that there is not a direct relationship between the proportion of bone in the spinal canal and neurological deficit [20]. The bone fragment may move through the spinal canal due to the dynamism during the fracture and return to the corpus. The bone fragment may move back to the corpus after hitting PLL, spinal cord and PLC depending on the size of the energy.

Cerebrospinal fluid (CSF) leak in burst fractures is related to lamina fractures in which the interpedicular distance is extended, and the spinal canal is narrowed [21]. Moreover, in our staging system, the proportion of dural injury was high in stage 2 and stage 3 patients. Although we did not find significant results, the relation between the number of bone fragments in the spinal canal and the dural injury was observed to increase. It was observed that if the grade of the fracture increased, CSF leak also increased.

It is necessary to develop a new simple staging system to assess both radiological and clinical status at the same time for burst fractures that are the worst and most frequently encountered spinal traumas. This classification system will help clinical assessment of the situation. The possibility of secondary organ injuries will increase, and neurological status will worsen if the grade of trauma increases according to ASIA scoring system.

We think that the most important parameter is the extension of the interpedicular distance and the relation between the bone fragment in the canal and PLC.

In conclusion, in burst fractures, if the energy that is exposed increases, the fragment moves on and leads to neural injury and breaks the posterior component of the spine. We aimed both to simplify the classification in the burst fracture by our classification method and to give an idea about the neurological condition.

According to this classification system, secondary organ injury and neurological deficit can be an indicator of energy exposure. If energy is high, the clinical status will be worse. Thus, we can get an idea about and secondary organ injuries.

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Case Report

Palatal Melanoma: "The Silent Killer"

Georgi Tchernev[1,2*], Torello Lotti[3,4], Uwe Wollina[5]

[1]Medical Institute of Ministry of Interior (MVR), Department of Dermatology, Venereology and Dermatologic Surgery, Sofia, Bulgaria; [2]Onkoderma - Policlinic for Dermatology and Dermatologic Surgery, Sofia, Bulgaria; [3]Department of Dermatology, University of Rome "G. Marconi" Rome, Italy; [4]Department of Biotechnology, Delft University of Technology, 2628 BC, Delft, The Netherlands; [5]Städtisches Klinikum Dresden, Department of Dermatology and Allergology, Dresden, Sachsen, Germany

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***Correspondence:** Georgi Tchernev. Medical Institute of the Ministry of Interior Ringgold standard institution - Medical Institute of Ministry of Interior (MVR), Department of Dermatology, Venereology and Dermatologic Surgery, Sofia, Bulgaria. E-mail: georgi_tchernev@yahoo.de

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Abstract

Primary melanoma of the oral cavity is extremely uncommon tumour consisting approximately 0.2 - 8.0% of all melanoma cases and 0.5% of all oral malignancies. It has an aggressive behaviour and poor prognosis, with 5 – year - survival rate between 5 - 20%. The initial symptoms are often unnoticed, which lead to late diagnosis and worsening of the prognosis. Because of its infrequent occurrence, there is no well - defined classification and therapeutic protocol, in contrast to melanomas of another side. Early diagnosis and treatment are essentially linked to survival rate. We present a case of palatal melanoma in a 76 – year - old female patient, as we want to emphasise the importance of the early detection and accurate diagnosis of melanoma of oral cavity, to its influence of the therapeutic outcome.

Introduction

Primary melanoma of the oral cavity is extremely uncommon tumour consisting approximately 0.2 - 8.0% of all melanoma cases and 0.5% of all oral malignancies [1][2]. It has been called "a silent killer", because of its highlighted aggressive behaviour and early metastatic spread [1][3].

The main aetiology reason for the pathologic proliferation of malignant melanocytes along the junction between the epithelial and connective tissues is not established yet [3]. Although chronic irritation, tobacco, alcohol and formaldehyde exposure have been implicated as possible risk factors, it is considered that most of the melanomas of oral cavity arise de novo [4]. In contrast to cutaneous melanoma, no particular precursor lesion has been identified, and atypical melanocytic hyperplasia is considered as a proliferative phase [2]. Despite blue nevi, dysplastic nevi involve the oral cavity extremely rare, most often – the palate mucosa [5]. While the hard palate is the most common side of affection of melanoma of the oral cavity, the buccal mucosa is affected in one-third of the cases, as the maxillar gingiva is more frequently involved than the mandibular one [3]. Uncommonly, the tongue and mouth's floor could also be affected [6]. The onset of the disease is between 40 and 70 years, with male predominance in gender distribution [6].

The initial symptoms are often unnoticed, which lead to late diagnosis and worsening of the prognosis [1]. Patients usually seek medical help because of bleeding, pain or swelling, which are associated with vertical growth phase and progression of the disease [6][7]. Furthermore, because of the anatomic structure of the oral cavity, bone invasion and destruction are commonly seen in cases of melanoma of the hard palate [2].

Surgery is the first choice of treatment which often require subsequent reconstruction of the defects and advanced cases [3][4]. However, the 5 -year -

survival rate in advanced cases with bone destruction varies between 5 - 20% and only 6.6% more than five years, which emphasise the underhanded aggressive nature of this kind of tumours [2][7][8].

Case report

A 76 – year - old Caucasian female patient presented with two months history of moderate pain in the oral cavity, more pronounced on the left. Patient's history was free of comorbidities and medications, as well as of family history for dermatologic diseases. The conducted physical examination revealed unequally pigmented lesion, composed by nodular elements, with sharply demarcated, but irregular borders, and partially ulcerated surface, covering almost the whole hard palate, more pronounced in left. Satellite pigmentations with reticular characteristic were also established on the right side of the palate (Figure 1. a, b, c).

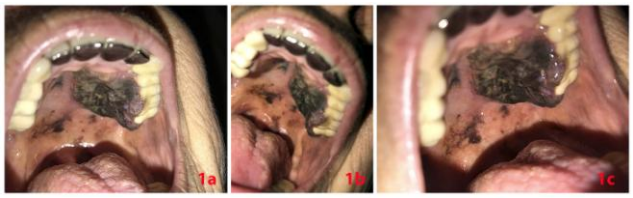

Figure 1: A, B, C - Clinical manifestation of melanoma of the oral cavity in 76-year-old female patient

The performed paraclinical blood tests established leukocytes (10.15 x 10^9/l) and monocytes count (8.8%) in the upper border of the normal range, slightly elevated glucose level in serum (6.18 mmol/l) and LDL - cholesterol (3.06 mmol/l). Imaging diagnostic procedures, including head and neck CT, obtained pathological lesion, affecting the mucosa, above the left half of palladium durum, thickened mucosa of the left maxillar sinus (due to palatial outgrowth and lamina perpendicular), 5 - 8 mm in depth, but the preserved bone structure, without a sign of destruction. This pathologic finding was also affecting the gingiva, through the alveolar border.

Enlarged lymph nodes with changes in their morphologic structure were also detected - two in left (level 2B), measuring approximately 29/40 mm and 16/24 mm and one in the right (level 2B), measuring 10/17 mm. Pathological soft - tissue nodule 15 mm in size was established, affecting the skin and subcutaneous tissue of right cheek. Cutaneous papillomatous lesion with benign characteristics was observed on left cheek. Abdominal CT examination revealed a hiatal hernia, cholelithiasis, a parenchymal and cortical cyst in left kidney. Enlarged abdominal and pelvic lymph nodes were not detected.

The diagnosis of palatal melanoma with lymph node involvement was made, based on these findings. No data for organ metastasis spread was presented. Clinically, the patient was staged in IIb (Westbury classification) and referred to maxillofacial surgery department for palatenectomy and subsequent reconstruction of the defect. Lymph node dissection was also planned. Patient's further follow up, and postsurgical screening was planned in the oncology department.

Discussion

Although extremely rare and aggressive in behavior, due to rapid hematogenous and lymph node metastatic spread, melanoma of the oral cavity could be asymptomatic flat macule for a long time (9). Because of its infrequent occurrence, there is no well - defined classification and therapeutic protocol, in contrast to melanomas of other side [2][3]. Although several authors team have been proposed different clinical and histological classification systems, unified criteria are not accepted worldwide. Therefore, Lopez et al. classified melanoma of the oral cavity, based on its clinical appearance into: 1) pigmented nodular type, 2) non - pigmented nodular type, 3) pigmented macular type, 4) pigmented mixed - type, and 5) non - pigmented mixed type [8]. Westbury has been proposed a clinical classification, similar to that for cutaneous melanoma, as follow: I - only primary tumor, II - metastasis presented (IIa - adjacent skin involved, IIb - regional lymph nodes involved, ab - adjacent skin and regional lymph nodes involved) and III - metastasis beyond regional lymph nodes [10]. According to these classification systems, our patients was classified as pigmented nodular type melanoma, with regional lymph node involvement. The lack of papillary and reticular dermis in oral mucosa, Clark's criteria for invasion, also could not be applied in cases of melanoma of the oral cavity [6]. Therefore, the classification of the Western Society of Teachers of Oral Pathology (WESTOP) have been implicated, based on histopathological pattern of the tumor, namely: (a) melanoma in situ, limited to the epidermis and its junction with the connective tissue; (b) invasive melanomas, which extend into the connective tissue and (c) melanomas with a combined pattern between invasive and in situ [6][11]. The level on invasion is determined as an independent predictor of survival, while the tumor thickness, vascular invasion, and necrosis have no significant influence on survival, as predictors of the survival rate, in contrast to cutaneous melanoma, which thickness determines the further diagnostic and therapeutic behavior in one hand, while vascular invasion is considered as the major predictor for distant metastatic spread [12]. Melanoma of oral cavity metastasizes early in its course in general [1]. Locoregional lymph node metastasis

occur in almost half of the cases during first 2 years of onset of the disease, while lungs, brain, bones and liver involvement affect up to 85% of the patients [3][13]. However, early diagnosis and treatment is essentially linked to survival rate. Diagnosis is usually made, based on the clinical appearance and histological findings, which could show a pleomorphic pattern that could mimic other tumours such as lymphomas, carcinomas, neuroendocrine carcinomas, sarcomas, and germ - cell tumours [14][15]. Positive inmunohistochemical examination for S – 100, HMB - 45, Melan A, tyrosinase and vimentin is usually helpful for confirmation, especially in atypically presented and apigmented melanomas [15][16]. However, it is postulated that mucosal melanomas may arise not only from melanocytes but also from Schwann cells in the mucous membrane [17]. The loss of heterozygosity at 12p13 and loss of p27K1P1 protein expression contribute to melanoma progression [17][18]. Surgery is the first choice of treatment, requiring collaboration with a maxillofacial specialist for reconstruction of the defect [19]. Carbon - ion radiotherapy is reported as an effective treatment option with acceptable toxicity in oral cavity's melanoma [20].

With the presented case, we want to emphasize the importance of the early detection and accurate diagnosis of melanoma of oral cavity, to its influence of the therapeutic outcome.

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Case Report

One Step Melanoma Surgery for Patient with Thick Primary Melanomas: "To Break the Rules, You Must First Master Them!"

Georgi Tchernev[1,2*]

[1]Medical Institute of Ministry of Interior (MVR-Sofia), Department of Dermatology, Venereology and Dermatologic Surgery, General Skobelev 79, 1606, Sofia, Bulgaria; [2]Onkoderma-Policlinic for Dermatology, Venereology and Dermatologic Surgery, General Skobelev 26, 1606 Sofia, Bulgaria

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Keywords: melanoma; congenital nevus; confocal; surgery; survival benefit

***Correspondence:** Georgi Tchernev. Medical Institute of Ministry of Interior (MVR-Sofia), Department of Dermatology, Venereology and Dermatologic Surgery, General Skobelev 79, 1606, Sofia, Bulgaria; Onkoderma-Policlinic for Dermatology, Venereology and Dermatologic Surgery, General Skobelev 26, 1606 Sofia, Bulgaria. E-mail: georgi_tchernev@yahoo.de

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Abstract

BACKGROUND: We present to the attention of the medical, dermatological and oncosurgical community data that serves to indicate the indispensability of optimisation of the algorithm and recommendations for diagnosis and surgical treatment of cutaneous melanoma. These recommendations could be referred to different subgroups of patients in different clinical stages as well as to patients with different initial characterisation (histological morphology) of the primary tumours. One step surgery is not a myth, even more, it could prove to be one of the best solutions for some patient collectives with advanced stages of melanoma.

CASE REPORT: We present a case of a 74 - year old patient with a congenital medium sized melanocytic nevus, located directly above the lateral part of the elbow joint. In one month and a half, an achromatic nodular formation evolves with a diameter of 2.7 x 2.3 cm, prominent over the skin level, painful by palpation and spontaneously bleeding. By the anamnestic, clinical and dermoscopic findings the patient was diagnosed with nodular melanoma associated with a congenital medium sized melanocytic nevus. A primary excision with a field of safety 0.5 cm in all directions was performed. After confirmation of the primary diagnosis (tumour thickness 8 mm with no ultrasonographic detection of enlarged lymph nodes), seven days later are - excision was performed with an additional field of surgical safety of 1.5 cm in all directions.

CONCLUSIONS: In this case remains unclear the following question: For what reason a preoperative high - frequent ultrasonography (HFUS) is not recommended to be used as it will allow only one surgical excision with the elimination of a tumour with a safety field of 2cm in all directions? The enigma about the obstacles preventing such a rational optimisation of the current diagnostic and therapeutic algorithm in patients with melanomas remains unresolved. One step surgery for cutaneous melanoma is widely used in many countries although it continues to be considered as a matter of dispute for some experts. Once again, by a clinical case and the following analysis, we would like to focus the attention of the dermatosurgical community on this crucial and highly significant problem. Innovations are very often resulting from the simplicity of logic, which unfortunately is not always accepted appropriately.

Introduction

Congenital melanocytic nevi (CMN) are benign proliferations of cutaneous melanocytic cells with incidence rate around 1% of the newborn infants [1]. They are composed of melanocytes which are grouped in focal nests in the epidermis, dermis or other tissues [2]. The definition “congenital” is expanded to melanocytic nevi that have occurred 6 months to 2 years after birth, according to different authors who explain this late occurrence with the insufficient melanogenesis or the extremely small size of the nevus postpartum [3]. Clinical classification of CMN is based on their size as following: small nevi (greatest diameter less than 1.5 cm); medium nevi (greatest diameter between 1.5 - 19.9cm) and giant nevi (diameter 20 cm or more) [4]. The most important concern related to the CMN is their malignancy potential [5]. There are many investigations that serve to evaluate the risk of malignant transformation, and at the current stage of knowledge, it is proven that the larger size of the lesion is associated with a significantly higher risk of malignant melanoma development [6]. The estimated lifetime risk for evolution in melanoma is a matter of controversies, but conforming to most of the reported medical data it is approximately 5%, depending on the size of the primary lesion (1 - 5% for small CMN, to 5 - 10% for giant GMN) [7].

There are several main problematic points in the management of patients with congenital melanocytic nevi: 1) The lack of organized and well - functioning centers for dermabrasio threatening of children in their first weeks to months after birth (concerning mainly giant congenital nevi) [8]; 2) The lack of well - trained dermatopathologists, who can quickly and accurately distinguish pseudomelanomas in infants from true melanomas (pseudomelanomas are dysplastic nevi, which in most cases are congenital small melanocytic nevi that are clinical, dermoscopically and histologically difficult for differentiating from real melanomas) [9][10]; 3) The lack of determination to more aggressive approach when it refers to medium sized melanocytic nevi, which are showing tendency of enhanced malignancy risk associated with increased age [11].

Last but not least, it should be taken into account the reluctance of some dermatologists to perform a preventive surgical resection of medium-sized congenital nevi, due to their insufficient competency level (national observations).

To establish the widespread so-called confocal laser microscopy, it is appropriate the following important facts be presented: 1,) Diagnosis melanoma is based on a clinical examination in 60% of the cases and up to 25% it is based on dermoscopic findings. In only 15% of the cases, confocal laser microscopy can give some clarity for the genesis of the lesions and whether they have to be surgically eliminated [13][14]. 2) Confocal microscopy has its limitations in certain areas of the human body [15][16][17][18].

Additionally, it has been found that the multifactorial genesis of melanomas, particularly in patients with dysplastic nevi syndrome shows various genetic mutations within a single patient, but also in every single lesion [19][20]. In simple terms, different nevi whether dysplastic or not, show diverse tendency and speed of nevus – to - melanoma evolving within the life of each patient. This means that two congenital or dysplastic nevi which seem to have completely identical clinical, dermoscopic, confocal - microscopic and even histological appearance, show entirely different malignisation tendency within an equal period in the same patient. It is interesting to be noted that the mutation analysis of several nevi in one patient shows significant differences [21][22][23]. This leads us to the conclusion that the personalisation of the medicine, in general, is inevitable even though it is still hard to achieve it by now. This particular reasoning underlay the logical statement that algorithms and high technologies could provide some advantages in the treatment of skin tumours, but they could by no means be equivalent or even a percentile equivalent of human logic. Melanoma guidelines suffer from lack of case – by - case personalisation and this leads to an inability of optimising the ultimate results.

Case report

A 74 – year - old female patient presented to the department of dermatologic surgery because of a nodular lesion with signs of malignancy, evolved within the borders of middle-sized congenital nevus. The lesion is located in the lateral brachial region of the right arm and has occurred one month and a half ago. The patient noticed rapidly increase in size and regular spontaneous bleeding. Local pruritus, pain and paresthesia were reported as additional subjective complaints.

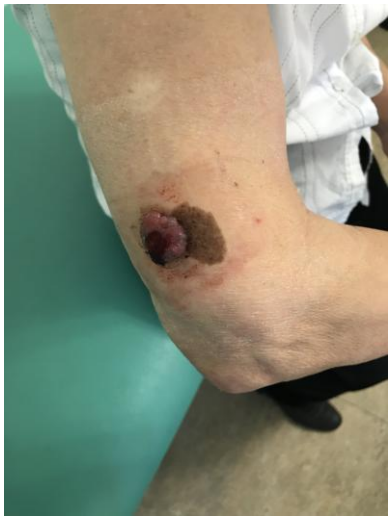

Figure 1: Clinical manifestation of nodular melanoma associated with congenital medium sized melanocytic nevus with Breslow thickness 8 mm, located in the lateral brachial region of the right arm of a 74 - year old patient

Clinical examination observed brown pigmented macula with a diameter of 6.3 x 4.1cm, irregular borders and uneven distribution of colour. On approximately half of its size, an elevated nodule with diameter 2.7 x 2.3 cm, asymmetrical shape, dark red colour and irregular borders with central bleeding erosion is situated (Figure 1).

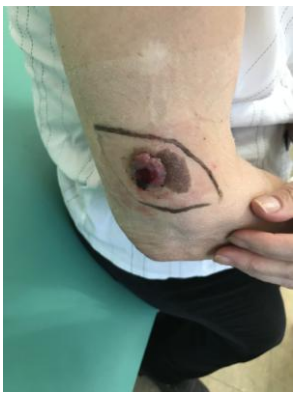

Figure 2: Preoperative surgical skin marking with 0.5cm filed for safety in all directions

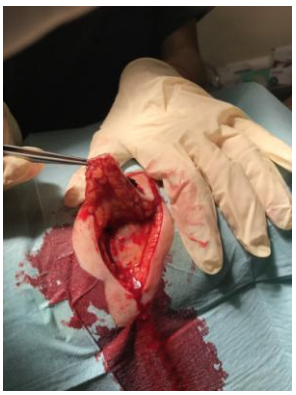

Figure 3: Elliptical surgical excision of the lesion under local anaesthesia

No enlarged lymphatic nodes were identified by palpation. Conducted paraclinical examinations revealed elevated ESR – 52 mm/h (< 39 mm/h); WBC – 12.01 /μl (3.5 - 10.5 /μl); Neu – 8.990 μl (1.900 - 7.900μl); GGT – 43 U/l (6.00 – 40 U/l); CRP – 5.70 mg/l (< 5 mg/l). Chest radiography detected poorly expressed emphysematous and fibrous changes.

Figure 4: Elliptical surgical excision of the lesion under local anesthesia

The right paracardial and basal regions are showing linear non homogenous infiltrative changes probably due to small pleural effusion or adhesions. Normal cardiac silhouette was found.

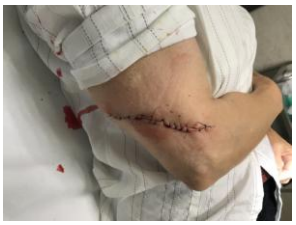

Figure 5: Wound closure with simple interrupted sutures

Ultrasound examination did not detect any axillar, cervical or inguinal enlarged lymphatic nodes. The liver was no focal changes, sharp borders and homogenous structure. The lesion was removed by surgical excision under local anaesthesia, with 0.5 cm field of safety margins in all directions (Figure 2 - 5).

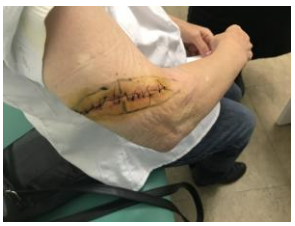

Figure 6: Preoperative surgical skin marking of the re-excision with 1.5 cm field for safety in all directions

Histological examination of the cutaneous lesion revealed nodular malignant melanoma with tumour thickness 8mm (Breslow), Clark IV, with no signs of spontaneous regression, high mitotic activity, epidermal erosion, insignificant lymphocytic stromal reaction and clear resection margins.

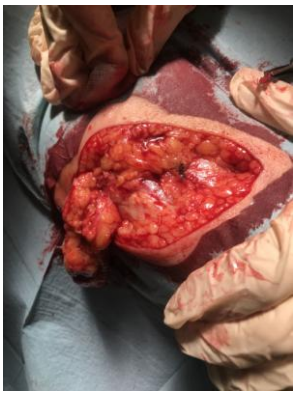

Figure 7: Wide elliptical surgical re-excision under local anaesthesia

The patient was diagnosed in stage IIC and underwent reoperation with 1.5 cm field of safety (Figure 6 - 9). Afterwards was referred for registration in oncologic dispensary for regular monitoring.

Figure 8: Wide elliptical surgical re-excision under local anaesthesia

Discussion

In the era of so-called personalised medicine, the current solutions for diagnosis and treatment of various diseases often are and should be challenged.

There are numerous factors that motivate the nation - following of certain guidelines but taking individual decisions for the therapeutic approach of a patient instead. Malignant melanoma should be considered as one of the most illustrative examples of such a non - standard model.

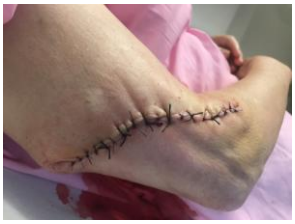

Figure 9: Wound closure with simple interrupted sutures

Critical reviews of the standard surgical treatment should not surprise the so-called experts because of four main facts and circumstances, as follows:

1) In the controversy with the great medical progress, we observe that even though pathogenesis of melanoma is multifactorial, the therapy is often (considered 2 years later) identical, regardless of the newly introduced target therapies.

2) It is unclear why melanomas over 8mm or 16mm do not evolve locoregional or distant metastases compared to significantly thinner melanomas, which show high metastatic tendency and extremely aggressive potential [24][25].

3) Rapidly changing therapeutic strategies for treatment of melanoma indicate a serious deficiency of orientation and a kind of helplessness among the medical community towards this never-ending problem.

4) Medical centres have different access possibilities which are reflecting in diverse approaches to patients in general. Why OMICS analyses are available for certain collectives, and not for others? Isn't this some high - tech personalised medicine which is available for a limited number and types of patients?

All these facts lead our minds to the logical question concerning not the difficulty of the pathogenesis or the target therapy, but the significantly more simplified surgical treatment: Isn't there any possibility for alleviation of the surgical treatment and reduction in the number of therapeutic interventions as well as the chances of incorrect assessment of the preoperative status? It is a simple question whether these factors can be somehow limited? And we believe that the answer is - definitely YES!

By the presented case, we would like to express our critical view regarding the lack of any individual approach in the recommendations of melanoma treatment in Europe, the US and worldwide at least for some collectives of patients. In the case of our patient two medium - sized surgical interventions were performed with a favourable outcome despite the initial 8 mm tumour thickness. In cases of melanoma, over 4 mm and no locoregional metastases a sentinel lymph node biopsy and lymphadenectomy are not recommended. Re - excisions, however, are. An open question remains - why in this initially clear clinical and dermoscopic case, guidelines do not recommend preoperative HFUS for detecting of the tumour thickness? Then, depending on the ultrasonographically measured thickness, only one single surgical excision could be performed?! In less thick melanomas this approach would lead to primary excision of the lesion with or without a sentinel lymph node biopsy at once, in a single surgical session. The surgical field of safety would be 1 cm or 2 cm in all directions, depending on whether the ultimately established thickness of the tumor is under or more than 2 cm [26]. This approach would be limited in cases of achromatic melanomas so they should be excluded from the category of tumors appropriated for this strategy. A possibility for their inclusion in the one - step melanoma surgery would be the use of confocal microscopy and/or cytological analysis in combination with immunohistochemical methods [27][28].

Although this concept would be considered as "frivolous" by many experts, the number of reduced surgical interventions and the optimization of the approach, in general, would lead to 1) Reduction of healthcare costs, 2) Limited possibilities of different mistakes by the therapists and patients (occurring between the two surgical interventions) and as an ultimate and most important outcome - 3) Long-term survival of the affected patient collectives would be expected.

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Case Report

Successful Craniotomy for Advanced Basal Cell Carcinomas with Cranial Bone Invasion and Dura Mater Infiltration - Unique Presentation in a Bulgarian Patient

Slavomir Kondoff[1], Atanas Drenchev[2], Torello Lotti[3], Uwe Wollina[4], Ilia Lozev[5], Ivan Pidakev[5], Ivan Terziev[6], Yavor Grigorov[7], Serena Gianfaldoni[3], Georgi Tchernev[8,9]*

[1]Okrajna Bolnica - Neurosurgery Sofia, Sofia, Bulgaria; [2]University Hospital Saint Anna - Neurosurgery Sofia, Sofia, Bulgaria; [3]University G. Marconi of Rome, Institute of Dermatology, Rome, Italy; [4]Städtisches Klinikum Dresden, Department of Dermatology and Allergology, Friedrichstrasse 41, Dresden, Germany; [5]Medical Institute of Ministry of Interior, Department of General, Vascular and Abdominal Surgery, General Skobelev 79, 1606 Sofia, Bulgaria; [6]University Hospital Tsaritsa Ioana, Department of Common and Clinical Pathology, Sofia, Bulgaria; [7]Department of Orthopedics and Traumatology, University Hospital Lozenetz, Koziak 1 Street, Sofia, Bulgaria; [8]Medical Institute of Ministry of Interior (MVR), Department of Dermatology, Venereology and Dermatologic Surgery, General Skobelev Nr 79, Sofia, Bulgaria; [9]Onkoderma - Policlinic for Dermatology and Dermatologic Surgery, General Skobelev 26, Sofia, Bulgaria

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Keywords: Surgery; Craniotomy; Basal cell carcinomas; Treatment outcome; Treatment approach

***Correspondence:** Georgi Tchernev. Medical Institute of Ministry of Interior (MVR), Department of Dermatology, Venereology and Dermatologic Surgery, General Skobelev Nr 79, Sofia, Bulgaria. E-mail: georgi_tchernev@yahoo.de

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Abstract

BACKGROUND: Basal cell carcinomas (BCC) located in the sun-exposed regions are a serious therapeutic challenge. Therefore early diagnosis and adequate therapy should be of a high priority for every dermatologic surgeon.

CASE PRESENTATION: We are presenting a patient with multiple BCCs, located on the area of the scalp, who had been treated several years ago with electrocautery and curettage after histopathological verification. However, the last few years the tumours have advanced, infiltrating firstly the tabula external and a year later the tabula interna of the cranium. A computed -tomography (CT) imaging and radiography of the skull were performed to reveal the definite tumour localisation, needed for planning an one - step surgical intervention. Both of the instrumental examinations confirmed the existence of osteolytic tumour lesions. Craniotomy with precise removal of the BCCs infiltrating the cranial bone in all of its thickness was performed. Partial resection of dura mater was also performed also because intraoperative findings established the involvement of the dura. Histopathological verification revealed bone and dural invasion with clean resection margins. The bone defect was recovered with hydroxyapatite cement. Reconstruction as the shape of the skull was carefully modified and adapted to its initial size and form. Layered closure of the skin and soft tissues were performed after the complete removal of the BCCs. The postoperative period had no serious complications.

CONCLUSION: Precisely managed therapy of BCC is curative in most of the cases as it ensures good prognosis for the patient.

Introduction

Basal cell carcinoma (BCC) is non - melanocytic skin epithelial tumour arising from the basal layer cells of the epidermis [1]. In the last few years, world statistics show rapidly increasing incidence rate as the lifetime risk is reaching nearly 30% [2]. Although BCC does not demonstrate significant metastatic tendency, its local destructive and infiltrative nature, as well as its tendency to receive turns, is into a serious medical problem, which should not be neglected [3]. Since exposure to UV - radiation is the main etiological factor of BCC, prevalent locations of the lesions are the face and the head, and scalp is the most commonly affected area [4]. Behind the acronym “SCALP” stands its five structural layers - skin, subcutaneous tissue, aponeurosis, loose areolar tissue, and periosteum. In cases of highly progressive local invasion, the tumour process infiltrates galea aponeurotica, periosteum, calvaria, superficial and deep layers of dura mater and the underlying brain [5] successively. At this stage, the

invasion of deeper tissues compromises treatment opportunities for achieving an optimal therapeutic result; it reflects on the long-time survival of the patient and increases healthcare costs as well [6].

Therefore, precise diagnostic approach and accurate therapeutic strategies are mandatory for prevention of any further complications which at a later stage could be fatal.

Case report

We present a 68 – year - old patient with multiple primary infiltrative BCCs in the scalp area initially treated 14 years ago with superficial contact X-ray therapy, end does 60 greys, followed by electrocautery (x2) several years later (Figure 1a). He presented to the dermatologic policlinic for diagnosis and therapy of two newly - formed pigmented lesions located in the left parietal region. Also, two chronic non - healing ulcerative wounds were observed in the same area which had occurred 6 years ago according to anamnestic data. An uncomfortable, itchy, burning sensation in the region was reported as a subjective complaint (Figure 1a - d). Somatic and neurological status as well as paraclinical assessment and chest X-ray examinations did not show any abnormalities. Profile radiography of the skull detected two osteolytic zones with irregular borders in the parietal region; no structural changes were observed.

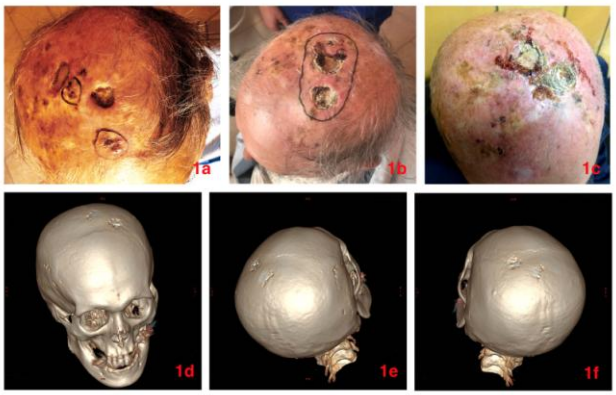

Figure 1: a) Clinical suspicion of 2 pigmented basal cell carcinomas, located next to the area of 2 ulcerated lesions. The ulcerated lesions are histologically confirmed as basal cell carcinomas; b) One year later wide expansion of the ulcerative lesions is observed with the addition of pain and bleeding; c) 4 months later 2 hyperkeratotic tumor formations with blood/yellow discharge have appeared; d - f) CT - examination of the lesions revealed progression in depth and involvement of tabula interna of the tumor process (one year earlier CT - examination detected tumor infiltration only in tabula external)

Cranial computed - tomography (CT) examination performed in June 2017 revealed two deformities in the form of tumour-mediated osteolysis, affecting the diploe of the tabula externa on the left parietal and parasagittal areas. Several months later, in November 2017 second cranial CT examination detected progression of the infiltrative process as two zones of osteolytic changes, affecting the tabula externa and the diploe of tabula interna (Figure 1d - 1f).

Complete excision with removal of periosteum and partial removal of the tabula externa was performed in collaboration with the neurosurgical team (Figure 2a, 2b). Intraoperative findings showed tumour infiltration of the parietal bone and the superficial layer of dura mater. This neoplastic formation was surgically removed in maximal safety margins. Thermal ablation of dura mater was performed as the tabula interna remained intact (Figure 2c - 2e). Hydroxyapatite cement was used for reconstruction of the cranial bone defect (Figure 2f - 2g). After meticulous haemostasis and layered soft tissue suturing, the surgical wound was covered with a sterile Bactrigrass dressing. The patient was referred to the plastic surgery department for reconstruction of the skin defect.

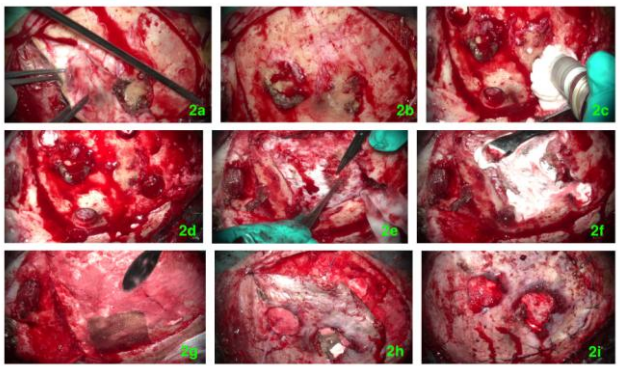

Figure 2: a) Careful dissection of the skin around the tumor with a wide margin of surgical safety; b) Skin defect as a result from complete dissection of the skin around the area of the tumors; c) In the 4 corners of a provisional rectangle surrounding the tumors 4 defects are situated via high-frequency drill with a set of specific heads. Dura mater remains intact. Severe bleeding was stopped with an electric knife; d) Clinical finding after locating of additional bone defects in the calvarium region; e) Careful removal of the cranial parts infiltrated by a tumour as well as part of dura mater with neoplastic involvement. Haemostasis; f) Applying hydroxyapatite bone cement for reconstruction of the cranial defect; g) Precise adaptation of the cement before hardening; h) Layered soft tissue closure after the surgical removal of the lesions; i) Postoperative status after adaptation

Histological examination revealed basal cell carcinoma invasion with clear surgical margins.

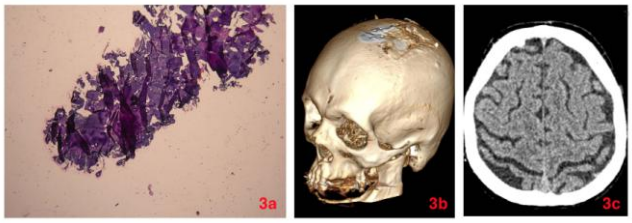

Figure 3: 3a – Histopathological data of basal cell carcinoma infiltrating the bone. Unsuccessful pathologic sample; 3b – Routine postoperative CT-imaging of the patient is showing relatively good adaptation of the cement to the normal cranial structure; 3c – Early postoperative CT of the patient. Inapparent finding. CT 3 days after surgical approach with cranial bone resection because of postoperatively reduced sensitivity of the right part of the body, suspected for ischemic insult

Discussion

We present to the dermatological and oncosurgical community a clinical case of a patient with basal cell carcinoma who developed recurrent neoplastic lesions with the progressive invasion of the skull due to incorrect treatment. The main principle for treatment of any malignant lesions including BCC is radical surgical elimination insufficient field of surgical safety [7].

As it is stated in the National Comprehensive Cancer Network (NCCN), the aim of treatment for BCC is the elimination of a tumour with maximal preservation of function and physical appearance [8]. The therapeutic strategy should be individualised to every patient according to the size, location and depth of a tumour as well as comorbidity and additional examination findings [9]. However, in any case, there is a simple rule of great importance that should always be followed when it refers to surgical management, and it is the definitive requirement of radical surgical approach. Any ignorance of this principle is a potential triggering factor for neoplastic development [10]. For this particular reason in most cases radical surgical excision with histopathological evaluation and regular dermatological follow up is the first line treatment for BCC [11]. In cases of more difficult to treat lesions, Mohs micrographic surgery is considered an eligible and reasonable option [12].

There are various alternative non - surgical methods for the treatment of BCC [13]. Radiation therapy is a standard therapeutic option for patients with contraindications for surgery, but it can also be used as adjuvant therapy [14]. However, according to the Guideline recommendations on BCC, it is not recommendable as first-line treatment if surgical excision is possible [15]. Curettage and cautery, as well as cryosurgery and laser ablative therapy, show variable recurrence rates and may be considered as a good treatment only for low-risk BCC [16]. Local therapy with chemotherapeutic and immune - modulating agents such as topical Imiquimod 5% or Fluorouracil may be indicated in some cases of small and superficial BCC [17]. Topical photodynamic therapy is another option, appropriate for superficial and thin nodular BCC in patients with large or multiple lesions and those in sites of high cosmetic importance [18].

A Hedgehog (Hh) pathway inhibitor (Vismodegib, Sonidegib) can be used for locally advanced BCC in patients with contraindications for surgical or radiation therapy as well as for post-treatment recidives and metastatic forms of BCC [19].

Unfortunately, according to the anamnestic data of our patient it can be concluded either that he had been treated several times with an inadequate treatment modality or that the electrocauterisation was not performed in enough margins.

Although BCC in the area of the head is commonly seen, the risk of involvement of the skull and dura mater is extremely rare with an estimated incidence of 0.03% [20]. According to a PubMed search, there are only 13 cases of BCC of the scalp with intracranial tumour invasion described in the world literature till now. Local excision of the scalp in combination with craniectomy with dural resection (if needed) is the standard surgical treatment in such cases [21]. It is followed by reconstruction of the bone defects (cranioplasty) using fascial graft for dura mater, and bone cement (calcium hydroxyapatite) and titanium mesh for the skull [22]. Skin and soft tissue defects are reconstructed using island flaps, rotational flaps or free tissue transfer [23][24]. Depending on the depth of tumour invasion, curettage of the tabula externa is a less traumatic therapeutic option which does not need any further osteoplasty. It is indicated in limited cases where the tumour process has superficial spreading and evolves only part of the cranium's thickness [25]. Both surgical techniques require clear surgical bone margins [26]. Surgical management of BCC is a serious challenge for the operating team in cases of neglected patients or histopathological subtypes of BCC with aggressive behaviour, represented as rapid growth into large sizes [27].

It is important to keep in mind that an infiltrative tumour process can be inapparent for a long time which reaffirms that every lesion should not be evaluated but removed concerning all principles of the good oncosurgical practice [28]. Precisely managed therapy of BCC is curative in most of the cases as it ensures good prognosis for the patient [29]. However, it shows high recurrence rate with risk of extensive invasion if there is not complete surgical removal [30].

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Case Report

Bullous Tinea Incognito in a Bulgarian Child: First Description in the Medical Literature!

Georgi Tchernev[1,2*], Ivan Terziev[3]

[1]Medical Institute of Ministry of Interior (MVR), Department of Dermatology, Venereology and Dermatologic Surgery, Sofia, Bulgaria; [2]Onkoderma, Policlinic for Dermatology and Dermatologic Surgery, Sofia, Bulgaria; [3]University Hospital Tsaritsa Yoana, Common and Clinical Pathology, Sofia, Bulgaria

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Keywords: Tinea incognito; Bullous Tinea; Therapeutic approach; Complete remission; Imitator

***Correspondence:** Georgi Tchernev. Medical Institute of Ministry of Interior (MVR), Department of Dermatology, Venereology and Dermatologic Surgery, Sofia, Bulgaria; Onkoderma - Policlinic for Dermatology and Dermatologic Surgery, Sofia, Bulgaria. E-mail: georgi_tchernev@yahoo.de

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Abstract

For the first time in the world medical literature, we describe a rare form of cutaneous dermatophytosis – a bullous form of Tinea incognito, classified by clinical picture, histopathological findings and an isolated infectious agent from the microbiological culture. After a thorough review of Medline/PubMed's relevant literature, we could not find similar cases of patients with Tinea incognito who are clinically presented with bullous lesions at the same time. Local application of corticosteroids in infants with unknown lesions may lead to progression of the underlying disease and may cause some serious problems in differential diagnosis aspect, while the clinical expression remains completely masked. Exactly for this reason, right at the beginning of the clinical complaints, a skin biopsy should be obligatorily performed in parallel with microbiological swabs. If there is no improvement after the local corticosteroid application, then diagnosis revision and change of the strategy of clinical behaviour would be appropriate to be done. The systemic treatment that we performed with Fluconazole 50 mg in combination with the local antimycotic agent for a 2-week period led to complete remission.

Introduction

Tinea incognito is practically a classical mycosis treated with a topical corticosteroid, which leads to demasking of clinical symptoms and often to the imitation of another type of skin disease [1]. Microsporum Canis is a dermatophyte fungus in which cats and dogs are recognised as the natural hosts [2].

M. canis is also easily transmitted to humans, causing lesions to the glabrous skin (tinea corporis) and the head (tinea capitis) [2]. Depending on the severity of clinical symptoms, systemic treatment could last between 2 weeks and approximately a month. It is recommended that topical therapy is given for at least 3 weeks.

Case Report

The case of a 3.5 – year - the old female patient is at this moment reported, presented in “ONKODERMA” dermatology, venereology and dermatological surgery ambulatory for a newly developed painful plaque localised laterally on the integument. Symptoms occurred approximately 4 weeks ago. The initial complaints of the patient were related to the recent occurrence of several itchy small papules which, following the assigned treatment with 0.1 % Methylprednisolone aceponate cream and systemic antihistamine – desloratadine 5mg once daily for 7 days, rapidly increased in size and reached confluence each other. When changing the topical

therapy to clobetasol propionate 0.5 %/g containing cream twice daily, additional blister formation and rapid increase in the initial size of the lesion in the peripheral direction (Figure 1a, 1b) were observed. During the clinical examination, a plaque formation of 8 cm to 5.3 cm were found, with the impression on the periphery of the lesion of 1) its bullous character at the peripheral edge of the entire lesion in the distal direction, and 2) secretion of serous to slightly yellowish color when mechanically induced rupturing of the blisters (Figure 1a, 1b).

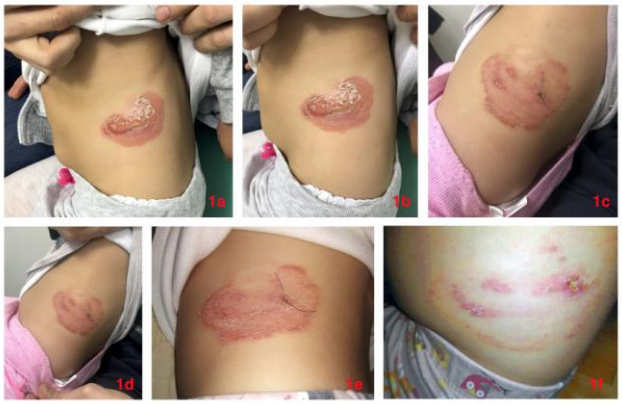

Figure 1: a, b: Clinical picture of a 3.5 – year - old child with Tinea incognito, manifested as a solitary vesiculobullous plaque laterally on the integument; c, d, e: Significant improvement in the clinical status after 1 - week treatment with Flutrimazole 1% solution in combination with Miconazole nitrate/hydrocortisone containing cream. The lesions were dry, no nodules observed. Elevated peripheral edge of the lesion and the fine diffusive desquamation characteristic of dermatophytosis are primarily observed; f: Week 2 of the treatment. Lack of fine diffusive desquamation, residual stripy erythemas in the periphery

Centrally, ruptured nodules with dried exudate and remnants of the extemporaneous agents were observed (Figure 1a, 1b). The patient was diagnosed with the working diagnosis of Tinea incognito, a bullous variant, and a bullous variant of mycosis fungiodes and Sweet syndrome was also discussed as a possible differential diagnosis. The histopathological findings were non-specific (HE staining, Figure 2a) and demonstrated: nonspecific dermal inflammatory infiltrate, as the inflammatory cells expressing CD3, CD4 and CD8 equally, and no mucosal fungoides - specific clonal expansion (Figure 2a - 2d). No c - kit and CD30 expression, no CD - 20 expressions. Blankophor staining was negative, PCR testing in lesion tissue scales was also negative, microbial swabs were negative for bacterial growth. The culture identified Microsporum Canis as an infectious agent.

The patient received systemic treatment with Fluconazole 50 mg daily for 2 weeks in combination with Flutrimazole 1 % solution in combination with Miconazole nitrate/hydrocortisone containing cream 2 times daily for a total of 7 days (Figure 1c - 1e). Subsequently, the topical therapy was changed to Flutrimazole 1% cream twice a day over a three-week period (Figure 1f). Complete remission was achieved.

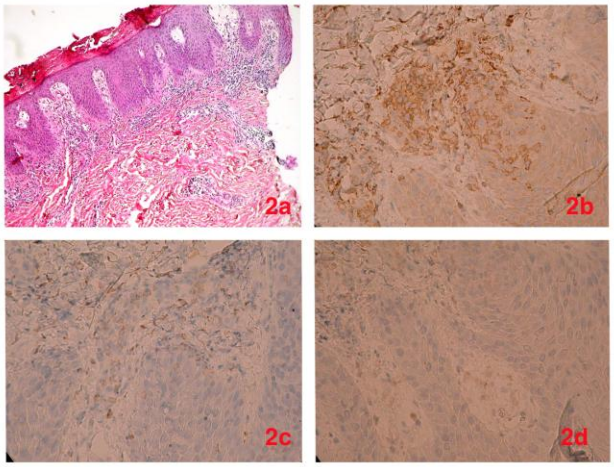

Figure 2: a: Non - specific dermal inflammatory infiltrate, HE staining; b: Inflammatory cells expressing CD3; c, d: Inflammatory cells equally expressing CD4 and CD8. No evidence of T - cell lymphoma clonal expansion

Discussion

The cases of patients with a bullous type of Tinea incognito described in the literature are few, with infectious dermatosis being the most common cause: Microsporum Canis, Microsporum gypseum, Trichophyton mentagrophytes, Trichophyton rubrum and Trichophyton violaceum [3] [4] [5] [6].

The complex diagnostic approach in the initial phase of the disease is crucial for the correct diagnosis, as well as for avoiding unnecessary risks that arise secondary to the progression of the infection as a result of the inadequate initial therapy.

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Dental Science

Oral Hygiene Index in Early Childhood Caries, Before and After Topical Fluoride Treatment

Olga Ratko Kokoceva-Ivanovska[1*], Olivera Sarakinova[1], Efka Zabokova-Bilbilova[1], Aneta Nikola Mijoska[2], Natasha Stavreva[2]

[1]Department for Pediatric and Preventive Dentistry, Faculty of Stomatology, Ss Cyril and Methodius University of Skopje, Skopje, Republic of Macedonia; [2]Department for Prosthodontics, Faculty of Stomatology, Ss Cyril and Methodius University of Skopje, Skopje, Republic of Macedonia

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Keywords: OHI-index; Early childhood caries; Initial lesion; Superficial lesion; Fluoride treatment

***Correspondence:** Olga Ratko Kokoceva-Ivanovska. Faculty of Stomatology, University "Ss Cyril and Methodius" Skopje, Skopje, Republic of Macedonia. E-mail: olga.kokoceva@gmail.com

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Abstract

BACKGROUND: Circular caries occurs in the earliest age of the children (1 - 1.5 year), immediately after the eruption of the deciduous teeth. During this period, children are too young to be able to properly implement oral hygiene. Consequently, it is at a negligible level, with plenty of soft plaque on the deciduous tooth surfaces.

OBJECTIVE: The main objective of this clinical trial was to determine the correlation between oral hygiene shown with Oral Hygiene index, and the initial stages of circular caries (initial lesion and superficial form), before and after topical fluoride treatment.

MATERIAL AND METHODS: For determination of the OHI - index we used the method of Green - Vermillion. It was determined two times in 117 patients, during the first visit and immediately before physiological replacement of deciduous teeth. Patients were two to three years old and diagnosed with initial stages of circular caries. Amino fluoride solution was applied once a week, during six months.

RESULTS: We obtained statistically significant improvement of OHI - index at the end of the test, among treated subjects from both major groups.

CONCLUSION: It can be concluded that the level of oral hygiene is correlated with the progression of changes in enamel. Topical fluoride treatment has a positive impact on reducing ECC.

Introduction

The circular cavity appears in the earliest age of the child (1 - 1.5 year), immediately after the eruption of deciduous teeth. The characteristic of this decay is that it occurs circularly in the gingival third of the tooth, and is called circular cavity [1]. Jacobi described it first in the 1862 year, and today it is also known as baby bottle caries or nursing bottle caries [2]. Meanwhile, latest scientific literature adopted term Early Childhood Caries (ECC) [3].

There are different data for prevalence of the disease depending on the geographic territory, and they vary from 3 to 45 %. ECC is also widely present in pre-school children in Macedonia, with 17.9% in children n aged 1, 5-3 in central areas of the capital Skopje, which according to WHO is high prevalence [4].

American Academy of Pediatric Dentistry defined early childhood caries (ECC) as the presence of one or more decayed (noncavitated or cavitated lesions), missing (due to caries), or filled tooth surfaces in any primary tooth in a child 71 months of age or younger [5]. In children younger than three years of age, any sign of smooth - surface caries is

indicative of severe early childhood caries (S - ECC). During this period the children are still too young to be able to implement oral hygiene properly. It is at a negligible level, with plenty of soft plaque on the tooth surfaces of deciduous teeth [6].

The disease has multifactorial aetiology like feeding and hygiene habits, while microbial investigations showed the presence of Mutans Streptococci (MS) and Lactobacillus. For some authors, the most important etiological factor is the defect of the structure of substances adamantine in deciduous teeth, whose mineralisation starts in the fourth month of the fetal life [7]. Although prenatally formed substantia adamantine is healthier and homogeneous with better structure, yet some systematic, infectious and chronicle diseases (diabetes, malnutrition) can have a negative impact [8]. Over 20 -50% of the mothers with pathological pregnancy have children with ECC. Premature children are with 37% higher prevalence of the disease.

Children who are breastfed have less ECC, but when prolonged it can also be concluded as a risk factor. Parents are recommended to avoid feeding bottle after the first year and to start using cups as soon as possible. Drinks with sugar (milk, tea and juices) and in between meal consumption of sugar-containing snacks or drinks should also be eliminated from the everyday diet. Infants should not be put to sleep with a bottle filled with milk or liquids containing sugars. Presence of ECC is also with higher risk of new carious lesions in the primary and permanent dentitions [9][10].

Early prevention of the disease is critical, and best treatments are brushing teeth with fluoride paste twice a day, and professionally applied topical fluoride treatments. The recommended professionally - applied fluoride treatments for children at risk for ECC who are younger than six years is five percent sodium fluoride varnish (NaFV; 22,500 ppm F) [11]. In recent decades circular cavity tends to be in an even greater prevalence and a problem for children, parents and us dentists. Therefore, we should devote special attention, in many ways.

The aim of this clinical study was to determinate the correlation between oral hygiene shown with OHI - index, and the emergence of the initial stages of the circular cavities: initial lesion (macula Alba) and superficial form, before and after topical fluoride treatment.

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Children who are breastfed have less ECC, but when prolonged it can also be concluded as a risk factor. Parents are recommended to avoid feeding bottle after the first year and to start using cups as soon as possible. Drinks with sugar (milk, tea and juices) and in between meal consumption of sugar-containing snacks or drinks should also be eliminated from the everyday diet. Infants should not be put to sleep with a bottle filled with milk or liquids containing sugars. Presence of ECC is also with higher risk of new carious lesions in the primary and permanent dentitions [9][10].

Early prevention of the disease is critical, and best treatments are brushing teeth with fluoride paste twice a day, and professionally applied topical fluoride treatments. The recommended professionally - applied fluoride treatments for children at risk for ECC who are younger than six years is five percent sodium fluoride varnish (NaFV; 22,500 ppm F) [11]. In recent decades circular cavity tends to be in an even greater prevalence and a problem for children, parents and us dentists. Therefore, we should devote special attention, in many ways.

The aim of this clinical study was to

determinate the correlation between oral hygiene shown with OHI - index, and the emergence of the initial stages of the circular cavities: initial lesion (macula Alba) and superficial form, before and after topical fluoride treatment.

Materials and Method

The study was clinical trial performed on patients from the Department of pediatric and preventive dentistry at the University Dental Clinic "Ss Paneteleimon" in Skopje, Macedonia. The patients (total number 117) diagnosed with initial stages of circular caries, aged two to three years old, and were divided into two groups. The earliest stage of the circular cavity was diagnosed in two ways:

-Observing the slightest change in the transparency of the enamel in the form of white patch with no cavitations as initial lesion - macula Alba;

- Inspection and sondage of the changes in the enamel in the form of an initial cavity diagnosed as a superficial form of a circular cavity.

In both groups regular check-ups were performed once a month, including following procedures: removing of the present soft plaque from the teeth; advice for improving patient's diet; advice for maintaining proper oral hygiene; determination of the index of oral hygiene - OHI and clinical monitoring of the initial stages of the circular cavity until physiological replacement of teeth.

Patient's parents were presented with the study protocol, with a complete explanation of the procedure, and their full written consent was obtained. They were also asked to fill out a questionnaire about the usual habits of maintaining oral hygiene and frequency of daily teeth brushing.

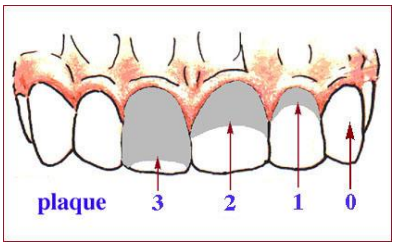

Figure 1: Method of scoring the presence of soft sediments

Determination of the Oral Hygiene Index (OHI)

OHI index (Oral Hygiene Index) shows patient's oral hygiene and express the presence of plaque on the surface of the teeth. OHI allows determination of a presence of the dental plaque, material-alba, and food residues. The most commonly used index for determining the soft sediments is a Green – Vermillion - Hirschman index (Figure 1).

There are three stages of soft plaque presence according to the method of Green - Vermillion (Table 1). This method simply allows us to investigate and determine the numerical presence of soft plaque and classified into four classes from 0 to 3. The index was determined twice in our patients: during the first visit to the clinic when we diagnosed the disease, and immediately before physiological replacement of deciduous teeth.

Table 1: Presence of soft plaque accumulation

Points	*Presence of soft plaque*
0	no soft plaque presence
1	1/3 of the tooth surface covered with soft plaque
2	1/3 to 2/3 of the tooth surface covered with soft plaque
3	more than 2/3 of the tooth surface covered with soft plaque

Aminofluoride application

Aminofluoride solution (Aminfluorid otopina®, Belupo, Croatia) with 12.140% of ZV,N,Na-tri- (2 - hydroxyethyl) - Na - octadecyl - 1,3 - di-aminopropane-dihydrochloride (I) and 1.135% of 1 - amino – 9 - octadecene hydro fluoride (II) (which corresponds to a total fluoride content of l.000%), with pH 3.8, was used for topical fluoride treatment, once a week for 6 months. Both fluoride components are surface active, adhere closely to enamel, and provide long-term contact.

After a thorough cleaning of the teeth with polish paste and brush, we applied the solution with cotton for 2 minutes. Patients were advised not to take any food and liquids in next 30 minutes.

Statistical Analysis

Presented data were statistically analysed with Statistical program SPSS for Windows 7. We were using standard deviation, Student - t test, Wilcoxon Matched Pairs Test and Mann Whitney U test.

Results

We analysed the effect of teeth brushing (twice a day, for at least two minutes) for removing dental plaque with a questionnaire. The analysed data for oral hygiene maintaining habits and daily frequency of brushing teeth in children showed that most of our examinee (56%) did not brush their teeth at all. Only 32% of the patients brushed their teeth once a day and just 12% twice a day. The results from the questionnaire about oral hygiene habits in our patients are presented with the pie in Figure 2.

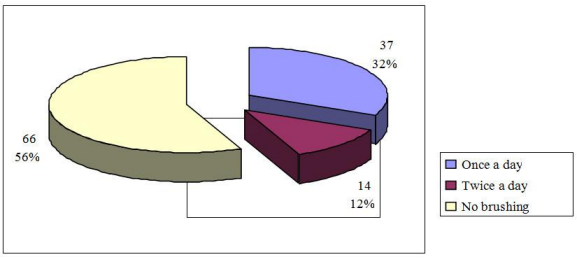

Figure 2: Daily habits for maintaining of oral hygiene and brushing teeth frequency in children

The values for OHI index were calculated during the first visit of the patients at the beginning of the investigation, and before the physiological change of the teeth. Patients with initial changes (Macula Alba) were selected, and the values of their index are shown in Table 2.

The first group of the patients was treated with amino fluoride varnish, and the control group was the patients whose parents did not accept fluoride treatment but wanted regular checkup and plaque removal. The total number of the examinee with the initial lesion was 61, of which 31 untreated and 30 treated with a topical fluoride treatment.

Table 2: Values of OHI-index in untreated and treated subjects with initial lesion (MaculaAlba)

Initial lesion (macula alba) examinee (N = 61)							
Non-treated 31				Treated 30			
Evaluation period	OHI-index			Evaluation period	OHI-index		
	0-1	1.1-2	2.1-3		0-1	1.1-2	2.1-3
First visit %	3	7	21	First visit %	2	11	17
	9.68	22.58	67.74		6.67	36.67	56.67
Before physiological replacement	15	11	5	Before physiological replacement	21	9	0
	48.39	35.48	16.13		70.00	30.00	0.00

In both patients groups (untreated and treated) with *initial lesion*, there was statistically significant difference of OHI - index compared with the first visit and at the time for physiological replacement of teeth (Wilcoxon Matched Pairs Test: Z = 4.197; p = 0.00027: Wilcoxon Matched Pairs Test: Z = 4.622; p = 0.000038). In the period before the physiological replacement of teeth or the end of the examination, examinees got evident significant OHI-index improvement.

The values of OHI index were also calculated in patients diagnosed with a *superficial form* of circular cavities untreated and treated with topical fluoride treatment, and they are presented in Table 3. We examined 53 patients, of those 30 were treated and 26 non - treated (control group).

Table 3: Values of OHI - index in untreated and treated subjects with superficial form

Examinee with superficial formation (N = 56)							
Non-treated 26				Treated 30			
Evaluation period	OHI-index			Evaluation period	OHI-index		
	0-1	1.1-2	2.1-3		0-1	1.1-2	2.1-3
First visit %	0	7	19	First visit %	1	8	21
	0.00	26.92	73.08		3.33	26.67	70.00
Before physiological replacement %	12	10	4	Before physiological replacement%	18	10	2
	46.15	38.46	15.38		60.00	33.33	6.67

In both groups with superficial form patients with high OHI index (2.1 - 3) were most present, with approximately 70% of an examinee.

The patients treated with topical fluoride treatment had significant improvement of OHI - index in the period before the physiological replacement of teeth at the end of the test (Wilcoxon Matched Pairs Test: Z = 4.540; p = 0.00006).

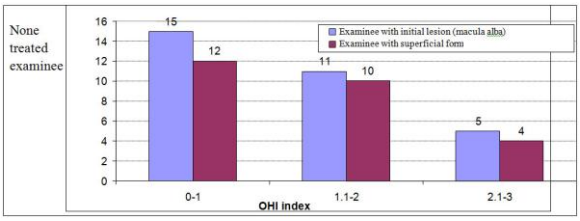

Figure 3: Comparison of untreated subjects with initial lesions and superficial form of caries

Figure 3 is related to a comparison of OHI index between the untreated subjects with initial lesions and superficial form of circular cavities, prior physiological replacement teeth (at the end of the test). We got a statistically significant improvement of OHI index, respondents from initial lesion group (Mann-Whitney U Test: Z = 2.366, p = 0.01796).

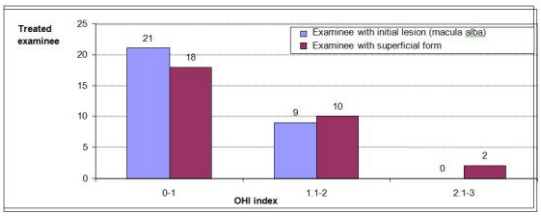

Figure 4: Comparison of treated subjects with initial lesions and superficial form of caries

Figure 4 shows a comparison of OHI index in examinee treated with topical fluoride treatment, in the same period (before physiological replacement of teeth, the end of the test). Among the patients with initial lesions and superficial form of circular cavities, we found a statistically significant difference (Mann - Whitney U Test: Z = 2.803; p = 0.0050) of OHI - index. The index is significantly lower in subjects with the initial lesion. There were no patients with the initial lesion and OHI - index (2.1 - 3).

Discussion

The results from the questionnaires for oral hygiene maintaining and daily frequency of brushing teeth in children were correlated with the test results of Louloudiadis, Maatouk and Markova [12][13][14][15]. Their investigation showed low oral hygiene habits and a statistically significant increase

of the ECC, which means that the number of affected teeth in the mouth of one sick child is increasing over the years.

According to some authors diagnosing early stages of this type of disease, prevention with fluoride treatment, together with the application of other preventive measures provide maximum benefits. Most importantly they have to be applied in the initial stage because of the fast development of the circular cavities. In this phase, by removing the cause for carries (dental plaque) on the one hand, and taking maximum precautions (good oral hygiene and topical fluoride treatment) on the other hand, we create conditions for dominating of remineralisation process to the demineralisation [16].

Professional applying of topical fluorides is effective in caries prevention, but the mechanisms are not yet well understood. Calcium fluoride (CaF_2) is probably main deposit product on enamel, and it possesses cariostatic mechanism. CaF_2 releases F ions that are subsequently incorporated into enamel as fluorhydroxyapatite (FHAP) or fluorapatite (FAP) [17].

In the first stage of the initial lesion, with no cavity presented yet, changes begin to occur in the subsurface layer of enamel. Preventive methods can completely repair and demineralise the lesion at this stage with complete extinction of the white spot - restitution ad integrum [18].

Vulovich in his in - vitro study has simulated acid demineralisation enamel area and used abrasive fluoride toothpaste directly to it. It was concluded that the mechanical effect of brushing reduced demineralisation of the enamel surface. Caries control measures must be established as the first step towards caries reduction, which will cause long-term changes in the oral environment, with the aim of transforming it from cariogenic to non - cariogenic [19].

His findings completely correlate with our investigation, because most of the initial changes in our patients were completely restored after regular controls with topical fluoride treatment. The positive cariostatic effect was also achieved by maintaining regular oral hygiene and improved hygiene and dietary regimen in the control group of the patients not treated with a topical fluoride treatment. Removal of the cryogen plaque inhibited the process of demineralisation, which resulted in biological repair of macula Alba, and stopped further progression of a carious process in the already created cavity [20][21][22].

Beside local benefits of the fluoride treatment in the process of remineralisation of the enamel, it also influenced the soft plaque reduction. In the group of patients with topical fluoride treatment, it showed a positive effect on the index of oral hygiene (OHI - index). The process of remineralisation occurs when the pH of dental plaque rises. The presence of fluoride reduces the critical pH by 0.5 pH.

Results in the present study demonstrated that education of the parents of children with high risk of developing caries is also very important part of our investigation. Our findings are similar to those saying that traditional health education may be insufficient to change parents' behaviour about their at-risk children. While some parents of children with ECC are unaware of the aetiology of this disease, others are well motivated, and the results in their children are unavoidable [23][24].

The important part of the investigation was advising parents about the importance of dietary regime. Bad diet and nutrition may interfere with the balance of tooth demineralisation and remineralisation in several ways. A diet rich in sugars and other fermentable carbohydrates, which are metabolized to acids by plaque bacteria, result in low pH and the growth of the acidogenic and aciduric bacteria (mutans streptococci). On the other side, a diet lower in added sugars and fermentable carbohydrates and high in calcium-rich cheese may favour remineralisation [25][26][27].

In the developed countries as a result of the effective and well-timed implementation of the primary preventive measures, the Early Childhood Caries has a relatively low prevalence of 3% [15]. In undeveloped countries, lack of information on the adequate way of feeding and no solid oral hygiene is the reason for the prevalence of the Early Childhood Caries is up to 45% [28-30].

From the analysis of the results obtained, it can be concluded that: - the level of oral hygiene is correlated with the progression of changes in enamel; - oral hygiene and fluoride treatment significantly influence in lowering of the soft layers; and - for the treatment group of patients, topical fluoride treatment has a positive effect on the index of oral hygiene (OHI).

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Dental Science

Evaluating the Amount of Tooth Movement and Root Resorption during Canine Retraction with Friction versus Frictionless Mechanics Using Cone Beam Computed Tomography

Mohamed Makhlouf[1*], Amr Aboul–Ezz[2], Mona Salah Fayed[2], Hend Hafez[1]

[1]National Research Centre, Orthodontics, Cairo, Egypt; [2]Cairo University, Orthodontics, Giza, Egypt

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Keywords: Canine retraction; Sliding mechanics; Sectional mechanics; NiTi coil spring; T-loop; Friction mechanics; Frictionless mechanics

***Correspondence:** Mohamed Makhlouf. National Research Centre – Orthodontics, Cairo, Egypt. E-mail: mmakhlouf@gmail.com

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Abstract

BACKGROUND: The current study was carried out to compare the amount of tooth movement during canine retraction comparing two different retraction mechanics; friction mechanics represented by a NiTi closed coil spring versus frictionless mechanics represented by T - loop, and their effect on root resorption using Cone Beam Computed Tomography (CBCT).

METHOD: Ten patients were selected in a split-mouth study design that had a malocclusion that necessitates the extraction of maxillary first premolars and retraction of maxillary canines. The right maxillary canines were retracted using T - loops fabricated from 0.017 X 0.025 TMA wires. The left maxillary canines received NiTi coil spring with 150 gm of retraction force. Pre retraction and post retraction Cone Beam Computed Tomography were taken to evaluate the amount of tooth movement and root resorption using three-dimensional planes.

RESULTS: T - loop side showed statistically significant higher mean anteroposterior measurement than NiTi coil spring side, indicating a lower amount of canine movement pre and post a canine retraction. Concerning the root resorption, there was no statistically significant change in the mean measurements of canine root length post retraction.

CONCLUSION: The NiTi coil spring side showed more distal movement more than the T-loop side. Both retraction mechanics with controlled retraction force, do not cause root resorption.

Introduction

Orthodontic treatment objectives may indicate extraction of the first premolar either for the relief of crowding, reduction of dento-alveolar protrusion and improving the facial esthetics, or correction of inter arch mal-relationships through dental camouflage. Hence canine retraction is one of the main procedures carried out during orthodontic treatment. Since the canine retraction procedure takes the longest duration of the entire orthodontic treatment, the main goal of this stage is to achieve a rapid and controlled canine retraction with minimal anchorage loss [1].

Two main canine retraction mechanics are known; Friction (sliding) mechanics or frictionless (sectional) mechanics. The friction created between archwire and bracket when pulling the canine distally using sliding mechanics may be influenced by many factors. Among those factors; surface conditions of archwire and bracket slot, wire section, torque at the wire - bracket interface, type and force of ligation, use of self - ligating brackets, inter-bracket distance, saliva, and influence of oral functions [2]**.** Various techniques for canine retraction have been introduced including Nickel Titanium closing coil, Elastomeric chains, and lace backs. On the other hand, frictionless mechanics imply the use of the sectional method as the use of Burstone's T - loop, Rickett's spring, or Gjessing's spring.

Researchers were interested in investigating the effect of different force levels on the rate of canine retraction using sectional springs. And many authors have described various designs of canine retraction springs, their suitability and efficiency [3][4].

Ziegler et al. (1989) [5], reported a more controlled tooth movement with less distal tipping with sectional mechanics than with the sliding mechanics.

The technique and efficiency of tooth movement with sliding mechanics have been studied by Drescher et al. (1989, 1990) [6][7] and a rate of retraction ranging from 0.21mm/month [8] to 0.81 mm/month [9], according to the used technique and retraction force were reported by researchers.

So far, the debate is ongoing for the best mechanics for canine retraction and has not been yet resolved. With the advancement of Cone Beam Computed Tomography (CBCT), more detailed and accurate measurements of tooth movement in the three planes of space are now possible.

Consequently, this study aimed to use CBCT in evaluating the rate of tooth movement during canine retraction when using sectional mechanics versus continuous mechanics.

Method

The sample for this study comprised of Ten adult patients in a split-mouth design (seven females, three males) with Class I or II division 1 malocclusion, and a treatment plan that necessitates the extraction of maxillary first premolars and retraction of permanent maxillary canines with moderate to minimum anchorage demand.

The levelling and alignment was performed using series of levelling archwires (0.014, 0.016, 0.016 X 0.022 - inch nickel titanium archwire) until reaching 0.016 X 0.022 - inch stainless steel archwire to begin the space closure phase. The interval between each archwire was between three to four weeks interval.

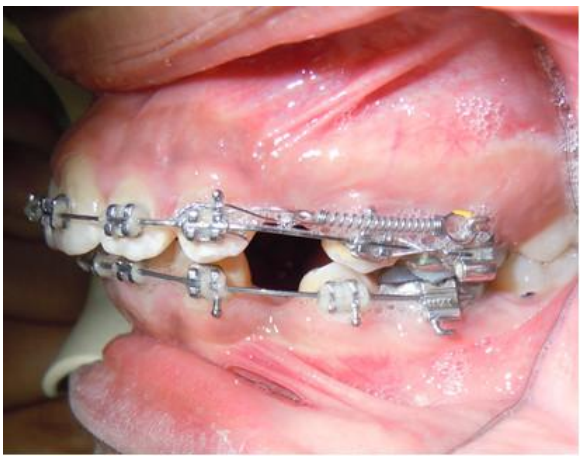

Figure 1: The left side with NiTi coil spring

After the levelling and alignment phase, the canine retraction was performed on the left side using a NiTi coil spring (Jinsung, Korea) and on the right side using a T-loop retraction spring after cutting the wire distal to the maxillary right lateral incisor to resemble an area of frictionless mechanics on the right maxillary canine. The necessary anchorage was made according to each treatment demands.

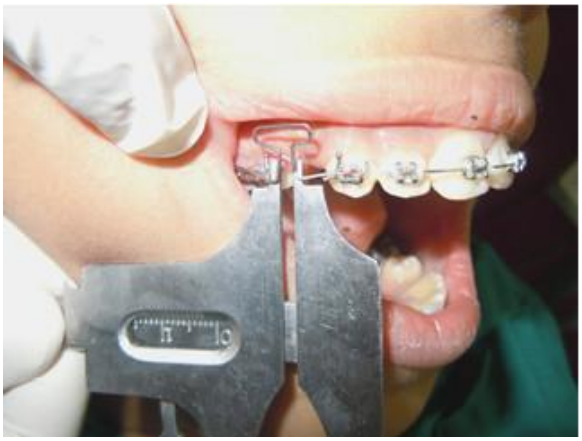

Figure 2: Insertion of the T-loop with activation of 3 mm

Before retraction, a lower alginate impression (Kromopan via L. Longo 18 - 50019 Sesto Fiorentino-Frenze-Italy) was taken and poured in dental stone (®ORTOGUIX III, Protechno, Spain) for fabrication of splint made of a thermoplastic material of 1.5 mm thickness as recommended by Ghoneim, 2010 [11].

T - loop closing coil spring was fabricated from a straight 0.017 X 0.025 TMA wire according to Nanda [12] using a fabrication template for standardisation, and pre-activated according to Marcotte [13], the angle between the mesial and distal arms of the T-loop was standardised to be 47 degrees. Anti-Rotation bends were made at both mesial and distal arms. The T-loop was inserted and ligated into the right maxillary canine using ligature wire, and a3 mm activation was achieved using the Boley gauge.

On the right side, a closing coil spring 8 mm in length was attached to the first molar, and a force of 150 grams was used for retraction. The maxillary left premolar and first molar were ligated together as an anchoring segment; the left maxillary canine was distally ligated with conventional ligature wire to prevent distal rotation during retraction.

The patients were seen every four weeks. The force was measured and activated to keep it constant all over the retraction phase. The post retraction CBCT was taken after four months and treatment was continued according to the treatment plan for each patient.

The following points, lines and planes were identified on each CBCT image:

SPH (Sphenoid - ethmoidal): A point representing the junction of the sphenoid and ethmoid bones and is located in the anterior cranial fossa.

PTMr – PTMl (Pterygomaxillary): the lowest point of each teardrop-shaped Pterygomaxillary

fissure at both sides.

U3IPr – U3IPl (maxillary canine incisal point): the tip of the incisal edge of each maxillary canine.

U3RPr – U3RPl (maxillary canine root point): the apex of the root of each maxillary canine.

FP (Frontal plane): established by SPH, PTMr and PTMl points.

The canine anteroposterior movement was measured as the perpendicular distance from (U3IPr or U3IPl) to the FP (Frontal plane), and calculated using the following equation:

Canine distalization = U3 APpost – U3 APpre

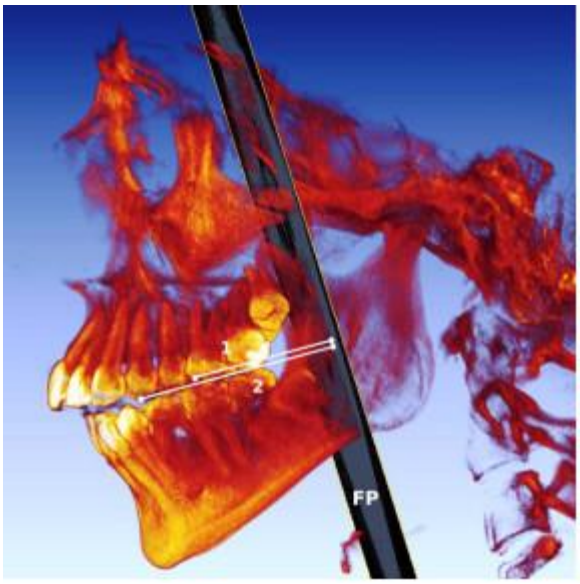

Figure 2: CBCT volumetric image showing the maxillary canine anteroposterior position measurements about frontal plane

The maxillary canine length was measured as the perpendicular distance from (U3IPr or U3IPl) to (U3RPr or U3IRPl) and calculated using the following equation:

Vertical length = U3 RESP post – U3 RESP pre

Statistical analysis

Data were presented as mean, standard deviation (SD) and standard error (SE) values. Data were explored for normality using Kolmogorov-Smirnov and Shapiro - Wilk tests; the results revealed that all measurements were normally distributed (parametric data). Levene's test was used to test the homogeneity of variance between the two sides. Non - significant results of Levene's test indicate homogeneity of variance. Paired t-test was used to compare between parametric data in the left and right sides as well as to compare between the data pre - and post-treatment.

The significance level was set at P ≤ 0.05. Statistical analysis was performed with IBM SPSS Version 20 for Windows.

Results

Comparing the anteroposterior position of the canine pretreatment showed none statistically significant difference between both sides (Table 1).

Table 1: Descriptive statistics & significant changes for the maxillary canine anteroposterior measurement pre - retraction on both sides

Side Measurements	NiTi coil spring side			T - loop side			*P*-value
	Mean	SD	SE	Mean	SD	SE	
AP (mm)	51.1	4.1	1.3	51.1	4.8	1.5	0.976

** Significant at P ≤ 0.05 using paired t-test;* **(AP)** Antero-posterior canine position.

Concerning the NiTi coil spring side, there was a statistically significant decrease in the mean measurement of the anteroposterior (AP) position of the canine post-retraction indicating distal canine movement. There was no statistically significant difference in canine length pre and post retraction (Table 2).

Table 2: Descriptive statistics & significant changes for the maxillary canine anteroposterior measurement and Canine length pre - & post - retraction with the Ni-Ti coil spring

Period	Pre-retraction			Post-retraction			*P*-value
Measurements	Mean	SD	SE	Mean	SD	SE	
AP (mm)	51.1	4.1	1.3	48	3.8	1.2	0.001*
RESP (mm)	26.4	2.1	0.7	26.2	2.1	0.7	0.637

** Significant at P ≤ 0.0 using Paired t-test* **(AP)** Antero-posterior canine position; **(RESP)** canine length.

On the other hand the T - loop side showed non - statistically significant change in the anteroposterior position and length of the canine (Table 3).

Table 3: Descriptive statistics & significant changes for the maxillary canine anteroposterior measurement and canine length pre- & post- retraction with the T - loop

Period	Pre-retraction			Post-retraction			*P*-value
Measurements	Mean	SD	SE	Mean	SD	SE	
AP (mm)	51.1	4.8	1.5	50.8	4.7	1.5	0.642
RESP (mm)	27.5	1.8	0.6	26.4	2.4	0.8	0.067

** Significant at P ≤ 0.05 using paired t-test;* **(AP)** Antero-posterior canine position; **(RESP)** canine length.

On comparing between both sides post retraction, T - loop side showed statistically significantly higher mean (AP) measurement than NiTi coil spring side, indicating a lower rate of canine movement than the NiTi coil spring side (Table 4).

Table 4: Descriptive statistics & significant changes for the maxillary canine anteroposterior measurements post-retraction in the two sides

Side	NiTi coil spring side			T-loop side			*P*-value
Measurements	Mean	SD	SE	Mean	SD	SE	
AP (mm)	48	3.8	1.2	50.8	4.7	1.5	0.010*

** Significant at P ≤ 0.05 using paired t-test;* **(AP)** Antero-posterior canine position

Discussion

The extraction of first permanent premolars for correction of various malocclusions has become an integral part of the orthodontic treatment procedures. Techniques of space closure are various. However they can be classified under two main mechanics; the sectional mechanics which involves frictionless tooth movement, and the continuous mechanics involving friction tooth movement.

A controlled tooth movement is always the goal of an orthodontist especially during the phase of canine retraction. Depending upon the relationship of the line of action of the force to the centre of resistance of the tooth, prediction of tooth movement in the three planes of space is possible [13]. A split-mouth technique was used in the present research aiming at standardisation of all variables as patient cooperation, oral hygiene and bone thickness. Canine retraction began after levelling and alignment; this was to eliminate any asymmetry between the two quadrants.

A standardised protocol was developed where the right canine was retracted using T - loop representing the sectional mechanics technique, and the left side was retracted using NiTi coil spring representing the continuous mechanics technique.

T - loops were constructed from 0.017 X 0.025 TMA straight wires, gable bends and anti-rotational bends were incorporated in the design. The selected design insured the delivery of high moment-to-force ratios and low horizontal force as reported by Thiesen et al. [14]. The spring design was fabricated as described by Nanda [12] and pre-activated as described by Marcotte [13]. The T - loop spring was activated 3 mm per visit to deliver approximately 150 gm of force, this activation protocol was recommended by Keng et al. [15].

Many retraction devices could be used to represent the continuous mechanics technique, However the choice of nickel-titanium closing coil springs used in this study was based on the fact that they do not exhibit rapid force decay such as seen with elastomeric chains or elastic modules, and deliver a constant light force which has been reported to be favorable in space closure [9][16]. It has been proven that excessive force application during space closure can produce adverse effects such as loss of incisor torque control and loss of tip and rotational control of upper molars with relative extrusion of their palatal cusps [16][17]. The low constant force of nickel-titanium springs may be more biologically compatible than the intermittent high forces delivered by elastomeric chains.

The force of 150 gm employed in the present study followed the recommendations of many authors who applied forces between 100 gm and 200 gm for canine retraction [18]. Boester and Johnston [19] found that 150 gm of retraction force gave the highest canine retraction rate Yet Ren et al. [20] have concluded that there is no evidence on an optimal force level.

In this study, cone beam computed tomography (CBCT), which is a three - dimensional tool was utilised in an attempt to overcome the limitations of the traditional two - dimensional projections. On discussing the obtained results from the three-dimensional analysis, the canine anteroposterior position before distalization and after distalization reveals the mean distance the canine travelled over the experimental period which can measure the rate of canine retraction at every side. The NiTi coil spring side showed a mean difference of 0.775 mm per month with a total distance of 3.1 mm in a period of 4 months, This rate of retraction comes into agreement with Dixon et al., [9] However other authors reported faster rates of canine retraction reaching 1.04 mm per month as reported by Nightingale and Jones [10], and 1.81 mm per month reported by Hyashi et al., [21]. Probably their higher rate of canine retraction could be due to the use of round cross-sectional wires with smaller diameters than the one used in this study.

The T - loop side showed a mean difference of 0.1 mm per month with a total distance of 0.3 mm in 4 months. The very low rate of canine retraction on this side was probably due to introducing a high moment – to - force ratio which was greater in value than the moment of force produced by activation of the T - loop to deliver 150 gm of retraction force, this ended up by achieving distal root movement with minimal crown movement. This justification comes into agreement with Thiesen et al. [14] who proved that T - loops constructed from 0.17 X 0.25 TMA wires yielded lower levels of horizontal forces, and that gable bends delivered high moment to force ratios.

Concerning canine length and root resorption, our statistically non - significant results are in agreement with those results in previously conducted studies held by Brusveen et al. [22] and Perona et al. [23].

In conclusion: **t**he NiTi coil spring side showed more distal movement than the T - loop side; and friction and frictionless retraction mechanics with controlled retraction force, do not cause root resorption.

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The Effect of Gaseous Ozone in Infected Root Canal

Nexhmije Ajeti[1], Teuta Pustina-Krasniqi[2*], Sonja Apostolska[3], Edit Xhajanka[4]

[1]Department of Endodontic and Dental Pathology, Dental Branch, Faculty of Medicine, University of Prishtina, Prishtina, Kosovo; [2]Department of Prosthetic Dentistry, Dental Branch, Faculty of Medicine, University of Prishtina (UP), Vice Rector for International Affairs in UP, Prishtina, Kosovo; [3]Department of Endodontic and Dental Pathology, Dental Faculty, University of Skopje, Skopje, Republic of Macedonia; [4]Faculty of Dental Medicine, Prosthodontic Department, Tirana, Albania

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***Correspondence:** Teuta Pustina-Krasniqi, Department of Prosthetic Dentistry, Dental Branch, Faculty of Medicine, University of Prishtina (UP), Vice Rector for International Affairs in UP, Prishtina, Kosovo. E-mail: teuta.pustina@uni-pr.edu

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Abstract

OBJECTIVES: During the treatment of chronic apical periodontitis and pulp necrosis the main role is to irrigate the root canal.

AIM: The aim of this in vivo study was to irrigate with 0.9% NaCl (Natrium Chloride), 2.5% NaOCl (Sodium Hypochlorite Solution, Sigma Aldrich - Germany) and 2% CHX (Chlorhexidine Digluconate Solution, Sigma Aldrich - Spain) combined with Gaseous Ozone (Prozone WH, Austria).

MATERIAL AND METHODS: This study was realised in the University Dentistry Clinical Centre of Kosovo (UDCCK), respectively in the Department of Endodontic and Dental Pathology, Dental Branch, Faculty of Medicine, Prishtina, Kosovo. The 40 subjects involved in this study belonged to both genders, in age between 15 - 65 years. The sample selection was randomised. The retroalveolar radiography for each patient was taken in the suspected tooth. As a therapeutic plan the authors decided to disinfect the root canal with the irrigants, as follows: 2.5% NaOCl, 2% CHX and gaseous ozone.

RESULTS: The statistical analyses were based on Kruskal - Vallis test, X - test, DF = 3, $r < 0.01$. In the isolated average number of the aerobe and anaerobe bacteria colonies, when gaseous ozone was used, there was the significant statistical difference.

CONCLUSIONS: When gaseous ozone was combined with irrigants 0.9%, 2.5% NaOCl and 2% CHX, it was concluded that the number of colonies of aerobic and anaerobic bacteria was reduced.

Introduction

The successes of endodontic treatments are influenced by the elimination of microorganisms from root canals [1]. Residual pulp tissue, bacteria, and dentine debris may persist in the irregularities of root canal system after meticulous mechanical preparation [2]. Also after, mechanical instrumentation, ex vivo in vivo evidence has revealed significant portions of the root canal walls untouched [3]. During and after instrumentation, the irrigants facilitate the removal of microorganisms, residual tissue and dentine debris from the root canal, using a driving mechanism [4]. Several irrigants solutions have antimicrobial activity, and actively kill the bacteria and smear layer when in direct contact with microorganisms. There are also other irrigating solutions with a cytotoxic potential, when meeting periapical tissue, thereby causing severe pain [5]. Sodium hypochlorite is the most commonly used irrigation solution. It is an excellent antibacterial agent able to dissolve necrotic and vital pulp tissue the organic components of dentin as well as a biofilm. The adverse effects of NaOCl may include unpleasant flavour, cytotoxicity [6], a potential of corrosion [7], but also possible allergic effects [8]. CHX by-glyconate is also widely used in dentistry, for its anti-microbial effect. One of the reasons for the CHX is the uniqueness of its use, namely the sustained antibacterial effect [9]. Nevertheless, similar to other agents, the impact of CHX is depended on the pH, and largely reduce the presence of organic matter [10]. CHX 2% may cause desquamation of the oral cavity mycosis,

discolouration of teeth, and it may have a toxic effect on epithelial cells [11] [12] [13]. For such reason, in endodontic treatment, one must use antiseptic means with antibacterial properties, but with the least side effects possible [12]. Irrigants may also be used in combination with other means of disinfection. Ozone has brought about a revolution in endodontic practice, regarding disinfection. The antibacterial effect of the ozone is a result of its action on cells, thereby damaging the cytoplasm membrane, as a consequence of osmosis of a dual bond, and the ozone effect on intracellular content, as a result of oxidisation [13].

Ozone is very efficient in antibiotic-resistant strains, and its effect increases in acidic pH. Ozone influences the cell immunity and humeral systems of the human organism. Ozone stimulates the proliferation of immune-competent cells and the immunoglobulin synthesis. It also activates the macrophage function against phagocytosis [14]. A higher concentration of ozone kills bacteria much faster, and it is 1000 times more powerful than any other agents against bacteria. One ozone molecule is equal to 3000-10000 chlorine molecules, thereby acting against microorganisms around 3500 times faster [15]. The aim of this clinical research study was: to determine the antibacterial effect of, gaseous ozone combined with 0.9 % NaCl, 2.5 % NaOCl and 2 CHX, in an infected root canal.

The aim of this study was to the determinate antibacterial effect of Gaseous Ozone, combined with 0.9% NaCl, 2 % CHX and 2.5% NaOCl.

Material and Methods

The research was performed in the University Dentistry Clinical Centre of Kosovo, respectively in the Department of Endodontic and Dental Pathology, Prishtina, Kosovo.

In this research 40 patients of both genders, in age between 15 - 65 years, were included. The sample selection was random. Upon taking the anamnesis and diagnosing for each patient, radiography was taken of the suspected retroalveolar tooth. To disinfect the root canal, the following irrigants were used: 2.5% NaOCl (Sodium Hypochlorite Solution, Sigma Aldrich - Germany), 2% CHX (Chlorhexidine Digluconate Solution, Sigma Aldrich - Spain) and gaseous ozone (Prozone WH, Austria).

Criteria for including patients in the study

The study only included patients diagnosed with Parodontitis apicalis chronic and Necrosis pulpae. To put diagnose and to come to the therapeutic plan, the retroalveolar radiography for each patient was taken in the suspected tooth. Patients included in the study must not be suffering from any other diseases such as allergic diseases, systemic diseases, respiratory systems, cardiovascular system, endocrines disorders of the thyroid gland. Further, the patients must not be under the effect of any other therapy, including antibiotics in the last six months, or be under treatment of chemotherapy.

The group was divided into three experimental groups and one control group.

Experimental group

Gr.1 (n = 10) - disinfecting the root canal with gaseous ozone, combined with 0.9% NaCl. Gr.2 (n = 10) - disinfecting the root canal with gaseous ozone, combined with 2.5% NaOCl. Gr.3 (n = 10) - disinfecting the root canal with, gaseous ozone combined with 2% CHX.

In each experimental group, three types of irrigants were used (0.9% NaCl, 2.5% NaOCl and 2% CHX). The technique was the same for all three groups, only the irrigation protocol for the three groups was changed. For this reason, this protocol of root canal irrigation shall be described specifically for each experimental group.

Control group

Gr.1 (n = 10) the root canal was irrigated only with 0.9% NaCl.

Gaseous ozone working technique and irrigation protocol using 0.9% NaCl

This research group included ten patients. Upon diagnosing the sterile Rubber Dam was placed. After the trepanning of the pulp cavity and before the instrumentation of the root canal, a primary sample of aerobic and anaerobic bacteria on the root canal was undertaken, with the aid of a sterile paper point (First measurement).

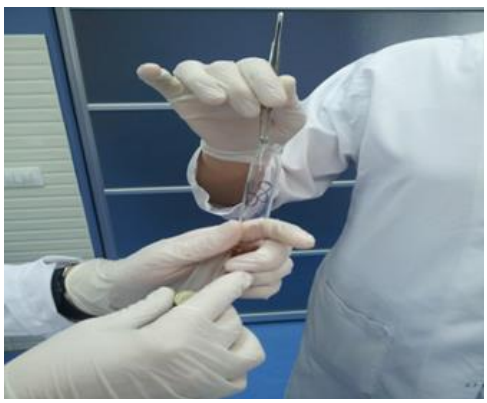

Figure 1: Placement of sterile paper point in tube

With the aim of cultivating the aerobic bacteria, the sample was taken from the root canal by sterile paper point. The sterile paper point placed in the root canal in a duration of 1 minute. This sample was rooted in agar and further dipped in a tube containing 9 ml Thioglycolate *(Thioglycolate медиум, Liofilchем Italy),* (Figure 1).

For cultivating anaerobic bacteria, another sample was taken from the root canal, using a sterile paper point, placed in the root canal at a duration time of 1 min, further planted in Schadler agar (*bioMerieux Sa, France*) and after dipped in a BHI containing tube 9 ml (*Brain Heart Infusion Broth Biolife, Italy)*. Upon the first sample, length of the root canal was determined to be 1 mm shorter than the real length of the canal. Further, instrumentation of the root canal was made by a conventional technique, with instruments K - files ≠ 15 - 60, depending on the volume of the root canal. After each instrumentation, the canal was irrigated with 5 ml of 0.9 %NaCl, and the final irrigation again with 5ml of 0.9% NaCl was made. After irrigating, the canal was drained with a sterile paper point, while the disinfection of the root canal used gaseous e ozone (Prozone, WH Austria), at a duration time of 6", 12", 18" and 24" (second, third, fourth and fifth measurement), (Figure 2, Figure 3 and Figure 4).

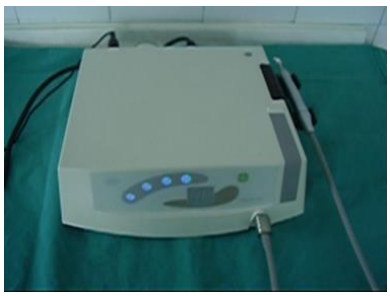

Figure 2: Prozone apart

Upon every Gaseous Ozone exposure, two samples from the canal are taken, one for the aerobic bacteria and the other for anaerobic bacteria, in the same conditions as in making the first sample of the root canal. *(Fifth measurement)*

Figure 3: Surgical aspirator

After instrumentation root canal, the 0.9 %, NaCl solution, was placed and the canal temporarily filled with phosphate cement. Schaedler plates, together with BHI - containing tubes, are placed in plastic bags, together with an indicator for anaerobic bacteria identification *(Anaerobic Indicator, bio - Mérieux SA, France)* and a Gen Bag generator. *(Gen Bag bio - Mérieux Sa, France).*

Figure 4: Application of Gaseous Ozone in root canal

This bag was hermetically closed with clips and immediately sent to the microbiological laboratory of the National Public Health Institute of Kosovo, for culturing of bacteria colonies. Further, samples were placed in a thermal state at a temperature of 37°, thereby incubating for 24 - 48 hours. The gram - positive and gram - negative anaerobic bacteria were determined by special cards *(Bio - Mérieux Sa, France)*, while their reading was made possible by the digital device *Vitek2 (Bio - Mérieux Sa, France)*.

Three days later, the patient was called for an examination, thereby removing the temporary backfill, also removing the 0.9% NaCl dipped fuse. Further, the root canal was drained with the sterile paper point, thereby taking the third sample from the root canal, under the same conditions of other samples*. (Sixth measurement)*

Gaseous ozone working technique and the irrigation protocol using 2.5% NaOCl

The group of this study involved ten patients. Upon diagnosing the sterile Rubber Dam was placed. After the trepanning of the pulp cavity and before the instrumentation of the root canal, a primary sample of aerobic and anaerobic bacteria on the root canal was undertaken, with the aid of a sterile paper point *(First measurement).*

With the aim of cultivating the aerobic bacteria, the sample was taken from the root canal by sterile paper point. The sterile paper point placed in the root canal in a duration of 1 minute. This sample was rooted in agar and further dipped in a tube containing 9 ml Thioglycolate *(Thioglycolate медиум, Liofilchем Italy)*. For cultivating anaerobic bacteria, another sample was taken from the root canal, using

a sterile paper point, placed in the root canal at a duration time of 1 min, further planted in Schadler agar (*bioMerieux Sa, France*) and after dipped in a BHIcontaining tube 9 ml (*Brain Heart Infusion Broth Biolife, Italy)*.

Upon the first sample, length of the root canal was determined to be 1 mm shorter than the real length of the canal. Further, instrumentation of the root canal was made by a conventional technique, with instruments K - files ≠ 15 - 60, depending on the volume of the root canal. After each instrumentation, the canal was irrigated with 5 ml of 2.5 % NaOCl. Inorganic tissue was removed using 5ml of 17 % EDTA *(Ethylenediamine tetraacetic, acid disodium salt dehydrate, Czech Republic*), and duration of time was 1 min and the final irrigation again with 5 ml of 0.9 % NaCl was made. After irrigating, the canal was drained with a sterile paper point, while the disinfection of the root canal used gaseous e ozone (Prozone, WH Austria), at a duration time of 6", 12", 18" and 24" *(second, third, fourth and fifth measurement)* (Figure 2, Figure 3 and Figure 4). Upon every Gaseous Ozone exposure, two samples from the canal are taken, one for the aerobic bacteria and the other for anaerobic bacteria, in the same conditions as in making the first sample of the root canal. *(Fifth measurement)* After instrumentation root canal, the 0.9%, NaCl solution, was placed and the canal temporarily filled with phosphate cement. Schaedler plates, together with BHI - containing tubes, are placed in plastic bags, together with an indicator for anaerobic bacteria identification *(Anaerobic Indicator, bio - Mérieux SA, France)* and a Gen Bag generator. *(Gen Bag bio - Mérieux Sa, France).* This bag was hermetically closed with clips and immediately sent to the microbiological laboratory of the National Public Health Institute of Kosovo, for culturing of bacteria colonies. Further, samples were placed in a thermal state at a temperature of 37°C, thereby incubating for 24 - 48 hours. The gram-positive and gram-negative anaerobic bacteria were determined by special cards *(Bio - Mérieux Sa, France)*, while their reading was made possible by the digital device *Vitek2 (Bio - Mérieux Sa, France)*.

Three days later, the patient was called for an examination, thereby removing the temporary backfill, also removing the 0.9% NaCl dipped fuse. Further, the root canal was drained with the sterile paper point, thereby taking the third sample from the root canal, under the same conditions of other samples *(Sixth measurement).*

Gaseous ozone working technique and the irrigation protocol using 2% CHX

The group of this study involved ten patients. Upon diagnosing the sterile Rubber Dam was placed. After the trepanning of the pulp cavity and before the instrumentation of the root canal, a primary sample of aerobic and anaerobic bacteria on the root canal was undertaken, with the aid of a sterile paper point *(First measurement).* With the aim of cultivating the aerobic bacteria, the sample was taken from the root canal by sterile paper point. The sterile paper point placed in the root canal in a duration of 1 minute. This sample was rooted in agar and further dipped in a tube containing 9 ml Thioglycolate *(Thioglycolate медиум, Liofilchем Italy)*. For cultivating anaerobic bacteria, another sample was taken from the root canal, using a sterile paper point, placed in the root canal at a duration time of 1 min, further planted in Schadler agar (*bioMerieux Sa, France*) and after dipped in a BHIcontaining tube 9 ml (*Brain Heart Infusion Broth Biolife, Italy)*. Upon the first sample, length of the root canal was determined to be 1 mm shorter than the real length of the canal. Further, instrumentation of the root canal was made by a conventional technique, with instruments K - files ≠ 15 - 60, depending on the volume of the root canal. After each instrumentation, the canal was irrigated with 5 ml of 2% CHX. Inorganic tissue was removed using 5ml of 17% EDTA *(Ethylenediamine tetraacetic, acid disodium salt dehydrate, Czech Republic*), and duration of time was 1 min and the final irrigation again with 5ml of 0.9% NaCl was made. After irrigating, the canal was drained with a sterile paper point, while for the disinfection of the root canal was used gaseous ozone (Prozone, WH Austria), at a duration time of 6", 12", 18" and 24" (second, third, fourth and fifth measurement) (Figure 2, figure 3 and Figure 4). Upon every Gaseous Ozone exposure, two samples from the canal are taken, one for the aerobic bacteria and the other for anaerobic bacteria, in the same conditions as in making the first sample of the root canal *(Fifth measurement).*

Figure 5: Bacterial colonies

After instrumentation root canal, the 0.9%, NaCl solution, was placed and the canal temporarily filled with phosphate cement. Schaedler plates, together with BHI - containing tubes, are placed in plastic bags, together with an indicator for anaerobic bacteria identification *(Anaerobic Indicator, bio -*

Mérieux SA, France) and a Gen Bag generator *(Gen Bag bio - Mérieux Sa, France).*

This bag was hermetically closed with clips and immediately sent to the microbiological laboratory of the National Public Health Institute of Kosovo, for culturing of bacteria colonies. Further, samples were placed in a thermal state at a temperature of 37°C, thereby incubating for 24 - 48 hours. The gram - positive and gram - negative anaerobic bacteria were determined by special cards *(Bio - Mérieux Sa, France)*, while their reading was made possible by the digital device *Vitek2 (Bio - Mérieux Sa, France)* (Figure 5).

Three days later, the patient was reexamined, thereby removing the temporary backfill, also removing the 0.9% NaCl dipped fuse. Further, the root canal was drained with the sterile paper point, thereby taking the third sample from the root canal, under the same conditions of other samples. (Sixth measurement)

Control group

The group of this study involved 10 patients. The same technique and procedure for instrumentation of the root canal and the sampling and submission to the microbiological laboratory were used. This group only differed with the difference in the protocol of irrigating the root canal. In this group, only the root canal irrigator of 10 ml of 0.9% NaCl was used. Two samples were taken before instrumentation: one for aerobic and one for anaerobic bacteria (First measurement). Immediately after the instrumentation was taken other two samples for aerobic and anaerobic bacteria (Second measurement), and three days after the instrumentation the similar two samples, as previous (Third measurement)

Results

In this study were included 40 patients of both genders and different ages from 15-65 years. The root canal was disinfected by applying GO combined with the following irritants: 0.9% NaCl, 2.5% NaOCl and 2% CHX. For every patient were taken before the instrumentation of the root canal (for aerobe and anaerobe bacteria). This was the first sampling. Eight samples were taken after the instrumentation of the root canal. Two of the eight samples were taken after the application of the GO (for aerobe and anaerobe bacteria), with the duration time of the 6". This was the second sampling. The third sampling was 12", the fourth sampling was 18", and the fifth sampling was 24". The last two samples (for aerobe and anaerobe bacteria), were taken only after instrumentation of the root canal. This was the sixth sample. In total for 40 patients were taken 480 samples from the infected root canal. Based on Kruskal - Vallis test, $r > 0.05$, χ – test = 7.748, DF = 3, showed that there was not any statistical significance in the average number of the isolated colonies of the aerobe bacteria between the clinically tested groups. Also, Kruskal - Vallis test, $r > 0.05$, χ - test = 0.426, DF = 3, showed that there was not a statistical difference in the average number of isolated colonies in the anaerobe bacteria (First measurement, Figure 6).

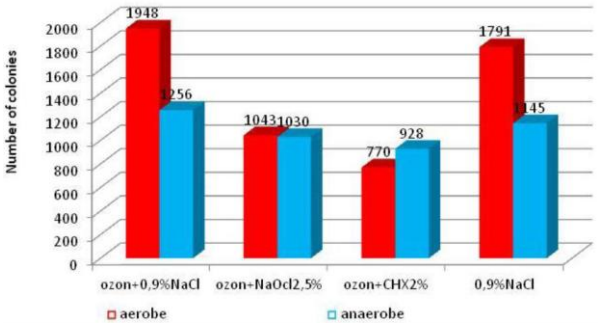

Figure 6: Average number of isolated bacteria colonies, aerobic and anaerobic bacteria, measured in all patients at first measurement

After the application of the GO (for 6 sec) mixed with the other different irritants, the statistical results as follows were founded: Kruskal - Vallis test, $p > 0.05$, χ – test = 19.304, DF = 3, $p < 0.01$ showed that there was a high statistical significance for the number of bacteria, isolated in the root canal. Whereas, the detailed analysis of Mann - Whitney test with inversion showed that there was not a statistical significance in between the group 1 - 2 and group 3 - 4 compared with the group 2 - 3 and 2 - 4 where the statistical significance was found. Gaseous Ozone combined with 2.5% NaOCl was most efficient in the reduction of aerobe bacteria compared with other groups.

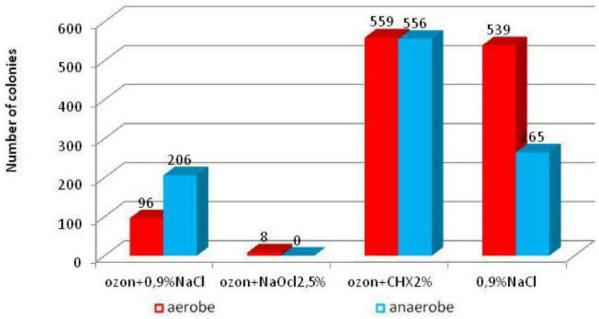

Figure 7: Average number of isolated bacteria colonies, aerobic and anaerobic bacteria, measured in all patients at second measurement

As concerned the anaerobe bacteria the Kruskal - Vallis test, χ –test = 10.495, DF = 3, $p < 0.01$ showed that exists the significant difference in the isolated average number of the anaerobe bacteria colonies. Whereas the detailed analysis with the

Mann - Whitney test showed, that does not exist any significant difference in between the groups 1 - 2, 1 - 3, 1 - 4, 2 - 3 and 3 - 4, compared with the group 2 - 4 where the significant statistical difference was found. The second measurement showed that when Gaseous Ozone was combined with 2.5 % NaOCl, decreased the number of isolated colonies of the anaerobe bacteria, compared with other testing groups (Second measurement, Figure 7).

Kruskal - Vallis test, χ – test = 17.29, DF = 3, $p < 0.01$ also showed that in the third measurement (Gaseous Ozone application at duration time 12"), exists the high statistical difference in the average number of aerobe bacteria, especially in the group 2. The statistical significance between the group 1 - 4, 2 - 3, 2 - 4 and 3 - 4 was shown and during the detailed analysis of Mann - Whitney test, compared with the group 1 - 2 and 1 - 3, which did not have any statistical significance in between.

As a concern, the colonies of anaerobe bacteria, the statistical results with the tests: Kruskal - Vallis test, χ – test = 110.724, DF = 3, $p < 0.01$ showed that exists a significant difference between the test group especially the group 2. The Mann - Whitney test showed that exists significant difference only between the group 2 - 4, compared with the groups 1 - 2, 1 - 3, 1 - 4, 2 - 3 and 3 - 4, where the statistical significance was not found. This measurement, also showed that the application of the Gaseous Ozone combined with 2.5 % NaOCl was more efficient in the reduction of the number of the colonies of anaerobe bacteria (Third measurement, Figure 8).

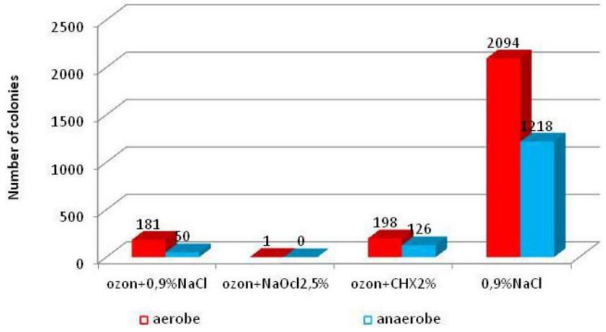

Figure 8: Average number of isolated bacteria colonies, aerobic and anaerobic bacteria, measured in all patients at third measurement

After the application of the GO combined with the different irritants, the statistical results were found: Kruskal - Vallis test, χ – test = 5.352, DF = 2, $p > 0.05$ showed that does not exist any statistical difference in the average number of the colonies of aerobe bacterias. Also, Kruskal - Vallis test, χ – test = 8.116, DF = 2, $p > 0.05$ showed that does not exist any statistical difference in the average number of the colonies of anaerobe bacterias between the tested groups (Fourth measurement, Figure 9).

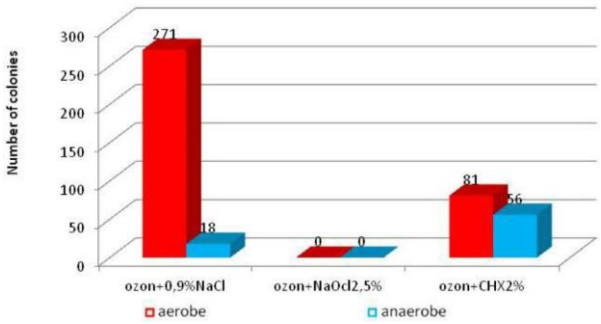

Figure 9: Average number of isolated bacteria colonies, aerobic and anaerobic bacteria, measured in all patients at fourth measurement

After the application of the Gaseous Ozone for 24" combined with different irrigants these statistical results were found: Kruskal - Vallis test, X – test = 0.886, DF = 2, $p > 0.05$ showed that does not exist any statistical difference in the average number of the colonies of aerobe and anaerobe bacterias (Fifth measurement, Figure 10).

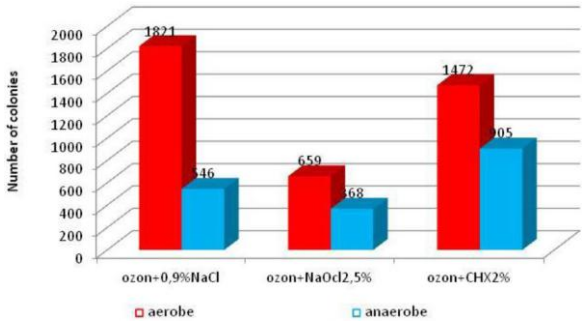

Figure 10: Average number of isolated bacteria colonies, aerobic and anaerobic bacteria, measured in all patients at fifth measurement

After the instrumentation of the root canal the statistical test: Kruskal - Vallis test, X – test = 7.23, DF = 2, $p < 0.05$ showed that exists the statistical significance, between the tested groups, especially in the second group. Mann - Whitney test showed that exists the statistically significant difference between the group 1 - 2 compared with the group 2 - 3 in the average number of the colonies of aerobe bacteria.

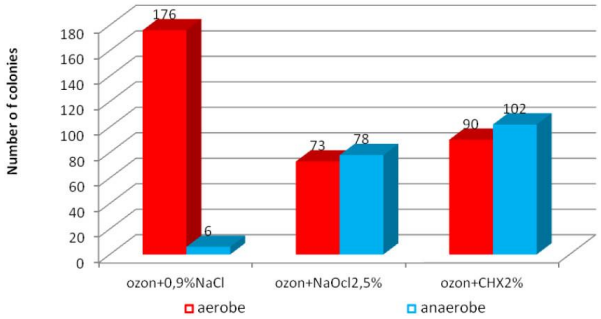

Figure 11: Average number of isolated bacteria colonies, aerobic and anaerobic bacteria, measured in all patients at sixth measurement

Also, during this measurement GO combined with 2.5% NaOCl affected in the reduction

of the number of aerobe bacterias. Whereas, as a concern, the anaerobe bacterias the Kruskal - Vallis test, X –test = 1.496, DF = 2, p > 0.05 did not show any statistical difference between the tested groups (Sixth measurement, Figure 11).

Discussion

In the literature, ozone is currently being discussed as a possible alternative antiseptic agent in dentistry because of its reported high antimicrobial power without the development of drug resistance [16]. Gaseous Ozone in the concentration of ~4 gm¯3. (Heal Ozone: Kavo, Biberach, Germany) Is already being used clinically for endodontic treatment. However, results of studies into its efficacy against endodontic pathogens has been inconsistent, and there is a less literature information regarding the most appropriate application time, concentration [17] and species of the bacteria. In our study, the antibacterial effect of the GO was estimated at periods of 6", 12", 18" and 24", combined with 0.9% NaCl, 2.5% NaOCl and 2% CHX. The results of this study showed that the Gaseous Ozone disinfection of the root canal, combined with 2.5% NaOCl, demonstrates a significant difference in reducing the aerobic and anaerobic bacteria colonies, compared to the use of Gaseous Ozone combined with 0.9% NaCl and 2% CHX. In a study of Alwadi et al., [18], in vivo conditions, the antibacterial effect was reported in the use of 0.5%, NaOCl, with or without using GO in the root canal. In such a study, they included 100 patients, and the root canal samples were taken before and after instrumentation of the root canal. According to the scholars, NaOCl and Gaseous Ozone influence the reduction of the bacteria colonies number in the infected root canal and that the ozone combined with NaOCl marks a significant difference when compared with the sole use of NaOCl. In this study, the gaseous ozone, at a concentration of 5 gm¯3 eliminated the number of aerobic and anaerobic bacteria colonies in the infected root canal, which also matches our study results. On the other hand, according to a study by Müller et al., [19], it was concluded that 5% NaOCl might reduce all bacteria from the infected root canal, for a different form of the application of Gaseous Ozone, photodynamic therapy and 2% CHX. The antibacterial effect of Gaseous Ozone was further confirmed by Virtej et al. [20]. The solution of 2.5% NaOCl, combined with Gaseous Ozone, at a duration time of 40", significantly reduced the number of aerobic and anaerobic colonies from the infected root canal. Also, an in vivo study of Jankovic et al. [21], which again matches our study results, is similar. In terms of duration of Gaseous Ozone application, our results have shown that the application of Gaseous Ozone at durations of 6" and 12" marks a significant reduction of aerobic and anaerobic bacteria colonies, when compared with the application of Gaseous Ozone at durations of 18" and 24" and compared with the number of bacteria colonies sampled before the instrumentation of the root canal. It is worth mentioning that with the extension of the application period of Gaseous Ozone combined with 2.5% NaOCl we came to entirely extinct the number of colonies of aerobic and anaerobic bacteria in the infected root canal when compared with Gaseous Ozone combined with 0.9% NaCl and 2 % CHX.

The number of bacteria colonies increased again after three days of disinfecting the root canal by using Gaseous Ozone. The increasing of colonies came as a result of failure to apply solutions for curing the infected root canal, which would help in further canal disinfection. Before the instrumentation, irrigation with NaOCl 2.5% and application of Gaseous Ozone, from the infected root canal was isolated four types of anaerobic bacterias; Clostridium clostridioforme, Clostridium bifermentans, Clostridium baratii and Actinomyces meyeri.

Whereas, after the application of these procedures, it was concluded that Clostridium bifermentans persisted in the root canal, even the application of Gaseous Ozone in a time interval of 6", 12" and 18". The disappearance was noted after application in a time interval of 24", whereas Actinomyces meyeri disappeared after 6" and Clostridium baratii completely disappeared after the application of Gaseous Ozone. Before the instrumentation, irrigation with CHX 2% and application of Gaseous Ozone, from the infected root canal was isolated four types of anaerobic bacterias; Lactobacillus, Actinomyces meyeri, Clostridium subterminale, Clostridium bifermentans and Clostridium butyricum. After the application of Gaseous Ozone in a time interval of 6" persisted only Clostridium butyricum, whereas the other bacterias completely disappeared.

Based on the results of this research, it may be concluded that: In treating the infected root canal with gaseous ozone, combined with irrigants 0.9%, 2.5% NaOCl and 2% CHX, reduce the number of colonies of aerobic and anaerobic bacteria. Statistical data show that the application of gaseous ozone, combined with 2.5%, NaOCl, has a better antibacterial effect against the number of aerobe and anaerobe bacteria colonies in the infected root canal when compared with 0.9% NaCl and 2% CHX.

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Angulated Dental Implants in Posterior Maxilla FEA and Experimental Verification

Hamed A. Hamed[1], Hamdy A. Marzook[1], Nahed E. Ghoneem[1], Mohamed I. El–Anwar[2*]

[1]Faculty of Dentistry, Mansoura University, Egypt - Oral and Maxillofacial Surgery Department, Mansoura, Egypt; [2]National Research Centre - Mechanical Engineering, Giza 12622*, Egypt*

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***Correspondence:** Mohamed I El - Anwar. National Research Centre - Mechanical Engineering, Oral & Dental Research division, Giza, Egypt. E-mail: anwar_eg@yahoo.com

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Abstract

AIM: This study aimed to evaluate the effect of different implant angulations in posterior maxilla on stress distribution by finite element analysis and verify its results experimentally.

METHODS: Two simplified models were prepared for an implant placed vertically and tilted 25° piercing the maxillary sinus. Geometric models' components were prepared by Autodesk Inventor then assembled in ANSYS for finite element analysis. The results of finite element analysis were verified against experimental trials results which were statistically analysed using student t-test (level of significance $p < 0.05$).

RESULTS: Implant - abutment complex absorbed the load energy in case of vertical implant better than the case of angulated one. That was reflected on cortical bone stress, while both cases showed stress levels within the physiological limits. Comparing results between FEA and experiment trials showed full agreement.

CONCLUSION: It was found that the tilted implant by 25° can be utilised in the posterior region maxilla for replacing maxillary first molar avoiding sinus penetration. The implant-bone interface and peri-implant bones received the highest Von Mises stress. Implant - bone interface with angulated implant received about 66% more stresses than the straight one.

Introduction

Trials for replacing missing teeth with root form implants back to thousands of years. Antiquities from ancient China and ancient Egypt show bamboo pegs and similarly shaped pegs from precious metals tapped into the bone for replacing lost teeth [1]. This way of thinking is translated into what we called dental implant. Dental implants support prosthesis like a crown, bridge, denture as a primary use for it. This support based on osseointegration, the process in which bone unite firmly with the surface of certain materials such as titanium or some ceramics biologically. The integration between bone and implant can bear the physical load for several years [1].

Although dental implants are considered as ideal manner for replacing missing teeth, the bone height from the alveolar crest to the sinus floor at the posterior maxillary region is usually insufficient due to sinus pneumatization, as well as to the lack of stability caused by maxillary bone loss at the edentulous sites required for osseointegrated implantation [2].

Tilting implants are an effective and safe substitute for surgery of augmentation of maxillary sinus floor and to maxillary sinus which is pneumatized. It can usually be conducted in patients with different systemic conditions which often have limitations for grafting of the bone. The angulated implants permit insertion that avoids anatomical structures like maxillary sinus [3].

High risks will be involved when restored prostheses are subject to non - axial loading. It is recommended to direct occlusal loads as close to the long axis of the fixture as possible. However, it is known that the loading on angled abutments is mostly off - axis, which raises the concern of how angled

abutments perform with such an unfavourable loading regimen [4].

The way in which loads are transmitted to the surrounding bone is the key factor for success or failure of the dental implant. Between different mathematical methods which can evaluate stress distribution within bone supporting dental implants, finite element analysis (FEA) is usually used in dentistry to evaluate the influence of clinical agents on the survival of implant placement, and also to predict the biomechanical status correlated with the different dental implant and alveolar bone conditions [5]. FEA allows the prediction of the stress distribution in the contact area of the implants with cortical bone, and around the apex of the implants in the surrounding bone. This method is advantageous for solving complex structural problems as it divides them into smaller and simpler interrelated sections through the use of mathematical techniques [6].

This study aimed to evaluate the effect of different implant angulations in posterior maxilla on stress distribution by finite element analysis and verify its results experimentally.

Materials and Methods

Two finite element models were specially prepared for simulating the clinical situation where a dental implant was placed into posterior maxilla in two different ways. The implant to be placed vertically (case study #1) and tilted by 25° inside the bone to avoid sinus penetration (case study #2).

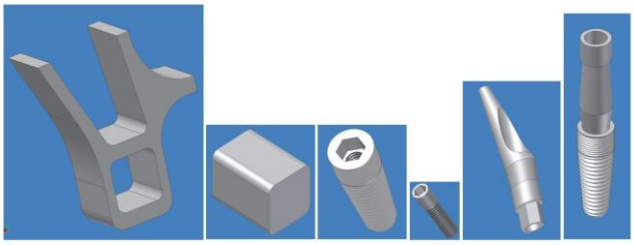

Figure 1: Screenshots of the two models' components on Inventor GUI

The finite element models' components (prescribed in the in - vitro study) as the abutments, screw, implant, cortical and cancellous bones were created on “Autodesk Inventor” Version 8 (Autodesk Inc., San Rafael, CA, USA) as presented in Figure 1. These components were exported as SAT files [7]. These components were assembled in ANSYS environment (ANSYS Inc., Canonsburg, PA, USA), where all used materials were assumed to be isotropic, homogenous and linearly elastic and its properties are listed in Table 1.

Table 1: Material properties used in the finite element model(s)

Material	Young's modules [GPa]	Poisson's ratio
Implant abutment complex	110.0	0.34
Cortical bone	13.7	0.30
Cancellaus bone	1.37	0.30

Set of Boolean operations between the modelled components were performed before obtaining the complete model(s) assembled. The meshing of these components was done by 3D brick solid element “187” which has three degrees of freedom (translation in main axes directions) [8]. The resulted numbers of nodes and elements are listed in Table 2, and cut sections in the meshed models are presented as screenshots from ANSYS in Figure 2.

Table 2: Number of nodes and elements in all meshed components

Volume	Vertical Implant Model (case #1)		Angulated Implant Model (case #2)	
	Number of Nodes	Number of Elements	Number of Nodes	Number of Elements
Cortical bone	18,738	18,549	20,345	21,555
Cancellous bone	14,928	14,465	27,663	25,303
Implant	49,958	45,193	32,353	29,282
Screw	364,884	283,868	1,857	1,876
Abutment	9,591	11,341	1,358	1,840

The extreme areas of the cortical bone were set to be fixed in place as a boundary condition. While the applied compressive load were set to be 200N, distributed equally on the abutment top area nodes. Solid modelling and finite element linear static analyses were performed on Workstation HP ProLiant ML150, with Intel Xeon 3.2 GHz processor (with 1MB L2 cache), 10GB RAM, using ANSYS version 14.5. The finite element analysis results were verified against experimental trials.

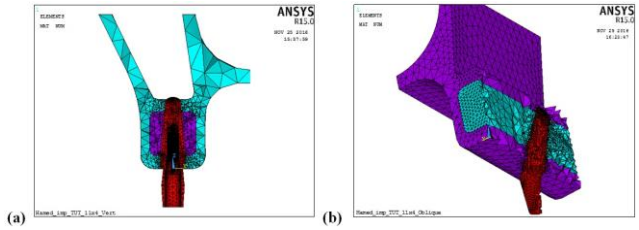

Figure 2: Screenshots of cut sections in the two models

The in - vitro study utilised six segments of bovine bone ribs. The samples were cleaned and removed of all soft tissue residues, then immersed in a saline and ethanol solution (1:1). Each rib received one implant, where vertically drilled to show the 11 mm depth of the implant site. The specific implant, rib and site to receive the implant preparation were chosen randomly. Accurate and atraumatic preparation of the bone at the implant site was done using the instrument set for 11mm length, 4mm diameter TUT - II implant system (TUT Dental Implant Co., Egypt). The six segments were divided into two groups; Group A (three bovine ribs received three straight implants) and Group B (three bovine ribs received three tilted implants). Fracture resistance test conducted using INSTRON® universal testing

machine (model 3345). All specimens were individually mounted in a jig then secured to the lower grips. While a load cell of 5 kN was used for force measurements that results were acquired using BlueHill software version 3.3 (by INSTRON®). A special stainless steel rod with a round end of 5 mm diameter was fixed in the upper moving grips to apply compressive load over the top of the abutment of each specimen till failure occurred in any component.

Results

The concentration of Von Mises stress was found on the surface of the crestal cortical bone around the implant neck except that for the bicortical implantation. Finite element results showed that implant-abutment complex absorbed the load energy in case of vertical implant better than the case of angulated one. That was reflected in cortical bone stress. Vertical implant transferred less - load to cortical bone (of order 22 MPa) by about 66% in comparison to angulated one (of order 67 MPa), while both cases showed stress levels within the physiological limits [9][10].

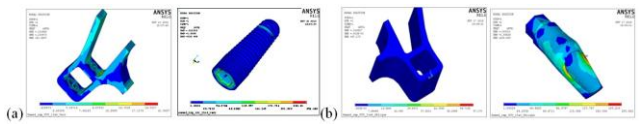

Figure 3: Sample of Von Mises stress distributions on both case studies (a) vertical implant; (b) angulated implant

Implant abutment connection received most of the load energy in both cases, where maximum Von Mises stress on implant appeared on this section. On the other hand, the maximum Von Mises stress appeared on abutment at different locations; at the connection with the implant in vertical implant case, and at thin walls around screw way to the implant in case of the angulated implant. Figure 3 demonstrates a sample of Von Mises stress distributions, while Figure 4 compares all components maximum, Von Mises, stress.

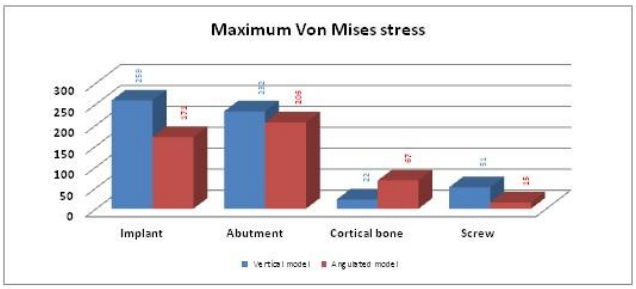

Figure 4: Maximum Von Mises stress comparison between the two cases

Applying the student t-test to the experimental trials' results of the fracture resistance for the two studied groups showed that there was a highly significant difference between the mean compressive loads of them, as shown in Table 3.

Table 3: Fracture load comparison between the two studied groups

Fracture Load	Vertical (control) (n = 3)	Angulated (n = 3)	T	P
Min. – Max.	449.1 – 524.4	381.4 – 421.4		
Mean ± SD.	496.3 ± 41.1	399.7 ± 20.2	3.651*	0.022*
Median	515.3	396.4		

t, p: t and p values for Student t-test for comparing between the two groups; *: Statistically significant at p ≤ 0.05.

Comparing results between FEA and experiment trials showed full agreement. The bone was strongly affected by the implant angulation because it was the first component to be fractured through the experimental verification. But in case of an angulated implant, the complex had endured less vertically applied forces to generate the similar level of stress on the cortical bone. This may be referred to abutment design, and its level of stress appeared on it. The more the abutment stress, the more the energy it absorbs, which reduce the energy or load transferred to the following parts of the system (implant and bone).

Discussion

The finite element analysis resulted in huge graphical representations (screenshots/pictures), each one present a type of deflection, strain, or stress. Commonly when Von Mises stress reaches critical values, all other types of stresses and deflections would be discussed to indicate the dominant effect from loading or system materials. In this study, the von Mises stress distribution on the bone away from the implant-bone interface was considered. This is because it was difficult to estimate the effect of stress distribution solely based on the stress pattern at the localised implant-bone interface in case of a severely atrophied bone. Also, stress on the implant/abutment complex was analyzed to estimate the risk of fracture in an angulated implant case.

The posterior maxillary area contains cancellous bones with low bone density and thin cortical bones, the quantity and quality of which are lower than those of the mandibular bone. Therefore, it is difficult for implants installed in this region to be stable. This is due to the small implant-to-bone contact area and the inferior bone quality [11].

Maminskas et al. [12] found out risks of mechanical impacts of peri-implant bone loss and prosthetic influence on bone stability. They concluded that peri-implant strain could be generated by nonaxial loading, cantilever prosthetic elements, crown/implant ratio, type of implant-abutment connection, misfits,

properties of restoration materials and antagonistic tooth.

The experimental trials where the implant was placed vertically was used as the control group. All tested samples showed the same mode of failure in cortical bone (where the implant was pushed into the bone) under different values of the applied load. Applying the student t-test to the experimental results of the fracture resistance test of the two studied groups showed that there was a highly significant difference between the mean compressive loads of them. Bone was mostly affected by the implant angulation as demonstrated through FEA stresses results too. Also, the angulated implant complex may endure more force to collapse in comparison to straight implant complex. Finally, comparing results between FEA (maximum Von Mises stress) and experiment trials (maximum load at failure) proved that the tilted implant 25° could be utilized in the posterior region maxilla for replacing maxillary first molar to avoid sinus penetration.

In previous studies [9][10][13] influence of implant - abutment angulations on stress distribution on central incisor were investigated. The conclusion of these studies was; cortical and spongy bone were insensitive to the crown material, and increasing abutment angulation from 15° to 25°, increases stress on cortical bone by about 20% and reduces it by about 12% on spongy bone.

Also, the cervical areas are the most critical on the abutments due to the force concentration that may be a reason for failures, i.e. increasing the abutment angulation had a negative influence on the fracture load.

Majority of finite element models in dental researchers [9][14] assumed perfect bond between assembled model components to simulate natural condition, in addition to assuming linear, static and isotropic material properties. The trend of the presence of higher Von Mises stresses in the bone around the angulated implant than bone around the straight implant did not differ by the bone levels [9][14]. Prominently, the highest level of stress was exhibited in the stepped area of the bones. In clinical situations, however, these phenomena are not likely to occur as the bone loss occurs continuously. In preliminary modelling, the maxillary bone was reconstructed from the data of other researchers [15][16] according to the anatomical area of the sinus.

For immediate loading, when the implant apex broke into or through the sinus cortical bone, the maximum displacements of the implant, particularly at the implant apex, were smaller than those did not reach sinus floor. Yan et al. [16] FE study on the association between implant apex and sinus floor showed that having the implant apex in contact with, piercing or breaking through the sinus floor cortical bone benefited the implant stability, particularly for immediate loading.

Finally, the results of this study were in agreement with the literature [9, 10, 13, 17] when abutments with 0, 15°, and 25° angulations were evaluated in the maxilla by 3D FEA, that the implants were recommended to be vertically aligned with axial loads.

Comparing results between FEA and experiment trials showed full agreement and found that the tilted implant by 25° can be utilised in the posterior region maxilla for replacing maxillary first molar avoiding sinus penetration.

Within the limitations of this study, it can be concluded that the highest bone stress was observed on the implant-bone interface and peri-implant bones, while the case of angulated implant showed higher Von Mises stress by about 66%. On the other hand, angulated implant complex components received more Von Mises stress by about 15 to 70% in comparison to the straight one's components.

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Dental Science

Knowledge and Preparedness of Dental Practitioners on Management of Medical Emergencies in Jazan Province

Ghassan M**.** Al-Iryani, Fareedi Mukram Ali[*], Naima H. Alnami, Sahar K. Almashhur, Maha A. Adawi, Abrar A. Tairy

College of Dentistry - Oral & Maxillofacial Surgery Jazan, Jazan, Saudi Arabia

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Keywords: Knowledge; Preparedness; Dental Practitioners; Medical Emergencies; Jazan Province (KSA)

***Correspondence:** Fareedi Mukram Ali. College of Dentistry - Oral & Maxillofacial Surgery Jazan, Jazan, Saudi Arabia. E-mail: faridi17@rediffmail.com

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Abstract

Medical emergencies are one of the most stressful situations the staff in a dental practice might encounter. The duty of care toward the attending patients obligates suitable preparedness to provide the necessary care if such emergencies ensue. Unfortunately, we found that 22% of the investigated dental clinics had no emergency kit available. Only 38% of the interviewed dentists felt confident to perform CPR, and 18% had no confidence to manage any medical emergency. An MCQ test of 20 questions examining the dentists' knowledge in medical emergencies was distributed, and the level of knowledge was found to be suboptimal. The average score of the interviewed dentists was 10.87 out of 20. Experience and specialty training had a negligible effect on the level of knowledge.

Introduction

A medical emergency is a medical condition requiring immediate treatment [1]. These emergencies require management by personnel who should ideally be suitably qualified to do so. Dependent on the severity of the emergency and the quality of any treatment given, it may require the involvement of multiple levels of care, from a first aider to an emergency physician through to specialist surgeons. The dentist should be able to initiate the primary management to avoid morbidity and mortality, and this warrants the need for basic knowledge and material preparedness to identify, access, and manage emergency situations in one's practice. Successful patient management relies on understanding the pathophysiologic processes and how to correct them [1][2][3][4][5][6]. Every dentist has high chance to be involved in the diagnosis and treatment of medical emergencies during their clinical practice. These emergencies can be directly related to dental therapy, or they may occur by chance in the dental office environment [3][4][7][8][9]. Changing demographics in the population, leading to increased longevity have resulted in more people having medical conditions which predispose to a medical emergency or are taking medications which may influence their dental management [10]. There is a lack of comprehensive studies on the incidence of medical emergencies in dental practices in Saudi Arabia.

There was a study by Mostafa et al. published in 2015 and looked into medical emergencies in dental offices in the eastern province of Saudi Arabia [11]. Never the less it is well documented that the Saudi population is suffering from a high incidence of Diabetes and cardiovascular diseases [12][13] which again may predispose to medical emergencies during dental treatment. No studies have been conducted in Saudi Arabia to investigate preparedness and knowledge of dentists in the management of medical emergencies.

Methods

This was a cross-sectional study conducted in Jazan province; Saudi Arabia. A questionnaire was formulated to assess preparedness, self - perception and knowledge of medical emergencies (appendix 1). This questionnaire was tested for content validity by three lecturers in the College of Dentistry, Jazan University, and then for readability by a pilot sample of dental practitioners. The study was approved by the Internal research Board at the college of dentistry, Jazan University. A cover letter was added to present the investigators and explain the study's objectives and to encourage participation. At the same time, it ensured the anonymity of the participants and the confidentiality of their data. The participants were supervised to affirm they did not get any assistance.

The participants had first to state the time since they obtained their bachelor degree in dentistry (years of experience) and whether they were specialists or general practitioners.

To assess preparedness, the following questions were asked:

1) Do you take detailed medical history for all your patients?

2) Do you have training in basic life support?

3) Do you have an emergency kit in your clinic?

To assess self - perception, the following questions were asked:

1) Are you confident to perform CPR?

2) Do you think you have the knowledge and skills to manage medical emergencies in your clinic?

To assess the knowledge we decided to avoid using "yes or no" style of questions due to the high probability of false positive replies. We prepared 20 multiple choice questions and focused them equally (five questions each) on the following four categories:

1) Identifying patient at risk of developing medical emergencies

2) Diagnosing medical emergencies

3) Pathophysiology of medical emergencies

4) Treatment and management of medical emergencies

The answers of the MCQs were graded by giving one mark for each correct answer, and no marks were given to wrong answers. The data from all parts of the questionnaire was then entered into excel office, and SPSS program was also used for the statistical analysis.

Results

One hundred dental practitioners participated and answered the questionnaire. Forty-five percent of the participants had less than five years of experience while 26% had from 5 to 10 years and 29% had more than 15 years. Specialists were 17% of participants while general practitioners were 83%.

For the assessment of preparedness 96% of participants claimed they always take a detailed medical history of all their patients. Ninety-five percent have had training in BLS, and 78% have an emergency kit in their clinics (Table 1).

Table 1: Assessment of preparedness

Question	YES	NO
Do you take detailed medical history for all your patients	96%	4%
Do you have training in basic life support	95%	5%
Do you have an Emergency Kit in your clinic	78%	22%

For the assessment of the practitioner's perception on their ability to manage medical emergencies, only 38% thought they are confident to perform CPR, and 82% believed they could manage medical emergencies (Table 2).

Table 2: Assessment of the practitioner's perception

Question	YES	NO
Are you confident to perform CPR	38%	62%
Do you think you have the knowledge and skills to manage medical emergencies in your clinic	82%	18%

In the assessment of knowledge, the average of scores was 10.87 out of 20. The highest score achieved was 16, and the lowest was 2. Twenty-seven of the participants scored less than 10, while only 3 scored 15 or more.

The distribution of scores was as in the following graph:

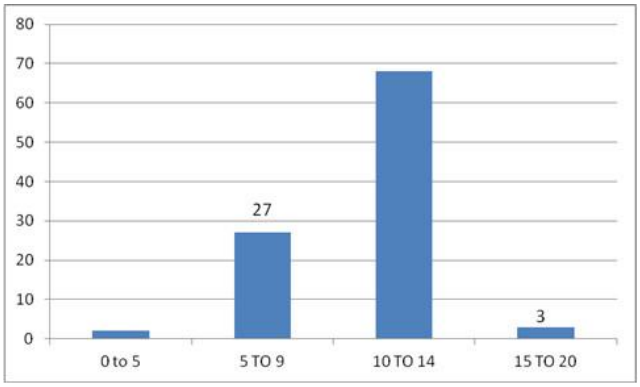

Figure 1: Distribution of scores

The mean scores (out of 5) in different categories of knowledge on medical emergencies were as the following chart:

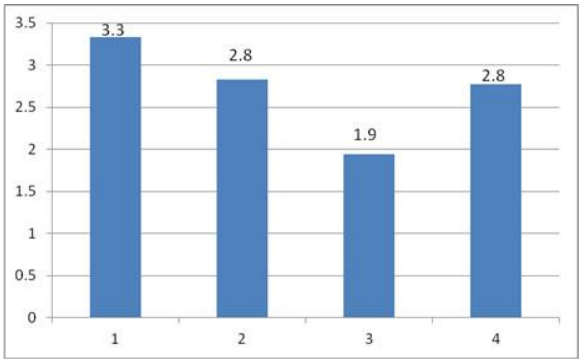

Figure 2: 1) Identifying patient at risk of developing medical emergencies; 2) Diagnosing medical emergencies; 3) Pathophysiology of medical emergencies; 4) Treatment and management of medical emergencies

Differences in scores among different groups were as follows:

Table 3: Knowledge between specialists and non - specialists

Knowledge category	Qualification	No	Mean	SD	the
Identifying patient at risk	Specialist	17	3.35	1.169	0.568
	Non specialist	83	3.33	1.250	
Diagnosing medical emergencies	Specialist	17	2.82	1.131	0.716
	Non specialist	83	2.83	1.080	
Pathophysiology of medical emergencies	Specialist	17	2.00	1.000	0.432
	Non specialist	83	1.93	1.022	
Treatment and management of medical emergencies	Specialist	17	3.06	0.966	0.036
	Non specialist	83	2.71	0.944	
TOTAL	Specialist	17	11.24	2.840	0.605
	Non specialist	83	10.80	2.560	

There were no significant differences in knowledge between specialists and non - specialists except in knowledge on treatment and management of medical emergencies were specialist's showed better knowledge (Table 3).

Table 4: Time since graduation (years of experience)

Knowledge category	Years since graduation	No	Mean	SD	the
Identifying patient at risk	Less than 5	45	3.71	1.325	0.018
	5 to 10	26	3.00	0.894	
	More than 10	29	3.03	1.210	
Diagnosing medical emergencies	Less than 5	45	3.04	1.107	0.178
	5 to 10	26	2.58	1.238	
	More than 10	29	2.72	0.841	
Pathophysiology of medical emergencies	Less than 5	45	1.89	1.112	0.894
	5 to 10	26	1.96	1.076	
	More than 10	29	2.00	0.802	
Treatment of medical emergencies	Less than 5	45	2.84	0.903	0.710
	5 to 10	26	2.77	0.951	
	More than 10	29	2.66	1.045	
Total	Less than 5	45	11.49	1.127	0.450
	5 to 10	26	10.31	1.039	
	More than 10	29	10.41	0.974	

Time since graduation (years of experience) had no significant effect on the knowledge except in knowledge on identifying a patient at risk, were dentists who had less than five years of experience scored better than those with a longer period of experience (Table 4).

Discussion

Having a detailed medical history is an important step to prepare the dentist for a possible occurrence of a medical emergency and even gives a better chance to prevent it. Ninety-six of the interviewed dentists claim they do take detailed medical history while only 4% do not. Taking medical history cannot be omitted and failing to obtain it is considered negligence if any complication did arise.

Most of our sample reported having training in BLS (95%) which was higher than other studies [14][15][16]. This is mainly because getting a practising license in Saudi Arabia requires attending and passing a course on BLS. Nevertheless, it was surprising to find that only 38% were confident to perform CPR. This was slightly lower than some other studies which found that dentists confident to perform CPR was 57% [14] and 46% [15]. The low rate of confidence despite the high number of those who have had training might be either due to the poor quality of training or the lack of frequent practice and refreshing courses.

In Saudi Arabia, it is mandatory by law to have a medical emergency kit in all dental offices. Only 78% claimed they have a kit which is similar to the percentage in a study in New Zealand where 80% of the dentists had a kit in their clinics [15]. It is worth mentioning that the surveyors did not see or check the emergency kits which make us suspect the situation is even worse than what was revealed through the questionnaire.

Eighty-two percent of our sampled dentists think they are capable of managing medical emergencies in their clinics. This was a very high level of perceived confidence when compared to other studies which showed less confidence among dentists [15][16]. We believe this high confidence contradicts with the low confidence in performing CPR and also with the low scores in the knowledge part of the questionnaire.

The knowledge scores of the dentists involved in this study in the assessment of knowledge were below average. There were 20 questions, and the overall average of scores was 10.87. Twenty-nine percent of the dentists scored less than 10, and no one managed to answer all questions correctly. The lowest scores were in the section on understanding the pathophysiology of medical emergencies. The average score was 1.94 out of the five. Successful patient management relies on understanding the underlying pathophysiologic processes and how to correct them [1].

The overall results of this study are in agreement and support other studies which indicated substandard readiness and low confidence among dentists in the management of medical emergencies [2][5][8][10][13][14][15][16][17][18][19][20][21].

Been a specialist or a general practitioner did not significantly affect the level of knowledge of medical emergencies. It was surprising to find that time since graduation also had no significant impact

on knowledge since it was expected that fresh graduates might have better knowledge on the topic.

In conclusion, the duty of care indicates the responsibility of the dentist to attend to and provide the initial management of any possible medical emergency in the premises of his practice within the acceptable standards. In Jazan Province it was clear through self-assessment, by measuring self - perception, and assessment by investigators that there is a defect in the preparedness and ability of dentists to manage medical emergencies.

The regulations must be observed and imposed to insure the material preparedness of dental offices to manage medical emergencies. More emphasis on the topic of medical emergencies should be made in the curriculum during the training. Continuous education and regular courses should be mandatory for all dentists.

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Dental Science – Case Report

Brown Tumour in the Mandible and Skull Osteosclerosis Associated with Primary Hyperparathyroidism – A Case Report

Danica Popovik-Monevska[1], Suzana Bozovik-Dvojakovska[1], Vladimir Popovski[1], Alberto Benedetti[1], Aleksandar Grchev[1], Filip Koneski[2*]

[1]Department of Maxillofacial Surgery, Faculty of Dental Medicine, Ss. Cyril and Methodius University of Skopje, Skopje, Republic of Macedonia; [2]Department of Oral Surgery, Faculty of Dental Medicine, Ss. Cyril and Methodius, University of Skopje, Skopje, Republic of Macedonia

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Keywords: Hyperparathyroidism; Brown a tumour; Mandible; Skull

***Correspondence:** Filip Koneski. Department of Oral Surgery, Faculty of Dental Medicine, "Ss. Cyril and Methodius" University in Skopje, Skopje, Republic of Macedonia. E-mail: dr.koneski@gmail.com

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Abstract

BACKGROUND: The hyperparathyroidism (HPT) is a condition in which the parathyroid hormone (PTH) levels in the blood are increased. HPT is categorised into primary, secondary and tertiary. A rare entity that occurs in the lower jaw in association with HPT is the so-called brown tumour, which an osteolytic lesion is predominantly occurring in the lower jaw. It is usually a manifestation of the late stage of the disease. Osteosclerotic changes in other bones are almost always associated with renal osteodystrophy in secondary HPT and are extremely rare in primary HPT. This article reports a rare case of a brown tumour in the mandible as the first sign of a severe primary HPT, associated with osteosclerotic changes on the skull.

CASE REPORT: A brown tumour in the mandible was diagnosed in 60 - year old female patient with no previous history of systemic disease. The x - rays showed radiolucent osteolytic lesion in the frontal area of the mandible affecting the lamina dura of the frontal teeth, and skull osteosclerosis in the form of salt and pepper sign. The blood analyses revealed increased values of PTH, calcitonin and β – cross-laps, indicating a primary HPT. The scintigraphy of the parathyroid glands showed a presence of adenoma in the left lower lobe. The tumour lesion was surgically removed together with the lower frontal teeth, and this was followed by total parathyroidectomy. The follow - up of one year did not reveal any signs of recurrence.

CONCLUSION: It is critical to ensure that every osteolytic lesion in the maxillofacial region is examined thoroughly. Moreover, a proper and detailed systemic investigation should be performed. Patients should undergo regular check-ups to prevent late complications of HPT.

Introduction

The main hormone that regulates the calcium metabolism in the body is the parathyroid hormone (PTH). It is secreted by the parathyroid glands, and its release is dependent on the plasma concentration of ionised calcium. The lower the concentration, the higher the secretion of PTH is. The primary function of PTH is to normalise the level of calcium in the blood. It demonstrates its activity by activation of osteoclasts, subperiosteal bone resorption, catalysing the vitamin D synthesis in the kidneys and increasing the reabsorption of calcium in the kidneys.

The hyperparathyroidism (HPT) is a condition where PTH is increasingly released from the parathyroid glands. It is categorised into primary, secondary and tertiary [1].

The primary HPT is, in most of the cases, a result of a gland adenoma, and rarely occurs due to malignancy [2]. It is characterised by hypercalcemia. The secondary HPT is a consequence of the hypocalcemia, where the glands increase the production of the hormone to mobilise the calcium from the bones to correct the condition [3]. The tertiary HPT is a condition developed after a long period of increased PTH secretion from the glands. Additionally, another form of HPT exists, known as a paraneoplastic syndrome or pseudohypo-parathyroidism, in which PTH is released into the bloodstream from ectopic parathyroid - like a gland.

A rare entity that occurs in the lower jaw is the so-called brown tumour, which is an osteolytic, cystic - like lesion filled with soft tissue composed of fibrovascular stroma and giant cells [4]. They are called brown tumours because of the colour that they

get as a result of the haemorrhage in the tissue, and the hemosiderin deposits [5][6] The frequency of occurrence of brown tumours in the primary and secondary HPT is 4.5% and 1.5 – 1.7%, respectively. The overall incidence is 0.1% [7]. It is thought that brown tumours are a late manifestation of HPT and nowadays they are not as often detected as before due to the early diagnosis of the endocrinologic condition. Osteosclerotic changes in other bones are almost always associated with renal osteodystrophy in secondary HPT and are extremely rare in primary HPT [8][9][10]. They are usually limited to the pelvis, spine, skull and ends of the long bones.

This article reports a rare case of a brown tumour in the mandible as the first sign of a severe primary HPT, associated with osteosclerotic changes on the skull with the so-called salt and pepper sign.

Case report

Sixty-year-old female referred to our clinic, complaining about discomfort and swelling in the front area of the mandible. The obtained medical data did not show any history of chronic or malignant conditions. According to the patient, the swelling in the vestibulum was gradually increasing with time. The patient did not complain about pain, stiffness or other subjective symptoms. The clinical investigation revealed solid, rounded formation in the vestibulum extended between the canine teeth in the lower jaw. There were no fluctuations, no tenderness and no colour or surface changes of the mass. No fistulas or pus were detected. All the lower incisors were mobile, indicating loss of the surrounding bone. Further examination tools were used: a complete blood analysis and panoramic x-ray were performed.

The blood analysis showed increased values of parathyroid hormone, osteocalcin and β - cross-laps (Table 1). No other significant alterations of the blood parameters were present. The x-ray showed a cystic-like radiolucent lesion in the mandible, extending from the roots of the second premolars from the one side to the second premolars to the other side (Figure 1). The lesion almost reached the lower edge of the body of the lower jaw. A severe reduction of the bone density was observed in the affected area, as well. Also, the lamina dura of the incisors and right canine was also affected. The similar lesion was detected on the x-ray in the left distal area of the mandible body, but that finding was not associated with any clinical signs or symptoms.

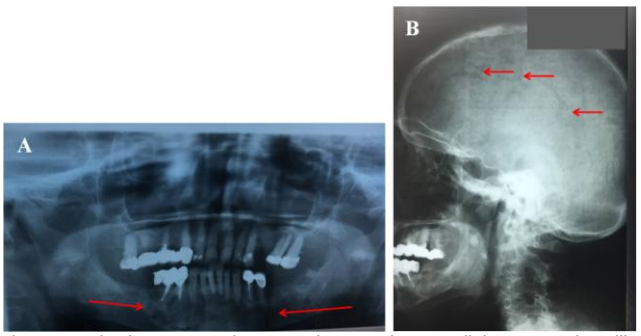

Figure 1: A) The panoramic x-ray shows a huge radiolucent cystic - like change in the frontal area of the mandible (red arrows), extending to the lower edge of the mandible and affecting the lamina dura of the lower frontal teeth; B) The red arrows on the lateral x-ray of the skull show osteosclerotic changes on the calvaria (salt and pepper sign)

The profile x-ray of the skull showed the lesions of the mandible from another perspective, but it also revealed osteosclerotic changes on the calvaria. These changes were addressed as salt and pepper sign.

Table 1: Laboratory tests and findings in the patient that lead to set the diagnosis

Tested parameter	Found value	Normal range
PTH	1312 pg/ml	15-65 pg/ml
Osteocalcin	56.4 ng/ml	15-46 ng/ml*
B - crossLaps	1.57 ng/ml	0.556 ng/ml*
Total Vit. D (25 - Hydroxyvitamin D)	30.88 nmol/l	25-110 nmol/l
Na^+	141 mmol/l	130-150 mmol/l
K^+	4.2 mmol/l	3.3-5.6 mmol/l
Ca^{2+}	1.64 mmol/l	1.16-1.29 mmol/l **

*in postmenopausal women; **in adults 60-90-year old.

Considering the obtained data from the blood and x-ray analyses, a working diagnosis of a brown tumour in the mandible as a result of HPT was set. Two-Phase scintigraphy of the thyroid and parathyroid glands was performed, and it showed accumulation of the Tc99m beyond the lower pole of the left thyroid lobe, extending sub-clavicular and posteriorly (Figure 2).

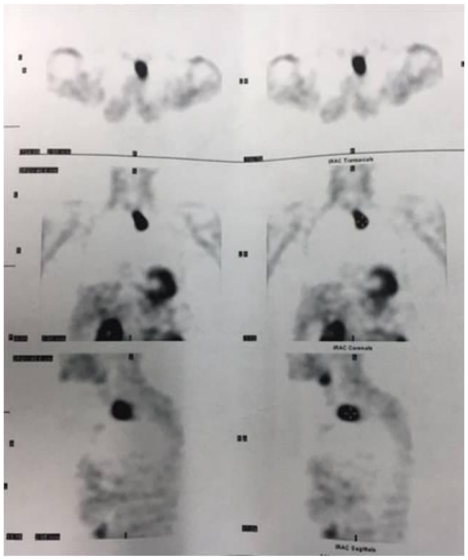

Figure 2: The scintigraphy of the parathyroid glands show accumulation of the isotope in the lower left parathyroid lobe, extending below the clavicle and posteriorly. This finding indicates a presence of gland adenoma

The extension was with a diameter of 4 cm, indicating a presence of adenoma of the parathyroid gland. In the absence of signs of renal failure and ectopic parathyroid glands, the HPT was determined as primary. The treatment plan included surgical removal of a brown tumour from the frontal area in the mandible, followed with excision of the gland adenoma (Figure 3).

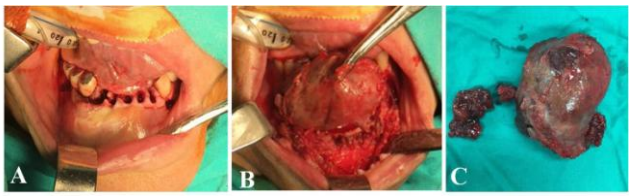

Figure 3: A) Preoperative look of the affected area. The lower frontal teeth were extracted due to severe mobility; B) Intraoperative view of a brown tumour (clamped lesion); C) A tumour was excised completely in one piece

We extracted the lower incisors and right canine due to the severe mobility. An intraoral buccal vestibular incision was made, and the mass was removed in one piece. The histological examination proved the working diagnosis of a brown tumour. The adenoma of the parathyroid gland was also removed, after which the level of PTH was gradually decreasing. No signs of recurrence of a tumour were noted in the following year.

Discussion

The clinical presentation of HPT in the early stages is not often abundant. Later, it may include renal calculi, osteoporosis, peptic ulcers, pancreatitis or neuropsychiatric symptoms [11][12][13][14]. Since HPT changes the bone metabolism, the bones are likely to become affected. Most involved bones are the ribs, clavicles, pelvic girdle and the lower jaws [15].

The brown tumours in the jaws associated with HPT represent reparative granuloma, rather than a true neoplastic process. These changes are due to the reduction of the bone density and its filling with granulation tissue. The lytic lesions on the bones, including the jaws and skull, as showed in our case, are signs of the terminal stadium of HPT. The technological advancements and the possibility of early diagnosis greatly decreased the incidence of these findings [16]. Thus, they are rarely seen in the developed countries [17]. However, this case showed that they still can represent a clinical entity that requires great attention.

A brown tumour associated with primary HPT is more frequently seen in the lower jaw, rather than in the upper jaw. The simultaneous finding in both jaws is extremely rare [18]. The females are three times more likely to be diagnosed with a brown tumour than the males when the disease is progressing to the late stage [3]. A brown tumour in this case, in addition to the bone resorption of the body of the mandible, affected the periodontal apparatus of the frontal teeth which lead to the severe mobility. This loss of the surrounding bone of the teeth was also demonstrated in previous studies and was a radiologic feature within 6 - 55% [15][19].

The diagnosis of a brown tumour cannot be solely set by the histological findings because they are not specific for this condition. The usual findings are mononuclear stroma cells accompanied by multinuclear giant cells [20][21]. These giant cells can be found in other lesions, like aneurismal bone cysts, giant cells granulomas, cherubism and Langerhans histiocytosis [22][23]. Therefore, the definite diagnosis should incorporate the clinical, biochemical, histological and radiological signs. The increased levels of PTH and osteocalcin indicated an increased release of the hormone from the glands. The level of calcium was also elevated due to the bone resorption, as an effect of the action of PTH. B - cross-laps, which are a specific marker for degradation of mature type I collagen during bone resorption, were also elevated. Furthermore, the scintigraphy showed increased uptake of the used isotope in the right parathyroid glands. The skull changes were a sign of general reduction in the bone density. All these findings, accompanied with the lytic lesions seen on the radiographs, depicted the condition of primary HPT.

The principle treatment of the primary HPT associated with bone changes and highly increased blood markers is partial or total parathyroidectomy. It is assumed that after the removing of the reason for HPT, the bone changes will spontaneously regress [13][14][24,25]. This claim is supported particularly for the small tumours and has been demonstrated in previous studies [19]. However, in cases when the tumour dimensions interfere with the function and every - day activities of the patient, the surgical removal is a reasonable solution [26][27]. This was the case with our patient. The growth of the lesion severely damaged the frontal teeth and was a reason for considerable discomfort during eating and even not taking any action. After the surgical removal of the jaw lesion and the gland adenoma, no signs of recurrence were detected in the follow up of one year.

This report shows that HPT may not be diagnosed until the late stages of the disease, despite the technological advancements and available diagnostic tools. The huge brown tumour in the mandible and the osteosclerotic skull changes were the initial signs of primary HPT in our patient. Therefore, it is critical to perform a detailed systemic investigation in the cases of suspect osteolytic lesions in the maxillofacial area. Moreover, rising the awareness and emphasising the importance of regular check-ups are also encouraged. These measures will

prevent the late complications of the disease that can be easily diagnosed in its early stages.

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Effectiveness of Acceptance and Commitment Therapy on Anxiety and Depression of Razi Psychiatric Center Staff

Mostafa Heydari[1], Saideh Masafi[1], Mehdi Jafari[2], Seyed Hassan Saadat[3*], Shima Shahyad[4]

[1]Department of Psychology, Kish International Branch, Islamic Azad University, Kish Island, Iran; [2]Health Psychology, Department of Clinical Psychology, Taleghani Educational Hospital, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran; [3]Behavioral Sciences Research Center, Lifestyle Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran; [4]Neuroscience Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran

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***Correspondence:** Seyed Hassan Saadat. Behavioral Sciences Research Center, Lifestyle Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran. Tel: +98-9125832937. E-mail: saadat350@gmail.com

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Abstract

AIM: Considering the key role of human resources as the main operator of organisations, the present research aimed to determine the effectiveness of acceptance and commitment therapy for anxiety and depression of Razi Psychiatric Center staff.

MATERIALS AND METHODS: This research follows a quasi-experimental type with pre-test, post-test plans, and control group. Accordingly, 30 people were selected through volunteered sampling among Razi Psychiatric Center staff. Then, they were randomly placed into two groups of 15 (experimental and control) and evaluated using research tools. Research tools consisted of Beck Anxiety and Depression Inventories whose reliability and validity have been confirmed in several studies. Research data were analysed using the analysis of covariance (ANCOVA).

Results: The statistical analysis confirmed the difference in the components of anxiety and depression in the experimental group, which had received acceptance and commitment therapy compared to the group that had not received any therapy in this regard (control group) ($p < 0.05$).

CONCLUSION: Acceptance and commitment therapy reduces anxiety and depression.

Introduction

With the increasing complexity of modern societies, the mission of organisations and institutions to meet the expectations of society becomes more sensitive and more important. Therefore, it can be acknowledged that our world is the world of organisations. What is now believed by the experts and consensus is the fundamental role of human resources as the main operators of organizations. In other words, the human gives organisations the life. Undoubtedly, the efficient and motivated workforce can have the most effectiveness to grow, develop, and achieve the planned objectives [1]. Work is an important part of the life of an individual. On the one hand, it can satisfy some basic human needs such as the physical and mental growing, social communication, creating a sense of worth, confidence, and competence, but on the other hand, it can be a major source of stress [2].

Some events during working days are interpreted as an extent of the threat to the physical and psychological well - being. The events that are perceived as stressful factors follow negative emotional responses, particularly anger or anxiety. Thus, these excitements cause behavioural and physical stresses. These pressures also increase the blood pressure, heart rate, and stress hormone secretion such as adrenaline by psychological arousal. Physiological changes in the short term can lead to physical symptoms such as a headache or stomachache. Finally, the continuous high heart rate and blood pressure will also cause heart disease. People must think well to be able to work properly and must be healthy to think well. Therefore, physical and

mental health can have a major impact on human resources productivity. Anxiety and depression are the important psychological issues that can cause physical and mental fatigue. Different interventions have been used to treat depression and anxiety. The point that should be considered is that interventions do not always have a positive effect and sometimes their effectiveness have been limited. Sometimes the interventions have been effective on some people and ineffective for others. Moreover, the new interventions regarding the occupation issues were less considered in Iran.

One of the interventions used in the field of depression and anxiety burnout is acceptance and commitment therapy. This therapy is one of the third wave interventions, which is based on universal consciousness (mindfulness) [3]. In this approach, the universal consciousness is the conscious awareness to experience here and now, with openness, interest, and acceptance. Pervasive consciousness includes living here and now, busy with work in progress and not getting distracted by thoughts. Also, in pervasive consciousness, the person allows thoughts and feelings to come and go what they are without trying to control. When we observe private experience (thoughts and feelings) with openness and acceptance, even the most painful of them are less threatening, and they seem more tolerable [4]. In acceptance and commitment therapy, depression conceptualisation is emotions related to past events such as death or losing something, which prevents normal reactions and adaptation to stressful life events. In above approach, the content of depressed person negative thoughts is not considered. The tendency to behave based on the content of thoughts is called "cognitive fusion", in which it is tried to eliminate the causes of depression that may not be helpful. "Defusion" is against the cognitive fusion, which mediates the consequences of depression, in which the visitors learn to clear their thoughts and make their actions are based on values [5].

According to what was said this study aims to investigate the effectiveness of Acceptance and Commitment Therapy on Anxiety and Depression of Razi Psychiatric Center staff.

Materials and Methods

The present research is quasi-experimental, and the applied plan in the research is pretest-posttest plans with two groups. Pretest and posttest plan was composed of the control group from the experimental group and the control group. Both groups were measured twice. The first measurement was performed by a pretest before the intervention, and the second measurement was performed after the end of required interventions. Table 1 shows the content of ACT sessions.

Table 1: Summary of contents in sessions based on acceptance and commitment therapy

Session	Description of session activities
First	Therapeutic relationship, the people acquaintance with the matter of therapy sessions and treatment contract
Second	Discovering and assessing inefficient strategies used in members to reduce anxiety and depression in different positions and evaluation of their effects, discussion of temporary and ineffective methods of using analogies, feedback and providing assignments
Third	Assisting people to accept painful personal events without conflict with them using analogies, feedback and providing assignments
Fourth	Explain to avoid painful experiences and knowledge of its consequences, training acceptance steps, changing language concepts using the of analogies, relaxation training, feedback and providing assignments
Fifth	The introduction of three - dimensional behavioural model to express the common communication behaviour/emotions, psychological and visible behavioural functions and discussion of trying to change behaviour based on them, feedback and providing assignments
Sixth	Explaining the concepts of roles and terms, viewing themselves as a context and contacting by analogies, understand the different sensory perceptions and mental separation, feedback and providing assignments
Seventh	Explaining the concept of values, creating motivation and empowering people for a better life, concentration exercises, feedback and providing assignments
Eighth	Training commitment to action, identifying behavioural patterns by values and commitment to act, summing up meetings, implementation after testing

The statistical population, sample, and sampling

The statistical population of the present research included all Razi psychiatric centre staff who have worked in 2015 - 16. The sample was selected through the voluntarily sampling method and randomly divided into two groups, the experimental group, and the control group. The number of people in each of two groups was 15 people.

The experimental group members participate in eight 90 minutes' sessions per week. No intervention was done in the control group.

Inclusion criteria

- The work experience of samples was considered five years and over in Razi psychiatric centre, with a Bachelor's degree level and above, in both sexes.
- The rate of burnout was evaluated using the Maslach Burnout Inventory at medium to high level.
- The sample must not have any history of mental illness.

Beck Anxiety Inventory

Beck Anxiety Inventory includes 21 items that there are four options for every phrase to answer. Each phrase reflects one of the symptoms of anxiety that usually people experience who are clinically anxious or are in a state of apprehension. The subjects sign their suffering from symptoms of anxiety last week in a column. Scoring includes not at all zero score, low one score, medium two scores, and severe

three scores. The anxiety scores range is from zero to 63.

Beck et al. (1988) expressed the reliability of the questionnaire in 1988 as much as 0.75 through retesting on 83 outpatients within a week. Federikh et al. (1992) reported an alpha coefficient as much as 0.94 for 40 outpatients. In a study on Iranian population, the Cronbach's alpha coefficient was 0.90 [6]. Also, the validity, reliability and internal consistency of the Beck Anxiety Inventory on Iranian population recorded as 0.72, 0.83, and 0.92, respectively [7].

Beck Depression Inventory

The inventory was first developed by Beck et al. (1961) [8]. BDI-II is a 21 - item self - report inventory, which is the revised form of BDI. It is applied to determine the severity of depression and depressive symptoms in psychiatric patients and determining depression in the general population. The scores of the inventory are placed up to 3 based on four options (0 - 3) for the absence of the specific indication to the highest degree of the sign in the scope.

Beck el al. reviewed studies that had used this tool and found that its reliability coefficient using retesting varied from 0.48 to 0.86 according to the distance between the frequency and the running. Beck el al. (1996) once again obtained retest reliability coefficient within one week as much as 0.93. Several studies have been conducted in Iran to measure the psychometric properties of BDI - II which its reliability was 0.78 and its validity were varied from 0.70 to 0.90 [9][10].

Research method

The anxiety and depression questionnaire was distributed among Razi psychiatric centre staff after preparation of research tools. People who have a moderate to high anxiety and depression were selected. Then, they completed Beck Depression and Anxiety Inventory. Among them, 30 people who have a moderate to high depression and anxiety were selected and randomly divided into two experimental and control groups of 15 people (This questionnaire was considered as a pretest for both groups). Those in the experimental group received Acceptance and Commitment therapy, but the control group did not receive this treatment. The method of treatment for the experimental group was eight 90 minutes' sessions per week. At the end of the weekly sessions (8 sessions), the questionnaires were given to the group again and anxiety and depression rate was recorded (post-test). Two months after the end of the session, a meeting was conducted on two groups, and their anxiety and depression were measured and recorded. The obtained scores by the pre-test and post-test scores as well as follow-up meeting scores to assess the effectiveness of the independent variables were analysed in the group. It is worth mentioning that intervention sessions are formed as a group that the summary of the content of each session is as follows:

Results

The descriptive findings of anxiety and depression are given in Table 2.

Table 2: The descriptive findings related to depression in the experimental and control groups in the pre-test, post-test, and follow - up

		Experiment	Control
Depression	Pre-test (n = 15)	21 ± 3.4	21.3 ± 3.0
	Post-test (n = 15)	17 ± 3.4	20.8 ± 2.8
	Follow-up (n = 15)	16.8 ± 3.0	20.3 ± 2.9
Anxiety	Pre-test (n = 15)	21.7 ± 1.2	22.6 ± 1.9
	Post-test (n = 15)	14.5 ± 1.9	21.7 ± 2.9
	Follow-up (n = 15)	14.0 ± 2.1	20.9 ± 2.2

Analysis of covariance was used to investigate this hypothesis according to the two-level class independent variable (experimental group and control group), the continuous dependent variable (anxiety and depression posttest scores) and independent variable (anxiety and depression pretest scores). Surveying data for the analysis of covariance showed that most of the assumptions are confirmed. Only the homogeneity of variances in some of the components was outside the criteria for which they have considered the alpha as much as 0.025. Covariance analysis results are reported below.

Table 3: Results of covariance analysis on the post-test anxiety scores in the experimental and control groups by controlling the pretest

Dependent variable	Source	Sum of squares	Df	Mean Square	F	Significance level	Impact rate
Anxiety posttest	Pretest	32.039	1	32.039	12.532	0.001	0.317
	Group	306.675	1	306.675	119.955	0.000	0.816
	Error	69.028	27	2.557			
	Total	10282.000	30				
Anxiety follow up	Pretest	39.414	0	39.414	11.782	0.002	0.304
	Group	271.201	1	271.201	81.072	0.000	0.750
	Error	90.320	27	3.345			
	Total	9601.000	30				

According to the above table results, there is a significant difference in the experimental and control

groups among anxiety (p = 0.000, F = 119.955). This table shows that there is a significant difference in posttest by removing the effect of pre-test scores among the adjusted average based on the group. In general, it can be said that acceptance and commitment therapy in post-test reduces anxiety. Given the size of this effect, the rate is significant. The follow - up results showed that treatment was stable by eliminating the effect of pretest (p = 0.000, F = 81.072). Therefore, it can be said that acceptance and commitment therapy significantly reduces anxiety in the long term.

Table 4: Results of covariance analysis on depression scores in the experimental group and control group by controlling the pre-test

Dependent variable	Source	Sum of squares	Df	Mean Square	F	Significance level	Impact rate
Depression posttest	Pretest	255.683	1	255.683	412.950	0.000	0.939
	Group	94.299	1	94.299	152.302	0.000	0.849
	Error	16.717	27	0.619			
	Total	11097.000	30				
Depression follow up posttest	Pretest	176.253	1	176.253	64.764	0.000	0.706
	Group	82.767	1	82.767	30.413	0.000	0.530
	Error	73.480	27	2.721			
	Total	10685.000	30				

According to the results, it can be said that there is a significant difference in the experimental and control groups among depression (p = 0.000, F = 152.302). This table shows that there is a significant difference in post-test by removing the effect of pre-test scores among the adjusted average based on the group. In general, it can be said that acceptance and commitment therapy in post-test reduces depression. Given the size of this effect, the rate is significant. The follow-up results showed that treatment was stable by eliminating the effect of pretest (p = 0.000, F = 30.413). Therefore, it can be said that acceptance and commitment therapy significantly reduces depression in the long term.

Discussion

In the current study, the effectiveness of Acceptance and Commitment Therapy on Anxiety and Depression was investigated and finding demonstrates that Acceptance and Commitment Therapy could reduce anxiety and depression. These results are consistent with findings of previous studies [16][11]. Nariman et al. showed that Acceptance/ Commitment Training have a positive effect on decreasing the social anxiety in students with specific learning disorder (SLD) [11]. Hosseinaei et al. demonstrated Group acceptance and commitment therapy (ACT) - based training decreases job stress but has no considerable effect on job burnout [12]. In the study, Lang et al. evaluated the efficacy of Acceptance and commitment therapy (ACT) for emotional distress among veterans of the conflicts in Iraq and Afghanistan. They found improvement following treatment in the whole sample across a variety of measures, including general distress and functioning and moderate to high levels of satisfaction with treatment [13].

Acceptance and Commitment Therapy has several basic components that are emphasising them at the different steps makes individuals accepting their problems and perceiving less anxiety and stress, which improves the health. This method is the limit range of mental flexibility, i.e. creating the ability for a practical choice among the various options that are more relevant rather than a practice, which is merely imposed to avoid thoughts, feelings, and disturbing memories [17]. In this therapy, it is initially tried to increase the subjects' psychological acceptance of subjective experiences (thoughts, feelings) and to reduce ineffective control practices mutually.

The patient is taught that any action to prevent or control these unwanted mental experiences are ineffective or inversed, which exacerbate them. The experience should be completely accepted without any internal or external reaction to remove them. The mental experience in patients includes things such as emotional ambivalence, frustration, chronic sadness, loneliness, loss of hope and a sense of continuity of generations, embarrassment, shame, guilt, and anger. Therefore, participants in the first step learned to accept the feelings without a reaction at first. In the second step, the psychological knowledge of subjects is added. This means that the individuals are aware of all mental states, thoughts, and behaviour in the present moment. In the third stage, the individuals are taught to separate themselves from the subjective experiences (cognitive isolation) so that they can act independently of the experience.

Fourth are the efforts to reduce the excessive focus on visualisation or personal story (as victims) that the individuals have made for themselves. Fifth is helping the individuals to understand their basic personal values and identify them to convert to specific behavioural goals (to clarify the values). Finally, motivating them to act responsibly towards the goals and values of the activities identified with the adoption of mental experiences. Finally, motivating them to act responsibly towards the goals and values of the activities identified with the adoption of mental experiences.

These thoughts can be subjective experiences related to events (trauma), or social anxiety and concerns. Thus, in the final stage, it is observed that participants accept their subjective experiences and they can act responsibly. The first direct consequence of accepting the feelings and emotions is reducing the negative thoughts, and responsible behaviour leads to an effective action instead of an anxious reaction.

To explain depression, it can be said that Acceptance and Commitment Therapy according to theorists is an important factor in creating and maintaining psychological trauma and increasing depression is an experimental avoiding. This means the exaggerated negative assessment of internal experiences (such as thoughts, feelings, and emotions) and the lack of willingness to experience, which leads to attempts to control or escape from them and can intervene in individual performance [17].

People who are more experimental avoiding experience, positive emotional experiences and lower mental health and feel that their lives are meaningless. However, the purpose of acceptance and commitment therapy is reducing the experimental avoiding and increasing psychological flexibility through accepting the inevitable distressing and unpleasant feelings, mindfulness cultivating to neutralise the excessive involvement by recognising and identifying personal values related to behavioural goals. Participants are encouraged to communicate with their experiences fully and without resistance while in motion toward worthy goals and accept them without judging their truth or falsity when the emergence.

This increases motivation to change despite obstacles and encourages individuals to achieve worthy goals in life. This will lead to depression improvement, especially in the field of psychology. Psychological flexibility and acceptance could improve the health status of people in different fields and help them to promote meaningful aspects of life and to increase valuable activities to help to improve the depression. Acceptance of thoughts as thoughts, feelings as feelings, and emotions as emotions (as they are, no more and no less), leads to weakening the cognitive fusions. Also, the adoption of internal events, when the individuals conflict with depressions and disturbances, allow them to develop their behavioural coffers. They can spend the obtained time to do targeted and valuable activities. Thus, depression is improved in this way.

This study has some limitations that restricted its application and generalisations. First, these findings are based on self - reporting of people. Secondly, the lack of large sample size of the population could be mention as other potential limitation. In the end, further studies with larger sample sizes are suggested to clarify the results of this study, additionally analyses the pattern of all aspects of the Acceptance and Commitment Therapy on anxiety and depression is recommended.

It can be said that Acceptance and Commitment Therapy could reduce anxiety and depression.

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Public Health

Stress Factors among Nurses at the Primary and Secondary Level of Public Sector Health Care: The Case of Slovenia

Jasmina Starc*

The University of Novo Mesto, Faculty of Health Sciences, Loko 2, Novo Mesto 8000, Slovenia

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***Correspondence:** Jasmina Starc. University of Novo mesto - Faculty of Health Sciences Novo mesto Na Loko 2 Novo mesto 8000 Slovenia. E-mail: jasmina.starc@guest.arnes.si

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Abstract

BACKGROUND: Working in nursing is mentally and physically demanding and is one of the most stressful professions.

AIM: To determine the basic causes of stress and examine the symptoms of stress among healthcare professionals at the primary and secondary level of health care.

MATERIAL AND METHODS: The research was based on the descriptive and causal non-experimental method of empirical research. The independent samples t-test was used.

RESULTS: The survey results have shown that those employed in nursing are exposed to stressful situations on a daily basis, most often involving psychological or physical violence in the workplace (M = 4.2), dealing with death (M = 3.9), lack of personnel (M = 3.9) and a high frequency of patients (M = 3.8). The following stress factors cause women greater stress than they do men: relationships among co-workers (t = 2.745; p = 0.006), psychological or physical violence in the workplace (t = 3.492; p = 0.001), and working with difficult patients (t = 2.427; p = 0.017).

CONCLUSION: To manage risks, employees and employers must work together and establish a suitable safety and organisational culture, which would enable them to manage and reduce stress.

Introduction

Stress affects all of us and is very common in organisations within the health care system, particularly in those that experience rapid changes and have a poor communication network [1]. Besides work processes, another important element is the organisation of work, and of resources, which can have a significant impact on the quality of work, on productivity, creativity, competitiveness, nursing care outcomes, and on ensuring patients' safety [2]. Other important elements are a professional liability, unfavourable working conditions, workload, working in shifts (this is unique to nurses working in secondary health care in Slovenia), working at night, poor interrelationships, a lack of information, accidental cuts and stabs, etc. [3][4][5].

Stress affects a person's psychophysical balance and their personality as a whole, which is reflected in their personal lives on the physical, emotional, psychological and social level, and, in the case of those employed in healthcare, also in their attitude towards patients [6]. In their line of work, nurses establish a wide range of relationships with patients and their relatives, which generate different types of tension that may lead to various stressful situations [7].

Despite the fact that nurses often notice a lack of respect from doctors, and that their knowledge and skills are underestimated [11], they are nevertheless expected to provide humane, compassionate, culturally sensitive, competent and ethical nursing care [12]. Hence, nurses have found themselves in an increasingly difficult, demanding and delicate situation, which is evident from the growing complexity of nursing interventions and the growing needs of patients on the one hand, and the demand for quality health care services on the other [13]. They begin to show emotional signs of stress (despair, concern, irritability, angry outbursts, dissatisfaction, oversensitivity, lack of self-respect and energy, fear, depression, etc.), as well as intellectual and mental

signs of stress (feeling of incompetence, incompletion of tasks, trouble concentrating, inability to think clearly, irrationality, unreasonable decisions, etc.) [14][15].

Due to their professional empathy towards those in need of help, nurses are exposed to stress factors on a daily basis. Events in their relative form trigger impulsive reactions, and after prolonged exposure, these often lead to the phenomenon known as burnout [18]. Situations which may lead to burnout in the workplace are: an excessive workload, which is increasing with the faster work tempo; intense and complex work; insufficient or strict supervision of work; an inadequate remuneration system; a lack of honesty; value conflicts; hindering professional development, etc. [13][19][20][21]. The burnout of nurses is also the result of particularly demanding, conflictive, harder-to-manage patients, as well as of the terminally ill, with whom the staff has frequent contacts and through whom they confront suffering and dying [22][24].

There are many stress and burnout prevention measures, strategies and techniques, which enable individuals to alter their perception, attitudes and behaviours to preserve their health and well-being. The main ones are [25]: cognitive restructuring, constructive monologue, time management, social support, assertiveness training, coaching, supervision, etc. Some authors [26][28][29][30] also highlight the role of organizations and the measures they take, such as: stress and burnout analysis and evaluation for the purpose of determining the factors of stress and burnout within the organization; programmes for improving the physical and mental fitness of employees; reallocation of employees to more suitable posts; an appropriate strategy of introducing occupational safety and health; social responsibility; establishing a suitable organizational culture; risk management; seminars and workshops on the topic of stress and burnout in the workplace, etc.

The main objective of this research study was to establish the basic causes of stress and examine the symptoms of stress and burnout among nurses in primary and secondary healthcare. The following research questions were asked:

1. Which factors most often cause nurses workplace stress?
2. How does stress affect the quality of nursing performance?
3. Which methods and techniques for reducing or eliminating stress do nurses use?
4. Are there any statistically significant differences between the two groups of respondents (women, men) in the factors that most often cause workplace stress?
5. Are there any statistically significant differences between the two groups of respondents (primary and secondary health care) in the factors that most often cause workplace stress?

Material and Methods

Study Design

The research was based on a descriptive and causal non-experimental work method. A questionnaire was used as the data collection method. The questionnaire was prepared based on a review of the literature [4][5][6][16][21] and adapted to the needs of the present study. The questionnaire consisted of nine closed-ended questions, which were divided into the following sets:

- Question about stress factors in the workplace: The respondents were offered 20 stress factors, which they rated on an attitude scale based on the level of stress they were causing them. The nurses rated the items on an attitude scale, ranging from 1 to 5, with 1 meaning “never”, 2 “rarely”, 3 “occasionally”, 4 “often” and 5 “always”.
- Question about the impact of stress on the quality of nursing performance: The respondents were offered 10 different methods/reactions to stressful situations in the workplace, which they rated on an attitude scale based on how the method/reaction was affecting their nursing performance. The nurses rated the items on an attitude scale, ranging from 1 to 5, with 1 meaning “never”, 2 “rarely”, 3 “occasionally”, 4 “often” and 5 “always”.
- Question about the methods and techniques for reducing or eliminating stress: The respondents were offered 11 methods or techniques for reducing or eliminating stress. They could circle multiple answers.
- Questions about the sample’s demographic characteristics: gender, age, education, length of service, the area of work, job satisfaction.

Participants

The survey was conducted among 370 nurses (14% male, 86% female) – according to data from Nurses and Midwives Association of Slovenia [34]. This ratio equals the Slovenian average of gender representation in nursing. 30% of them were between the ages of 31 and 40; 25% were under 30 and between 41 and 50, respectively; and 20% were 51 and over. 64% of them were nurses with a bachelor’s degree, 19% were nurses who have completed

secondary school, 12% had a master's degree in medicine or nursing, and 5% were nurses who have completed a short-cycle college. The majority (32%) had up to 10 years of service, 26% from 11 to 20 years of service, and 21% from 21 to 30 years of service and 31 or more years of service, respectively. 65% of them were employed in primary health care and 35% in secondary health care. 36% of them were very satisfied with the work they were doing; 56% of them were satisfied; 6% were undecided; 2% were dissatisfied, but no one was very dissatisfied.

Data Analysis

The survey was conducted online using the sampling method for social networks – snowball sampling. The method's strength lies in the fact that it is the best and cheapest way to contact the target population.

All respondents participated voluntarily and anonymously in June and in the first half of July 2017.

The research complies with the ethical principles of researching and protecting collected data (the personal data of respondents was not connected with the answers, which prevented us from identifying them with the published results; moreover, the data was used solely for research purposes and not for subsequent non-research purposes which would violate the dimension of information privacy).

Results were described with absolute and relative frequencies. To analyse the differences in stress factors between both groups of respondents (women – men; primary health care – secondary health care), the results were verified with the independent samples t-test. These differences were confirmed with a 5% probability of error.

The data were processed using the SPSS 23.0 statistical software package. The reliability of the attitude scale regarding the frequency of stress factors was confirmed by Cronbach's α with a value of 0.815; regarding the impact of stress on the quality of nursing performance the value was 0.780; and 0.770 regarding the methods and techniques for reducing or eliminating stress.

Results

The study began by taking a look at the factors that most often cause nurses workplace stress. The survey results show that the following factors cause them the greatest stress: psychological or physical abuse in the workplace (M = 4.2), being confronted with death (M = 3.9), a lack of staff (M = 3.9), and a high frequency of patients (M = 3.8). They are caused moderate stress by exposure to infection (M = 3.5), working at night (M = 3.2), their working hours (M = 3.1), working conditions (M = 3.1), low pay (M = 2.9), working with difficult patients (M = 2.8), and poor work organization (M = 2.5). They are caused minor stress by relationships between the management and employees (M = 2.2), a lack of material resources (M = 2.2), administrative work (M = 2.2), relationships among co-workers (M = 2.1), and a lack of training (M = 2.1). They consider the following to be the least stressful factors: the social security of their jobs (M = 1.8), diversity of work (M = 1.7), working overtime (M = 1.7) and work suited to their abilities (M = 1.5) (Fig. 1).

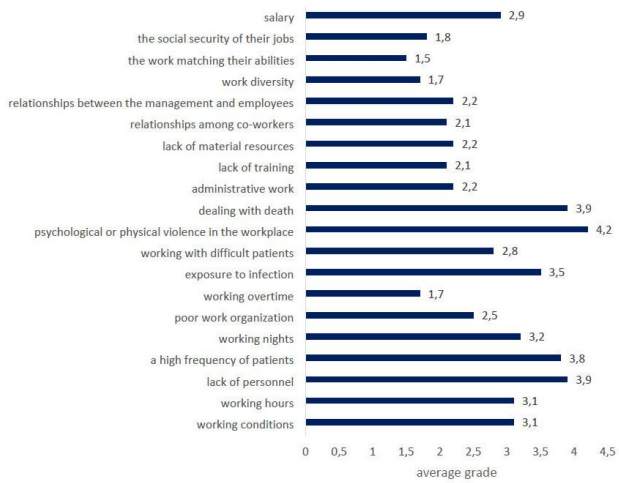

Figure 1: Stress factors

Afterwards, the study tried to determine how stress affects the quality of nursing performance. The results of the analysis (Fig. 2) show that stress affects nurses in different ways. Most of them have trouble concentrating (M = 4.1), become unmotivated to work (M = 3.4), and enter into conflicts with patients (M = 3.4).

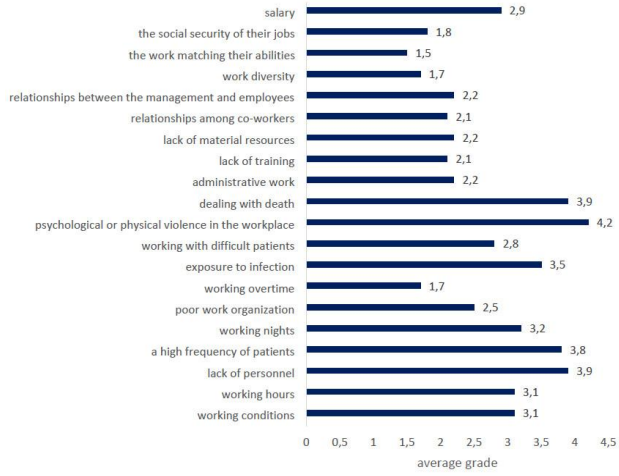

Figure 2: Impact of stress on the quality of nursing

Some of them also commit malpractice (M = 3.2), get angry towards patients (M = 3.2), become unfriendly towards patients (M = 3.2), and enter into conflicts with co-workers (M = 3.1). Despite stressful situations, only a minority of them fail to do their job (M = 2.9), rarely enter into conflicts with management (M = 2.2), and make unreasonable decisions (M = 2.1).

The study was also interested in the methods and techniques they use for reducing or eliminating stress. They could choose multiple answers (Fig. 3).

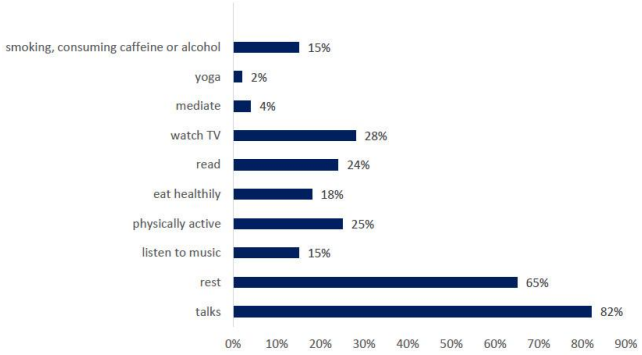

Figure 3: Methods and techniques for reducing or eliminating stress

The survey results show that nurses most often reduce or eliminate stress through talks (82%) and rest (65%). Some of them also decide to watch TV (28%), be physically active (25%), read (24%), eat healthily (18%), listen to music (15%) and relieve stress by smoking, consuming caffeine or alcohol (15%). Only 4% decide to meditate and 2% to do yoga.

An independent samples t-test was used to verify if there were any statistically significant differences between genders in the variables that measure individual dimensions of stress factors.

Table 1: T-test for checking for differences in stress factors between genders

Stress factor	Gender	n	M	t	*p*
Relationships among co-workers	Male	52	1.91	2.745	0.006
	Female	318	2.11		
Psychological or physical violence in the workplace	Male	52	3.90	3.492	0.001
	Female	318	4.51		
Working with difficult patients	Male	52	2.2	2.427	0.017
	Female	318	3.25		

The results of the analysis show that there are statistically significant differences between both groups of respondents (women, men) in the following factors: relationships among co-workers (t = 2.745; p = 0.006), psychological or physical abuse in the workplace (t =3.492; p = 0.001), and working with difficult patients (t = 2.427; p = 0.017). These factors are much more stressful for women than they are for men.

An independent samples t-test was used to verify if there were any statistically significant differences between nurses working in primary and secondary healthcare.

Table 2: T-test for checking for differences in stress factors between nurses working at the primary and secondary level of health care

Stress factor	Level of healthcare	n	M	t	*p*
dealing with death	Primary level	240	3.3	2.926	0.003
	Secondary level	130	4.5		
psychological or physical violence in the workplace	Primary level	240	4.4	2.454	0.003
	Secondary level	130	4.0		
working with difficult patients	Primary level	240	2.0	2.678	0.014
	Secondary level	130	3.6		
exposure to infection	Primary level	240	3.2	1.411	0.011
	Secondary level	130	3.8		
working nights	Primary level	240	2.5	1.702	0.009
	Secondary level	130	3.9		
a high frequency of patients	Primary level	240	3.6	2.941	0.003
	Secondary level	130	4.0		
lack of personnel	Primary level	240	3.6	3.131	0.002
	Secondary level	130	4.2		
working hours	Primary level	240	2.4	3.108	0.006
	Secondary level	130	3.8		

The results of the analysis show that there are statistically significant differences between nurses working in primary and secondary health care in the following factors: being confronted with death (t = 2.962; p = 0.003); psychological or physical abuse in the workplace (t = 2.454; p = 0.003); working with difficult patients (t = 2.678; p = 0.014); exposure to infection (t =1.411; p = 0.011); working at night (t =1.702; p = 0.009); a high frequency of patients (t = 2.941; p = 0.003); a lack of staff (t = 3.131; p = 0.002); working hours (t = 3.108; p = 0.006). These factors are much more stressful for nurses working in secondary health care.

Discussion

The first research question inquired about the factors that most often caused nurses stress in the workplace. It was discovered that nurses rated psychological or physical abuse, being confronted with death, a lack of staff, a high frequency of patients, and exposure to infection as highly stressful factors in the workplace. Other researchers [8][12][31] have reached similar conclusions, since the results of their research show that nurses experience stress due to: a lack of staff and the resulting excessive workload, highly demanding work and tasks, too high expectations from and inappropriate attitudes of superiors, poor work organization, and not getting along with co-workers. According to the latest Slovenian research study [23], conducted among nurses in secondary health care, the most stressful factors are low pay, poor interpersonal relationships in the workplace, and psychological or physical abuse in the workplace. Similar results were reached by researchers in China [17], where it was discovered that stress among head nurses and senior nurses was

caused by factors such as the nursing profession and work-related issues; time allocation and workload problems; working conditions and equipment problems; patient care issues; and management and interpersonal problems.

The management of workplace stress is one of the key elements in ensuring the health and well-being of nurses because healthy and satisfied employees are the capital of every health care institution. The signs of stress are difficult to recognise at first since they appear in different areas of their everyday lives [32]. Magnusson and Gooding [18] have established that stress has the biggest impact on those working in the helping professions because they are working in highly emotionally demanding situations, in which they are confronted with people's problems and pain on a daily basis.

All of these situations influence the quality of the work they are performing. The second research question inquired about how stress was affecting the quality of nursing performance. It was discovered that in stressful situations nurses have trouble concentrating, are unmotivated to work, and enter into conflicts with patients and co-workers. Jerčič and Kersnič [11] list the following effects of stress: an increase in workplace injuries and errors in judgement; feeling incapable of carrying out tasks correctly; diminished productivity; a decline in motivation; inability to carry out tasks correctly; poor job performance; and insensitivity to other people's needs. The present research study has reached similar conclusions, considering that the respondents have stated that the (low) quality of their nursing performance is affected by diminished concentration on work and a lack of motivation to work and that they commit malpractice, are unfriendly towards patients, and get angry.

Kaučič [12] warns that stressful situations, which may lead to workplace burnout, reduce the critical-thinking, problem-solving and decision -making skills of nurses. Those affected by stress try to overcome their problems by resorting to alcohol, narcotics and smoking, which makes matters even worse. Bilban and Pšeničny [2] add that the nurses who ignore signs of fatigue and stress and increase their activity further by "running" to workaholism, intensify their exhaustion into burnout. It is, therefore, necessary to teach nurses the skills to overcome stress, which act as protection against emotional exhaustion, stress and burnout [21]. Sometimes, they also need help from a psychologist [33]. There are many stress prevention strategies and techniques which enable individuals to alter their perception, attitudes and behaviours to preserve their health and well-being [9][25]. The survey results (third research question) show that the methods and techniques nurses most often use to reduce or eliminate stress are talks and rest, while they only rarely use yoga and meditation.

The study also answered the fourth research question about whether there were any statistically significant differences between the two groups of respondents (women, men) in the factors that most often cause workplace stress. The results of the analysis show that relationships among co-workers, psychological or physical abuse in the workplace, and working with difficult patients are much more stressful factors for women than they are for men. Jennings [10] reached similar results and added that the family - work conflict about stress, burnout, and well -being indicated the importance of considering both work and family spheres. Her study may have particular relevance for nursing because the profession is predominately female.

The fifth research question inquired about whether there were any statistically significant differences between the two groups of respondents (primary and secondary health care) in the factors that most often cause workplace stress. It was concluded that for the surveyed nurses employed in secondary health care the factors of being confronted with death, psychological or physical abuse in the workplace, working with difficult patients, exposure to infection, working at night, a high frequency of patients, a lack of staff, and working hours ($t = 3.108$; $p = 0.006$) were more stressful than for the nurses employed in primary health care. So far, no in-depth research has been conducted into the differences in stress factors among nurses employed in primary and secondary healthcare in Slovenia. After reviewing the literature, Ščuka [27] discovered that absenteeism is on the rise in the public sector in Slovenia; it is three times higher than absences from work in the commercial sector, with as many as 38% of employees experiencing excessive stress at work.

Study Limitations, Guidelines for Further Research and Suggestions

This study has certain limitations, which is why its results cannot be generalised to the entire population of nurses in primary and secondary healthcare; the participating nurses were selected in a way that does not guarantee representativeness. However, the research findings may serve as a starting point for further research into this field.

It would be sensible to conduct a longitudinal study, which would compare the impact of stress on the quality of nursing performance about the nurses' length of service and their use of methods and techniques for reducing or eliminating stress, both in primary and secondary healthcare. Considering that no in-depth research has been conducted into the differences in stress factors among nurses employed in primary and secondary healthcare in Slovenia, it would be wise to devote more attention to this segment of research in the future.

We must provide employed nurses with a safe working environment, improve their work method, clearly define the roles of individuals in a healthcare institution, improve the organisational climate and communication among employees, and shape strategies for improving motivation in the workplace. But above all, we must teach them how to identify and eliminate or reduce stressful situations, how to cope with stress, and offer them support when they are already exposed to stress and perhaps also suffering the consequences [14]. The greater the number of individuals in a nursing team who are in control of themselves and aware of their stress factors and of their reactions to them, the more they change the culture of the entire healthcare team. Nowadays, we are striving towards an organisational culture in which mutual respect and assertive behaviour are predominant [12].

In connection with the above, we propose that nurses in primary and secondary healthcare are provided compulsory and continuous supervision, lectures and expert workshops on raising awareness about which measures to take when stressful situations arise, led by experienced experts with psychotherapeutic knowledge. It may prove effective to improve working conditions, show moderation in standardizing the work of nurses and how they maintain control over their work, reduce the number of patients in the care of a single nurse, allow a suitable amount of time to administrative work (work on a computer), clearly define a nurse's competencies, etc.

The management of all healthcare institutions should clearly define strategies for reducing stress depending on the size of the institution and the financial resources at its disposal, e.g.: programmes for improving well-being in the workplace, introduction of relaxation techniques as part of job training (autogenic training, yoga), including a list of measures for ensuring proper safety at work and consequently protecting the health of nurses. Health care institutions, and patients, in particular, benefit the most from a healthy and rested nurse because her efficiency and productivity are increased, the number of work-related mistakes are reduced, sick leaves are rarer and shorter, and burnout does not occur. By introducing said strategies, we would not only reduce the stress-related illness rate among nurses but would also significantly raise the quality of their work.

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Epidemiological Profile of Acute Viral Encephalitis in a Sample of Egyptian Children

Bassant Meligy[1], Dalia Kadry[2], Iman H. Draz[1], Huda Marzouk[1], Nevine R. El Baroudy[1], Amira Sayed El Rifay[3*]

[1]Department of Paediatrics, Faculty of Medicine, Cairo University, Cairo, Egypt; [2]Department of Microbiology, Faculty of Medicine, Cairo University, Cairo, Egypt; [3]Departments of Child Health, National Research Centre, Cairo, Egypt

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***Correspondence:** Amira Sayed El Rifay, Departments of Child Health, National Research Centre, Cairo, Egypt. E-mail: amirasayed.ak@gmail.com

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Abstract

INTRODUCTION: Acute encephalitis syndrome (AES) is a considerable public health problem.

AIM: This study was designed to describe the aetiology, demographic features, clinical picture, short-term outcome and risk factors of mortality of children with viral encephalitis in Egyptian children.

METHODS: PCR detection of viruses in the CSF of pediatric patients admitted to the pediatric unit or ICU Cairo University Pediatric hospital presenting with encephalitis syndrome.

RESULTS: Of the 96 patients included in the study, viral etiological agents were detected in 20 cases (20.8%), while 76 patients (79.2%) had no definite viral aetiology. The most abundant virus detected was Enterovirus (EV) in fourteen (14.5%), two (2.1%) were positive for human herpes simplex virus 6 (HSV-6), one (1.0%), human herpes simplex virus1 (HSV-1), one (1.0%) Epstein Barr virus (EBV), one (1.0%), cytomegalovirus (CMV) and one (1.0%) with varicella-zoster virus (VZV). On the short term outcome, 22 (22.9) patients died, and 74 (77.1%) survived. Severity outcome among survival was vegetative in three cases (4%) severe in 9 (12.16%), moderate in 14 (18.9%), mild in 29 (39.2%) and full recovery in 19 (25.6%). Mortality risk factors for younger age, the presence of apnea, the need for mechanical ventilation and the presence of abnormal CT findings were all significantly associated with fatal outcome ($p < 0.05$).

CONCLUSION: Enterovirus was the most common cause of encephalitis among Egyptian children. Mortality was correlated with younger age and disease severity at admission. Sequelae were high among infected children.

Introduction

Encephalitis is a critical, potentially lethal condition that can cause a variety of viral, bacterial, parasitic, as well as from toxins and autoimmune reactions to vaccines [1].

Although viruses are considered as the most important etiological agents of encephalitis worldwide [2], viruses are often poorly understood the cause of encephalitis. In more than 60% of encephalitis, a demonstrable etiologic agent cannot be identified [3] [4] [5].

Acute viral encephalitis (VE) is an unusual presentation of viral infections which affects children and young adults mainly after viral invasion of the central nervous system presenting by acute onset of fever and a change in mental status (including symptoms such as confusion, disorientation, coma, or inability to talk) and/or new onset of seizures in a person of any age at any time of year [2] [6].

A range of neurotropic viruses is involved in acute viral encephalitis [7]. Moreover Every day new viruses are being associated with encephalitis of varying severity making specific diagnosis challenging [6].

Herpes Simplex virus (HSV) - 1 is the virus most commonly involved in sporadic fatal encephalitis; however, other viruses engaged in encephalitis include herpesviruses (HSV - 2, varicella-zoster, cytomegalovirus, Epstein - Barr, and HHV 6 and 7); paramyxoviruses (measles, rubella); orthomyxoviruses (influenza A virus); enteroviruses (EV 70 and 71, polio -, echo - and coxsackieviruses);

flaviviruses (West Nile, Japanese encephalitis, dengue and Zika viruses); retroviruses (human immunodeficiency virus); alphaviruses (Venezuelan equine -, eastern equine -, western equine - encephalitis); bunyaviruses (La Crosse virus); rhabdoviruses (rabies virus); parvovirus (B19); and astroviruses [8] [9] [10] [11] [12].

In the absence of pathologic evidence of brain inflammation, diagnosis of encephalitis can be inferred from an inflammatory response in the CSF or the presence of abnormal neuroimaging consistent with parenchymal affection and can be used as surrogate markers of brain inflammation [13].

The estimated incidence of encephalitis has a wide variability and is dependent upon age, demographics, climate, the presence of natural host for causative agent, and presence of epidemic illness [14].

Identifying the aetiology of encephalitis is challenging. The definition varies, and distinguishing encephalitis from meningoencephalitis or even meningitis or encephalopathy can be difficult [15].

Improving the diagnostic tools for encephalitis using PCR rather than tissue cultures has increased the yield of etiologies [16] [17].

Most studies performed to detect viral causes of childhood encephalitis are done in western or Asian countries, while the literature has a paucity of data in the Middle Eastern region [1] [9] [12].

This study was done to acknowledge the etiological viral causes of childhood encephalitis in Egypt and to demonstrate the clinical features, seasonal variations and short-term outcome of the cases.

Methodology

This two - year prospective cohort study conducted on children diagnosed with encephalopathy syndrome selected from those admitted to the pediatric unit or pediatric ICU Cairo University Pediatric hospital during a search period all over the whole year.

Ethical consideration

The study was designed to conform to the requirements of the latest revision of Helsinki Declaration of Bioethics [18]. The researcher obtained the approval of the medical ethical committee of the National Research Centre and the ethical committee of Research Committee of Pediatrics Department-Faculty of Medicine - Cairo University. Signed informed consents were collected from the legal guardian of the children before enrolment and after explanation of the aim and nature of the study.

Inclusion criteria

All pediatric patients from the age of 1 month to 13 years presented by encephalopathy syndrome were included in the study.

Acute encephalopathy was defined using The Consensus Statement of the International Encephalitis Consortium; Encephalitis was defined as acute encephalopathy fever with alteration of consciousness and/or with neurological deficit, secondary to central nervous system involvement lasting more than 24 hours, and not more than a one-week history [13].

Exclusion criteria

Other causes of encephalopathy as a brain tumour, vascular disorders, intoxication, or psychosis, traumatic brain injury, pre-existing neurological conditions as metabolic encephalopathy or epilepsy, febrile seizure.

Bacterial meningitis was excluded by the clinical picture of the patient with severe irritability and signs of meningeal irritation with positive CSF bacterial cultures, other infectious etiologies as brain abscess, acute organ dysfunction detected from clinical examination and lab finding.

Data collection

Demographic data as age, sex and season were included. Clinical data as full history and neurological manifestations were recorded. The place of admission whether hospital ward or intensive care settings or the need for mechanical ventilation was mentioned.

In addition to the routine lab investigations, CSF cell count, chemistry and culture were also done. Pleocytosis was defined with CSF cells >15 cells/mm^3 for infants aged 1 to 2 months, and >5 cells/mm3 for patients aged >2 months; lymphocytes predominance if > 60%, high CSF proteins if > 45 mg/dL, CSF glucose: serum ratio should be 60%.

Urgent non - contrast CT imaging of the brain was done whenever indicated, not all patients were followed by MRI due to the extreme difficulty in transferring patients to the radiology unit especially if clinically unstable or if the patient was on mechanical ventilation. However resonant magnetic imaging (MRI) was scheduled upon discharge to the ward.

Patients were managed symptomatically until a definite cause could be found. All patients received acyclovir on admission and continued for 14-21 days when diagnosed with herpes virus or stopped if diagnosed otherwise, and received the appropriate

treatment; discussing treatment is beyond the scope of our research.

The clinical course of the patient is followed for a short term during his hospital stay, and case lethality was recorded whether he survived or discharged.

The neurological outcome of the survived patients was classified according to pediatric cerebral overall category scale into, no squeal, mild, moderate, severe, vegetative and brain dead [19].

Laboratory investigations

Nucleic acid extraction, reverse transcription and PCR amplification

RNA extraction was done automatically using QIAamp MinElute Virus Spin Kit (QIAGEN) Cat. No (57704), on QIA cube machine. cDNA was synthesised by reverse transcription using RevertAidTM First Strand cDNA Synthesis kit Cat.No. (K1622 SG1300)(Fermentas, Ontario, Canada), accords to manufacturer's instruction. cDNA was stored at -80°C until processed.

In PCR, efficiency can be reduced by inhibitors that may be present in the clinical specimen An Internal Control (Meningitis ACE IC) is supplied. This allows the user both to control the nucleic acid isolation procedure and to check for possible PCR inhibition. The Internal Control is introduced into each specimen before nucleic acid extraction and is co-amplified with target nucleic acid from the clinical specimen. Also; the 8 - methoxypsoralen (8 -MOP) system is used to extinguish the template activity of contaminated DNAs. 8 - MOP is known to intercalate into double-stranded nucleic acids and form a covalent interstrand crosslink after photoactivation with incident light of a wavelength of 320 - 400 nm. The Seeplex Meningitis - V2, employs a "Dual Priming Oligonucleotide (DPOTM) technology, which provides freedom in primer design and PCR optimisation and maximises PCR specificity and sensitivity. Briefly, PCR amplification was performed using 5 µL of cDNA, 2 µL of 10X MV2 primer mixture, 3 µL of 8 - MOP and 10 µL of 2X Multiplex Master Mix (Seegene Inc.) in a total volume of 20 µL. The 2X Multiplex Master Mix contains dNTP and enzyme for the specific amplification of the pathogen's genome and 10X ACE PM is the primer mixture for the specific target amplification. The amplification protocol was as follows: initial denaturation at 94°C for 15 min, 40 cycles of denaturation at 94°C for 30 sec, annealing at 63°C for 90 sec, extension at 72°C for 90 sec, and final annealing at 72°C for 10 minutes. The amplified PCR products were electrophoresed in 2% agarose gels and stained with ethidium bromide.

Statistical method

Data were coded and entered using the statistical package SPSS (Statistical Package for the Social Sciences) version 23. Data were summarised using mean, standard deviation, median, minimum and maximum in quantitative data and using frequency (count) and relative frequency (percentage) for categorical data. Comparisons between quantitative variables were made using the non-parametric Mann-Whitney test [20]. For comparing categorical data, Chi-square ($\chi 2$) test was performed. The exact test was used instead when the expected frequency is less than 5 [21]. P - value less than 0.05 was considered as statistically significant.

Results

The study was conducted from first January 2015 to 31th of December 2016. One hundred twenty children admitted with criteria encephalopathy syndrome were enrolled in the study in either general pediatric units or intensive care units. Twenty four patients were excluded from investigation (as revealed other reasons for presentation, e.g. systemic lupus encephalitis, acute disseminated encephalomyelitis, brain abscess, or vasculopathy.

Ninety-six patients were eligible for the study, 60 (62.55%) were males, and 36 (37.5%) were females, 50 (52%) needed intensive care admission, while 43 (44.8%) needed mechanical ventilation.

Table 1: Demographic and clinical finding of studied cases

Age (months)			
	Mean ± SD	29.34 ± 28.29	
	Range	1-120	
The length of hospital stays (days)			
	Mean ± SD	15.42 ± 15.53	
	Range	2 -107	
		Frequency	Percent
Gender			
	Female	36	37.5%
	Male	60	62.55%
Seasonal variation			
	Summer	37	38.5%
	winter	30	31.2%
	Spring	16	16.7%
	Autumn	13	13.5%
Clinical data			
Coma		77	(80%)
Fever		68	(70%)
Seizures		64	(66%)
increased intracranial pressure		14	(14%)
irritability		13	(13%)
focal deficits		7	(7%)
Intensive care admission		50	52%
Mechanical ventilation need		43	44.8%
CT imaging finding			
Normal		67	69.8%
Brain Edema		23	24.0%
Hypodense areas of hypoxia.		6	6.25%
Severity outcome among survival*			
vegetative		3	4%
severe		9	12.1%
moderate		14	18.9%
mild		29	39.2%
Full recovery		19	25.6%
Mortality rates n			
Death		22	22.9
Survived		74	77.1

The age of patients ranged from 1 month-12 years (mean 29.34 + 28.29). The majority of patients presented during summer (37 or 38.5%), and winter (30 or 31.2%), while minority presented during spring (16 or 16.7%) and autumn (13 or 13.5%).

Clinical manifestations of the studied patients included: coma or disturbed conscious level in 77 (80%) patients, fever in 68 (70%) patients, seizures in 64 (66%) patients, increased intracranial pressure 14 (14%) patients, irritability in 13 (13%) patients and focal deficits in 7 (7%) patients.

The length of hospital stay was 2 -107 days (15.42 ± 15.53). CT imaging finding was normal in 67 (69.8%) of cases, while twenty -three (24.0%) revealed brain oedema. Six cases (6.25%) showed hypodense areas of hypoxia. On the short - term outcome 22 (22.9%) patients died, and 74 (77.1%) survived (Table 1).

CSF pleocytosis ranged from 0 - 300 (46 ± 59), lymphocytes predominated in 76 (79.2%), and polymorphnuclear cells in 19 (19.8%), CSF was acellular in one case that was neutropenic and acquired CMV infection. CSF glucose level ranged from 10 - 127 mg/dL (62.12 ± 24.06) and CFS proteins 5 - 305 gm/dL (47.7 ± 46.43)

Viral etiological agents were detected in 20 cases (20.8%), while 76 patients (79.2%) had no definite viral etiology. The most abundant virus detected was EV in fourteen (14.5%), two (2.1%) were positive for HSV - 6, one (1.0%) HSV - 1, one (1.0%) EBV, one (1.0 %)CMV and one (1.0%) with VZV (Table 2).

Table 2: Laboratory and CSF finding of studied cases

CSF finding	Frequency	Percent
Lymphocytes predominance	76	79.2%
Polymorphonuclear cells predominance	19	12.1%
Acellular	1	1%
CSF glucose level mg/dl		
	Mean ± SD	62.12 ± 24.06
	Range	10-127
CFS proteins level gm/dl		
	Mean ± SD	47.7 ± 46.43
	Range	5-305
Viral etiological agents	Frequency	Percent
Ev	14	14.5%
Hsv - 6	2	2.1%
Hsv - 1	1	1%
EBV	1	1%
Cmv	1	1%
Vzv	1	1%
No virus detected	76	79.2%

Severity outcome among survival according to pediatric cerebral performance category scale were vegetative in three cases (4%) severe in 9 (12.16%), moderate in 14 (18.9%), mild in 29 (39.2%) and full recovery in 19 (25.6%).

Table 3 demonstrate clinical picture according to the causative virus; there was no significant difference between clinical presentation, CSF finding or outcome.

Table 3: Demographic, clinical of studied cases according to the causative virus

	EV (*n* = 14)	HSV-1 (*n* =1)	HSV-6 (*n* = 2)	EBV (*n* =1)	CMV (*n* =1)	VZV (*n* =1)	No virus (*n* = 76)
Demographic data							
Age (median in months)	19	24	24	14	67	48	18
Sex, (n, percentage)							
male	10 (10.4%)	1(1%)	2 (2%)	1 (1%)			46 (48%)
female	4 (4.1%)				1 (1%)	1(1%)	30 (31%)
Season							
Summer	4 (4.1%)	1(1%)					
Winter	3 (3.1%)		2 (2%)				
Spring	4 (4.1%)						
Autumn	3 (3.1%)			1(1%)	1(1%)	1(1%)	
Clinical data							
Coma n (percentage)	12 (12.5%)	1(1%)	2 (2%)	1 (1%)	1 (1%)	1 (1%)	62 (64.5%)
Irritability n (percentage)	1 (1%)	0	1 (1%)	1 (1%)	0	0	10(10.4%)
Increased ICP n (percentage)	2 (2%)	0	0	0	0	0	12 (12.5%)
Motor n (percentage)	2 (2%)	0	0	0	0	0	6 (6.25%)
MV	5 (5.2)	0	2 (2%)	1 (1%)	1 (1%)	1 (1%)	32 (33.3%)
Mortality n (percentage)	5 (5.2%)	0	0	0	1	0	16 (16.6%)
Neurological deficits	3 (3.1%)						16 (16.6%)
0	2 (2%)	1 (1%)		1 (1%)			24 (14.58)
Mild	2 (2%)		1 (1%)				11 (11.45%)
Moderate	1 (1%)					1 (1%)	8 (8.3%)
Severe	1 (1%)		1 (1%)				1 (1%)
vegetative							

EV: enterovirus; HSV: herpes simplex virus, EBVEpstein Barr virus, CMV cytomegalovirus; VZV: varicella zoster virus; CSF: cerebrospinal fluid, ICPintracranial pressure, MV: mechanical ventilation.

Mortality risk factors assessment demonstrated that the younger age, the presence of apnea, the need for mechanical ventilation and the presence of abnormal CT finding as brain oedema and hypodense areas were all significantly associated with fatal outcome ($p < 0.05$) Table 4.

Five cases among EV group died making mortality within the group 35.7%. Another CMV case died, this case was brain dead on admission.

Table 4: Risk Factors for mortality

	the
Age	0.027*
Sex	0.380
Pleocytosis	0.099
Coma	0.108
Fever	0.762
Seizures	0.647
Apnea	0.001
Motor deficit	1
Mechanical ventilation	< 0.001*
Edema of CT	0.004*
Virus Type	0.392

* ($p < 0.05$) is considered significant

Discussion

Viral encephalitis is a significant cause of childhood morbidity and mortality. Up to 60% of cases of suspected viral encephalitis remain unexplained due to the failure of routine laboratory techniques to detect an infectious agent [22].

In our study, the incidence of confirmed cases of viral encephalitis was 20.6%. Although this percentage is lower than our studies to some extent [15] [23], but it is similar to many other studies [12]

[13] [24] Moreover with studies with large time scale and larger sample size as the prospective study held by California Encephalitis Project (CEP) all over 7 years and included 1570 cases of encephalitis which revealed only 16% of encephalitis cases had a confirmed or probable etiology [25].

Accurate diagnosis and immediate management of viral encephalitis are critical to reducing complications and fatality rates [26] A diagnostic gap is still present although the progress that had taken place with both traditional and molecular biological technologies in detecting the causes of viral encephalitis.

The current study aimed to acknowledge the etiological viral causes of childhood encephalitis in Egypt and to demonstrate the clinical features, seasonal variations and short-term outcome of the cases.

Many studies set in Western industrialised countries tend to focus on HSV, VZV, EBV, EV, respiratory viruses or bacterial encephalitis [25] [27]. In contrast, Asian countries are faced with a high incidence of arboviruses as Dengue virus and Japanese encephalitis virus [28] [29] [30].

As few data was found on viral encephalitis in developing countries and there was no clear published data on viral encephalitis in Egyptian children. Fourteen cases were caused by EV infections; which was the most abundant virus in the study; there was no specific temporal relation to any season of the year.

Two (2.1%) were positive for HSV - 6, one (1.0%) HSV - 1, one (1.0%) EBV, one (1.0%) CMV and one (1.0%) with VZV. This change of encephalitic viral epidemiology is related to the widespread of vaccination, making HSV-1 dethroned as the most common cause of viral encephalitis among children. Incidence of EV ranged from less than one, to 25% in previous studies [25] [28] [31] [32] [33].

The study addressed the clinical profile and short-term complication of the viral encephalitis. There was male preponderance in our study (62.55%); this was in keeping with most studies [32] [34]. Reasons for higher male incidence could be due to more exposure to the causative agents or the underlying genetic makeup of a male. Clinical manifestations among different etiologies were mostly imperceptible, except for rabies patient who developed laryngospasm and hydrophobia after exposure to a dog bite. As previously mentioned in other studies, in the current study coma was the most common presentation [35] [36].

CSF pleocytosis is suggestive of an inflammatory process of the brain, the lack of CSF pleocytosis, however, does not exclude encephalitis. It is recognised that the CSF may be devoid of cells in immunocompromised patients [37] or early during infection [38].

Forty-five percent to 55% of children with viral encephalitis will show a CSF lymphocytic pleocytosis, although within the first 48 hours this may be neutrophil-predominant [39].

Case fatality in our study was 22.9%, and this was significantly associated with younger age, the presence of apnea, the need for mechanical ventilation and was highly significant with the presence of abnormal CT finding. All of these risk factors indicate disease severity.

In general the overall case fatality of viral encephalitis is between 0% and 29% [27] [32] [40] [41].

Mechanical ventilation was a risk factor of fatality in Mailles and his colleagues in France, 2007 [23]. While younger age and low Glasgow Coma Score (GCS) were risk factors [28]. Immunocompromise was a major mortality factor in the study population in England [42]. Patients with normal neuroimaging studies were more likely to recover than patients with abnormal neuroimaging ($P = 0.008$.) The risk of death or severe damage in patients less than 1 year of age was 5.0 - fold ($p < 0.001$) greater than that of older children [43]. Among survivals, there were neurological sequelae in 74.32%, in the present study. That was higher than other studies which reported sequelae in 25 – 69% [35] [36].

The limitations of our study were a high proportion of unknown causes, which may be due to a later presentation of cases beyond the detection timeframe of PCR, the elimination of other extra - CSF sites for molecular sampling, the presence of a viral cause that is deficient in our labs diagnostic assays, the presence of non - infectious cause of encephalitis as autoimmune encephalitis, or the presence of a new virus. Owing to the high prevalence of EV in the study group, it is recommended to include PCR for EV in the routine workup of encephalitis.

In summary, enterovirus was the most common cause of encephalitis among Egyptian children. There was a high percentage of unknown cases. Mortality was correlated with younger age and disease severity at admission. Sequelae were high among infected children.

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Female Genital Mutilation in Sudan

Adel Hussein Elduma*

National Public Health Laboratory, Ministry of Health, Khartoum, Sudan

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***Correspondence:** Adel Hussein Elduma, National Public Health Laboratory Ministry of Health, Khartoum, Sudan. E-mail: dumanet@yahoo.com

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Abstract

BACKGROUND: Female genital mutilation or female circumcision (FGM) is a serious health problem in Sudan. This procedure is harmful to women and causes many complications during pregnancy and childbirth.

OBJECTIVE: This study aims to determine the female genital mutilation (FGM) and its associated factors in Sudan.

SUBJECTS AND METHODS: Data from Sudan Multiple Indicator Cluster Survey (MICS - UNICEF) was used in this research. The survey was carried out in 2014 and included women aged between 14 – 49 years. A logistic regression model was used to find an association between dependent and independent variables.

RESULT: Total numbers of 21947 women were included in the survey and out of the 6249 (28.5 %) from urban and 15698 (71.5%) from rural areas. The prevalence of female circumcision was 89%. Women who had circumcised daughters were 32.1 %. The highest prevalence of FGM was reported from South Kordofan state with 7.8%, and lowest was in Red Sea state (7.6%). A significant association was observed between circumcised women and their marital status, daughter circumcision, and the level of education.

CONCLUSION: The practice of female genital mutilation is spread all over the country. Poor women with low level of education are at high risk for this phenomenon. More efforts have to be provided to end this dangerous practice.

Introduction

The WHO defines the female genital mutilation as procedures that intentionally alter or cause injuries to the female genital organs for non - medical reasons. No doubt, this procedure can cause many complications such as problems in urination, bleeding, infections as well as a complication of childbirth leading to newborn deaths [1]. Female genital mutilation or female circumcision or cutting is common practice in Africa and Middle East [2]. There is no clear evidence to indicate where female genital mutilation/ circumcision practices have been reported or performed. According to the previous literature, Egyptians were found practising male and female circumcision/ around the middle of the fifth century B.C. But Infibulations or Pharaonic circumcision, the most prevalent type in Africa. Some studies indicated that the Infibulation or Pharaonic circumcision title was applied by Sudanese when this practice spread from Upper Egypt to the North Sudan where was called as Sudanese circumcision [3].

There are five types of female genital mutilation in Africa namely Mild Sunna which include the pricking of the prepuce of the clitoris with a sharp instrument. The second is called Modified Sunna in which partial or total removal of the clitoris is applied. Furthermore, the third type is called clitoridectomy / Excision in which the removal of all or part of the clitoris is performed plus a partial or all removal of the Labia minor. Moreover, the Infibulations or Pharaonic circumcision includes clitoridectomy, excision of Labia minor and the inner wall of the Labia major. The fifth type is introcision where enlargement of the vaginal orifice with a sharp instrument is practised [4].

WHO has a different definition of female

genital mutilation and classified it into four categories namely clitoridectomy, excision, infibulations and type four includes all other harmful procedures [5]. Globally, more than 200 million of girl and women have been suffered from FGM in Africa, Middle East and Asia [6]. Reasons for practising FGM are varied from one region to another and include a mix of socio-cultural factors within families and communities. In Africa, people considered FGM as a vital cultural heritage for women to grow properly, as marriage requirements and control women's' sexuality. Indeed, people, who are practising this harmful practice they do not consider it as a violation of right or can have dangerous consequences for women life [7]. Consequently, in communities where FGM is practising, it is often driven my belief that it maintains the virginity of women, which can provide safe marriage [8][9]. Moreover, they believe that FGM is hygiene practice and maintain women clean and beautiful. Some people are living where the FGM is common in the community enforced to do it due to social norms as a prerequisite for marriage [10].

Sudan is a developing country located in east North Africa with an area of 1.9 $km^{2,}$ and the estimated population is 36.2 million in 2014 based on the last census conducted in 2008. The country has major young with more than 15 million children below the age of 18 years and 4.5 million below the age of five years [1]. Furthermore, the country has 18 states with different ethnic groups and different socio-cultural practice. FGM is widely practised in Sudan particularly among girls aged between 6 and 12 years old. The FGM is usually performed by the midwife without any anaesthesia or antibiotic [11]. This study used the Multi Indicators Cluster Survey data to determine the distribution of the female genital mutilation and the related factors in different areas of Sudan.

Sudan national survey data on female genital mutilation was obtained from the Multiple Indicator Cluster Survey (MICS - UNICEF). The data was a household on female gentile mutilation of women aged 14 to 49 years in Sudan, 2014. Consequently, data were collected by using community survey questionnaire (Multiple Indicator Cluster Survey). The questionnaire was designed to collect data from 40 areas in each state. Furthermore, two stages clustering sampling technique was used to collect data. So, rural and urban areas were identified as the main sampling strata. Probability proportional to the size of enumeration areas was systematically selected from the strata. Variables included in the survey were resident areas where it is rural or urban, women educational level, wealth index quintile, women female genital mutilation experience, and if the women had ever heard about FGM or not. Women deemed to be eligible to be included in the study were those who aged between 15 - 49 years old.

Statistical analysis

Data were computed and analysed using the Statistical Package for Social Sciences (SPSS - IBM 20). Both descriptive and analytical statistics as chi-square statistical test, multiple logistic regression models were performed. Binary logistic regression was used to examine the relationship between predictors and female genital mutilation. Subsequently, variables significant in the binary logistic analysis (with $p < 0.05$) were included in the multiple logistic regressions. Consequently, multiple logistic regressions were used to determine which predictor was independently associated with women circumcision. Then, daughter circumcision, residence (rural-urban), education level, wealth index, marital status, and age of circumcised daughter were fed in the model as independent variables.

Results

Total numbers of 21947 women were included in the survey from all 18 states in Sudan. A total number of 6249 women (28.5 %) from urban, and 15698 (71.5 %) from rural areas were included in this survey. All surveyed women indicated that they have heard about female genital mutilation during their life. The prevalence of female circumcision among the women age 14 to 49 years in Sudan was 89 %.

Table 1: Basic characteristics of surveyed women

Item	No	%
Area		
Rural	15698	71.53
Urban	6249	28.47
Total	*21947*	*100*
Maritalstatus		
Currently married	20898	95.22
Previously married	1049	4.78
Total	*21947*	*100*
Education		
No education	11157	54.69
Primary	6696	30.51
Secondary	3248	14.80
Higher education	386	3.81
Total	*21487*	
Wealthindex		
Poorest	4929	22.46
Second	5246	23.99
Middle	4768	21.73
Fourth	3713	16.92
Richest	3273	14.90
Total	*21947*	
Women circumcision		
Circumcised	19451	89.1
Not circumcised	2406	10.9
Total	*21947*	
Ever heard about circumcision		
Yes	21974	100
No	0	0
Total	*21947*	

The majority of women involved in this survey (95.2 %) are currently married. The proportion of the

circumcised women with no education was 54.7 %. Meanwhile, it was 3.8 % among circumcised women with higher education (University and above). The prevalence of female circumcision was higher among women living in rural areas (71.5 %) compared to those who live in urban areas (28.5 %) (Table 1). On the other hand, the study indicated that only 32.1 % of daughters were circumcised.

The highest prevalence of FGM was reported from South Kordofan state (7.8 %), followed by East Darfur with the percentage of 7.6. The lowest prevalence of female genital mutilation was in Red sea state which was 3.0 % (Table 2).

Table 2: Distribution of circumcised women in different states

No	State	No of circumcised women	%
1	Red Sea	648	3.3
2	Kassala	926	4.7
3	Gadarif	1099	5.6
4	Khartoum	1093	5.6
5	Gezira	1246	6.4
6	White Nile	1228	6.3
7	Northern	1049	5.3
8	River Nile	968	5.0
9	Sinnar	1066	5.5
10	Blue Nile	977	5.0
11	North Kordofan	1154	5.9
12	South Kordofan	1523	7.8
13	West Kordofan	989	5.1
14	North Darfur	1369	7.0
15	West Darfur	756	4.0
16	South Darfur	1324	6.7
17	Central Darfur	640	3.2
18	East Darfur	1486	7.6
Total		19541	

The logistic regression analysis indicated an association between marital status and women circumcision with Crude Odds Ratio (COR = 1.27) and 95 % C.I (1.054 – 1.522). The analysis revealed that women with low level of education practising circumcision more when compared to women with high level of education. Also, the difference of women circumcision in the rural and urban area was statistically significant COR = 1.229 and 95 % CI (1.115 – 1.355). An association also was observed between women circumcision and wealth index where the poorest group had a high rate of circumcision. Daughter circumcision was strongly associated with women circumcision with COR = 19.1, 95 % C.I (14.62 – 24.87) (Table 3).

Table 3: Logistic regression analysis of the circumcised women predictors

Predictor	Adjusted Odds Ratio	p. value	Confidence Interval
Education			
Non	Reference		
Primary	0.286	0.000	0.255 - 0.321
Secondary	0.142	0.000	0.115 – 0.176
Higher education	0.194	0.000	0.136- 0.277
Marital status			
Formerly married	Reference		
Currently married	1.27	0.001	1.054 – 1.522
Daughter circumcision			
Not circumcised	Reference		
Circumcised	19.1	000	14.62 – 24.87
Wealth index quintile			
Poorest	Reference		
Second	1.29	0.000	1.151 – 1.464
Middle	1.697	0.000	1.509 – 1.901
Fourth	0.620	0.000	0.531 – 0.725
Richest	0.268	0.000	0.207 – 0.321
Residence			
Rural	Reference		
Urban	1.229	0.000	1.115 – 1.355

Multiple logistic regressions were used to examine the association between women circumcision as the dependent variable and the five predictors which were significant in the logistic regression. The multiple logistic regressions revealed that four predictors were statistically significant when entered into the model. Only the residence predictor was not statistically significant (Table 4).

Table 4: Multiple logistic regression predictors for circumcised women

Predictor	Adjusted Odd ratio	p. value	Confidence Interval
Education			
Non	Reference		
Primary	0.264	0.000	0.231 - 0.302
Secondary	0.133	0.000	0.103 – 0.172
Higher education	0.180	0.000	0.117- 0.276
Marital status			
Formerly married	Reference		
Currently married	1.5	0.001	1.184 -1.917
Daughter circumcision			
Not circumcised	Reference		
Circumcised	36.8	000	27.96- 48.54
Wealth index quintile			
Poorest	Reference		
Second	1.423	0.000	1.237 - 1.635
Middle	2.614	0.000	2.259 - 3.026
Fourth	1.543	0.000	1.257 - 1.893
Richest	0.897	0485	0.662 - 1.216
Residence			
Rural	Reference		
Urban	1.032	0.645	0.902 - 1.181

Discussion

This study was conducted using data from the Multiple Indicator Cluster Survey (MICS) UNICEF, to estimate the prevalence of the Female genital mutilation/ circumcision and the related risk factors among women aged 15 – 49 years old in Sudan. The analysis of the data indicated that the prevalence of the female genital mutilation in Sudan is still high (89 %) particularly in rural areas. This result is almost similar to study conducted in Sudan 1998 which included three areas namely Shendi in River Nile state, ALhaj Yousif in Khartoum and Juba in Sudan. The overall prevalence was 89 % indicating that female genital mutilation still a big problem in Sudan since the prevalence is the same [12].

Furthermore, the analysis of this survey shows that the female genital mutilation was decreased among young women because the percentage was low among daughters. This finding is in agreement with other study conducted in 2003 to assess the attitude of Khartoum University students and found that 56.8% of female students had undergone female genital mutilation [13]. Another study conducted in Khartoum in 2006 reported that 20% of girls aged between 4 to 9 years practised female genital mutilation [14]. Moreover, the result of this study is inconsistent with a study conducted in Africa and involved many countries where the prevalence of female genital mutilation is high. Additionally, the study reported that the prevalence of

female genital mutilation/circumcision was 91% in Egypt in North Africa, 86% in Mali in West Africa, 74 % in Ethiopia in East Africa, and was relatively low in southern part of Africa [7]. Also, a previous study conducted in Senegal using MICS data reported a 39.9 % of women included in the household survey reported had undergone female genital mutilation. Our study reported that 32.1% of surveyed women mentioned that they had daughters was undergone female genital mutilation. This percentage was high when compared with the same study conducted in Senegal where 9.4 of women stated that their daughters had experienced female genital mutilation [15]. Likewise, this result regarding daughter circumcision was in agreement with another study where 79.55% of mother they had undergone female genital mutilation while only 19% of their daughter had experienced female genital mutilation [16]. Besides, another study conducted in Egypt supported this finding where the decrease in the prevalence of female genital mutilation among adolescent women was statistically significant [17].

Our study reveals that female genital mutilation is prevalent among women living in rural areas compared to those who live in urban areas. Thus, this result is supported by a study conducted in Ethiopia where the majority of circumcised women from rural area residents [18]. As a result, the analysis revealed that the prevalence of female circumcision was high among the women with low level of education. The logistic regression analysis revealed that increase in the women education related to the decreases with female genital mutilation. So, this finding conforms with other studies reported from both Ethiopia and Egypt where the low education level is related to a high rate of FGM [19][20].

A high prevalence of female circumcision was among the poorest and the second poorest women indicating that the least wealth groups are associated with female circumcision. Moreover, women with high wealth index were less likely to practice FGM indicating that the economic situation in the community has a role in this phenomenon. Contrarily, a study conducted in Ethiopia reported that women with richer wealth index categories had a higher prevalence of female genital mutilation compared to women in the poorest category [21]. Also, living in urban areas was associated with high rate of women female mutilation in the univariate analysis, but when entered in the multivariate logistic regression it was not significant. So, the prevalence of female genital mutilation is similar in both urban and rural area. A separate study conducted in Egypt indicated that women living in the rural area had a low prevalence of female genital mutilation [22]. No doubt, Female genital mutilation has a connection with many complications that threatening the life of women during childbirth. These complications include difficulty in a vaginal examination during pregnancy, vaginal Vistula, and postpartum hemorrhagic [23][24]. According to WHO report 2015, the maternal mortality rate is still high in Sudan and accounted for 311 female deaths per 100,000 live births [25]. As a consequence, female genital mutilation has numerous harmful practices that may have a direct or indirect effect on maternal health. Findings of one study conducted by the WHO participated women who had undergone female genital mutilation indicated that they suffered from the high risk of adverse effect during labour and giving birth [26]. Many studies reported the consequences caused by female genital mutilation in Sudan. Reconstructive surgery was done for a woman aged 24 years old suffered from the large vulval mass for the last 6 years due to female genital mutilation. Also, a girl age 7 years old presented with necrotising fasciitis case as a result of female genital mutilation was reported from Kassala New Hospital in eastern Sudan [27][28]. In different study assessed the knowledge and the risk factors associated with HIV among the participants attending the counselling and testing centre in Sudan, they reported that female circumcision is one of the risk factors associated with HIV [29].

Information education and communication initiative "Saleema" was launched by National Council of Child Welfare in collaboration with the UNICEF - Sudan to eradicate FGM in 2008. "Saleema" is an Arabic word means "the whole, healthy in body and mind, unharmed, pristine and perfect God-given condition" [30]. In spite of the effort made by the government, civil society organisation and UN agencies, still, the female genital mutilation is practising in Sudan. So, more efforts are needed to end this harmful phenomenon through exploiting different sources such as media, community leaders and policy stakeholders.

In conclusion, female genital mutilation is still a serious problem in Sudan not only in rural area, but also among people living in urban areas. Elimination of poverty and improvement of education level will contribute to end this harmful phenomenon. Empowerment of people and polices is highly needed and effort should made to end terrible social and cultural norms which indigenize this practice.

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