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Basic Science

Her2/neu Protein Expression and Oncogene Amplification in Gastric Carcinoma with Clinico-Pathological Correlation in Egyptian Patients

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Abstract

AIM: Amplification of the *Her2/neu* gene and overexpression of the Her2/neu protein in gastric carcinoma (GC) is a golden criterion for target therapy with trastuzumab (Herceptin). We aim to evaluate the immunohistochemical protein expression and amplification of the oncogene Her2/neu by FISH technique in the epithelial gastric carcinoma and to compare their association with different clinicopathologic parameters aiming at identifying positive cases that may benefit from targeted therapy.

MATERIALS AND METHODS: This study was done on eighty-five tumour tissue samples from patients with GC as well as thirty non-malignant lesions (Gastritis, intestinal metaplasia, adenoma with low-grade dysplasia, adenoma with high-grade dysplasia). All were immunohistochemically stained with Her2/neu antibody.

RESULTS: All equivocal and some selected GC cases were submitted for FISH technique to detect *Her2/neu* gene amplification. By immunohistochemistry twenty-three cases (27%) were defined as positive for *Her2/neu* gene amplification and/or protein overexpression. The levels of Her2/neu positive (3+), Her2/neu equivocal (2+) and Her2/neu negative (1+/0) were measurable in 14.2%, 32.9% and 52.9% of the samples, respectively. FISH showed that *Her2/neu* gene was amplified in 22 cases, 10 Her2/neu positive (3+), 11 (39.3%) Her2/neu equivocal (2+) and 1 Her2/neu negative (1+) cases with IHC staining those who can benefit from anti Her2/neu target therapy. *Her2/neu* was overexpressed positivity (3+) more in intestinal type and mixed carcinoma, and moderately differentiated tumours. None of gastritis, intestinal metaplasia or adenoma with low-grade dysplasia cases showed positivity for Her2/neu (3+). The Her2/neu positivity (3+) was associated with both adenocarcinoma cases and high-grade dysplasia (P = 0.002).

CONCLUSIONS: The results highlight the necessity of FISH test for further categorization when gastric cancer cases are equivocal (2+) by IHC to determine eligibility for the targeted therapy. Stepwise increase in the expression of Her2/neu was seen in low-grade dysplasia, high-grade dysplasia and carcinoma cases implying its role in cancer evolution. Overexpression of Her 2/neu in GC patients can be promising in selecting those who can get benefit from anti-Her2/neu target therapy.

Introduction

Gastric cancer (GC) is a major cause of cancer death worldwide, especially in developing countries. The incidence of GC varies from country to country, probably as a result of genetic and environmental factors [1]. In Egypt, GC is the 12th most common cancer in both sexes representing 1.6% of the total cancers. It is the 12th leading cause of cancer death representing 2.2% of the total cancer mortality [2]. This is shown in many Egyptian populations- based cancer registries [3]. At the Egyptian National Cancer Institute hospital-based registry 2002–2010, GC is the 14th most common cancer representing 1.8% of cases in both sexes [4].

There is a need to stratify patients with GC into the appropriate screening, surveillance, or treatment programs. Although histopathology remains the most reliable and less expensive method, numerous efforts have been made to identify and validate novel biomarkers to accomplish the above goals. In recent years, several molecules have been identified and tested for their clinical relevance in GC management, with the exception of Her2/neu, none of the biomarkers is currently used in clinical practice, and some of them were described in single studies [5].

Despite ongoing advances in the treatment of gastroesophageal cancer, prognosis remains poor. The best promise to improve this poor survival is provided by new targeted agents. Of these, human epidermal growth factor receptor 2 (Her2/neu) is currently in the spotlight [6].

Her2/neu is a proto-oncogene located on chromosome 17q21 and a member of the human epidermal growth factor receptor (EGFR) family. It encodes an 185 kD transmembrane tyrosine kinase receptor protein that, through dimerization with other family members, regulates signal transduction in cellular processes including proliferation, differentiation, and cellular survival [7]. Many studies have indicated a role of Her2/neu in the development of various types of human cancer. Her2/neu is amplified, in about 10–20 % of breast carcinomas [6], in GC have been shown in studies. Her2/neu positivity can be detected in approximately 20% of patients, which is a characteristic associated with poor prognosis [8]. Since the costs for trastuzumab therapy are high and side effects are significant, accurate selection of eligible patients for this therapy is crucial. Her2/neu status is mainly assessed by immunohistochemistry (IHC) and chromogenic (CISH) or fluorescence *in situ* hybridization (FISH) [6]. To eliminate discrepancies observed between IHC and FISH, Hofmann et al. [9] established an IHC scoring system specific for GC. In an international consensus meeting, modifications to the breast scoring system were made mainly based on the more frequent basolateral (incomplete) membrane staining and heterogeneity in GC.

The aim is to evaluate the immunohistochemical protein expression and amplification of the oncogene Her2/neu by FISH technique in the epithelial gastric carcinoma and to compare and detect their association with different clinicopathologic parameters aiming at identifying positive cases that may benefit from targeted therapy.

Material and Methods

Eighty-five cases of carcinoma of the stomach were collected from archived tissue samples from the Surgical Pathology Department of Theodor Bilharz Research Institue (TBRI) for patients who had undergone total, subtotal or partial gastrectomy was evaluated for Her2/neu status using IHC analysis **.** In addition to this, 9 endoscopic gastric biopsies for patients with gastritis were also included in the study. None of the patients had undergone prior preoperative radiation, chemotherapy or targeted therapy.

Of the 85 cases, 40 cases were selected with intestinal-type gastric adenocarcinoma with different grades and stages and were compared using immunohistochemical analysis to 30 other tissue samples in the form of 4 cases of intestinal metaplasia, 10 cases of adenoma with low grade dysplasia, and 7 cases of adenoma with high grade dysplasia that were isolated from juxta tumoral tissues in addition to the 9 endoscopic gastric biopsies with gastritis. Based on the outcome of the immunohistochemical analysis of Her2/neu protein in the 85 cases of gastric carcinoma, 40 cases were evaluated for the Her2/neu gene status using the FISH technique, all cases with equivocal Her2/neu protein expression (score 2+) to detect gene status and document eligibility for targeted therapy (28 samples), 10 randomly selected cases with positive Her2 protein expression (score 3+) and only 2 randomly selected cases negative for protein expression (score 1+).

Histopathological study

Paraffin blocks containing representative samples of the tumours were selected by reviewing all of the available H&E stained slides. The Lauren classification of gastric cancer was followed to subgroup all cases in histopathology according to Lauren [10].

The patient demographics and pathologic information were retrieved from each pathology report including Personal data*,* Clinical data; Tumor localisation, type of surgery, tumour type and grade, depth of invasion, the number of lymph nodes resected and the number of lymph nodes with metastases and final pathological diagnosis. All stomach cancers with a midpoint in the stomach lying more than 5 cm distal to the EGJ, or those within 5 cm of the EGJ but not extending into the EGJ or esophagus, are staged using the gastric (not-EGJ) cancer staging system set out in the 7th edition of the American Joint Committee on Cancer staging, and cancers whose midpoint is in the lower thoracic esophagus, EGJ, or within the proximal 5 cm of the stomach (cardia) that extend into the EGJ or esophagus are staged as adenocarcinoma of the esophagus [11].

Tumours are graded according to WHO grading system into well differentiated, moderately differentiated and poorly differentiated [12].

Immunohistochemical technique

Immunohistochemistry for Her2/neu was performed on sections cut from the paraffin blocks with a commercially available rabbit monoclonal Anti-human Her2/neu antibody (CELL MARQUE SP3, USA). Briefly, samples were sectioned at 4 µm onto positively charged slides (Superfrost plus, Menzel-Glaser, Germany) and the slides were stained on an automated platform the Dako autostainer Link 48. Heat-induced antigen retrieval was used for 30 min at 97°C in the high-PH EnVision™ FLEX Target Retrieval Solution and the primary antibody was used at a dilution of 1 in 100.

Interpretation of immunostaining

Immunostaining of Her2/neu was performed and scored according to the revised scoring criteria of Hofmann et al. (9) used in the ToGA trial, which was based on the intensity of membrane staining and quantity of positive neoplastic cells on a scale of 0-3. A score of 0 or 1+ was considered negative while scores 2+ was considered equivocal and 3+ was positive. Heterogeneous Her2/neu expression was considered if the tumour shows the presence of both Her2/neu-positive and Her2/neu-negative subpopulations of carcinoma cells in a single tumour [13]. All immunostained slides were analysed using Zeiss microscope with high resolution (Axioscope, Germany) at different powers (x 50, x 100, x 200, x 400).

Gene expression of Her2 by fluorescence in situ hybridization

FISH analysis was used on a representative proportion of the tissue, using the Path Vision kit (Abbott Laboratories. Abbott Park, IL, USA). All samples with positive HER2 protein expression were evaluated using labelled probes for both fluorophores, i.e., Vysis CEP 17 17p11.1-q11.1 Alpha Satellite DNA Spectrum Green, and Vysis LSI HER-2/neu 17q11.2-12 Spectrum Orange.

Paraffin was removed from 4 µ tissue sections by washing the slides in xylene for ten min, then in 100% alcohol for five min twice, then air drying them. Slides were then immersed in pretreatment solution at 80°c for 15 minutes, and then tissue sections were digested with protease solution by immersing slides in solution at 37°C for five minutes. Slides were air dried for 2–5 minutes. Tissue sections were post-fixed in 10% neutral buffered formalin at room temperature for 10min before dehydration in ascending grades of alcohol and air drying, Tissue sections were denatured in the denaturation solution at 72°C for five min. Then in ascending grades of alcohol 70%, 95%, 100% alcohol for one min. each, air dried for 2–5 minutes. Probes for the pericentromeric region of chromosome 17 (Spectrum Green™) and the locus-specific probe for Her2 (Spectrum Orange™) were used. For each section, one µl of each probe was added to seven µl hybridization mix (50% formamide, 2 × SSC, 10% dextran sulphate) and one µl deionized water and denatured in a water bath at 72°C for five min and then hybridised overnight at 37°C. Post-hybridization washes were done by immersing slides in pre-warmed 2X SSC/0.3% NP-40 at 73.1ºC, for two mins. Slides were air dried in darkness. Slides were mounted in 10µl diaminido phenyl indole (DAPI)/ antifade. Control sections of normal stomach and Her2 amplified breast tumours were included in each run.

Interpretation of FISH

Slides were viewed with a fluorescence microscope (BX51 upright, Olympus Corp, Japan), where three areas were identified and in each area, 20 nuclei were assessed. Chromosome 17 copy number and HER2 copy number were assessed for each of the 20 nuclei at 1000 magnification. A ratio of chromosome 17 copy number over HER2/neu copy number was obtained from the 60 nuclei. HER2/neu was classified based on the value used in breast cancer diagnostics into 'amplified' (Her2/neu/centromere 17 ratios >2) 'not amplified' (Her2/neu/centromere17 ratio <2) or polysomy (more than 2 green signals.

Statistical analysis

SPSS for Windows, version 18 was used for statistical analysis (IBM corporation, Armonk, new York, USA). Means of different groups were compared using one-way ANOVA. Comparison between percent positive cases was calculated by Chi-Square test. A p-value of 0.05 was considered of statistical significance.

Results

Her2/neu expression by IHC

The patients' clinicopathological character-critics of the 85 cases gastric carcinoma are listed in (Fig. 1; Table 1). Twelve cases (14.2%) were scored as positive for Her2/neu membrane staining (3+), 32.9% were equivocal (2+), and 52.9% were Her2/neu negative (0/1+) (Table2). Her2/neu protein expression by IHC in adjacent non-tumor normal appearing mucosa was detected in 11/85 (12.9%) of gastrectomy specimens. The Her2/neu expression was membranous (score 1+ or 2+) (Fig. 2; Tables 2-3).

There was a higher Her2/neu positivity (3+) in the cases with intestinal type adenocarcinoma and

mixed type in relation to diffuse type carcinoma. The positivity (3+) in the mixed type was detected in intestinal differentiation, not in diffuse areas. The mixed type was 20% showed positivity (3+), followed by the intestinal type (13%) with no statistical difference between different types.

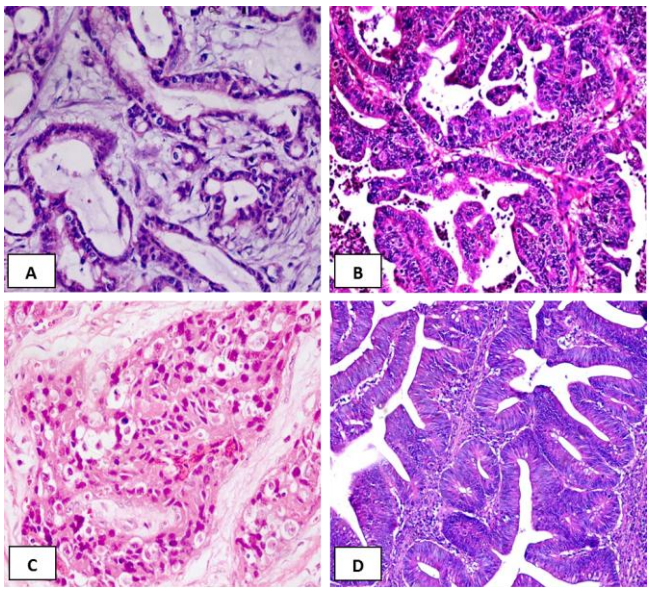

Figure 1: H&E stained gastric adenocarcinoma sections. A) case of well differentiated gastric carcinoma; malignant cells show mild nuclear anaplasia and arranged in well-formed glands (H&E x 400); B), case shows moderately differentiated carcinoma, shows papillary architecture with fibrovascular cores covered by malignant epithelial cells show moderate nuclear anaplasia (H&E x 200); C) case of poorly differentiated gastric carcinoma, malignant cells show moderate nuclear anaplasia (H&E x 400); D) case of adenoma with high-grade dysplasia, showed irregular glands with marked nuclear stratification (H&E x 200)

Her2/neu positivity (3+) was 18% in the moderately differentiated tumours and 9.7 % of the poorly differentiated tumours with no significance difference between them.

Table 1: The relation between the pathologic parameters and Her2/neu protein positivity by IHC in carcinoma cases

Pathologic data	Number (%)	Her2/neu (IHC) (score 3+		P value
		Positive	%	
Histologic type				
Adenocarcinoma; intestinal type	54 (63.5%)	7	(13%)	0.567 (insignificant)
Mixed	20 (23.5%)	4	(20%)	
Diffuse	11 (13%)	1	(9%)	
Histopathological Grade				
Well differentiated	4 (4.7%)	0	(0 %)	0.272
Moderately differentiated	50 (58.9%)	9	(18%)	(insignificant)
Poorly differentiated	31 (36.4%)		(9.7%)	
Depth of invasion				
T1	0	3	(0%)	0.499
T2	14 (16.5%)	4	(28.6%)	(insignificant)
T3	45 (52.9%)	5	(11.1%)	
T4	26 (30.6%)	3	(11.5%)	
Lymph node status				
N0	21 (24.7%)	3	(14.2%)	0.724
N1	11 (12.9%)	2	(18.2%)	(insignificant)
N2	25 (29.4%)	4	(16%)	
N3	28 (33%)	3	(10.7%)	
Location of the tumour*				
GEJ	14 (16.6%)	1	(7.1%)	0.575 (insignificant)
Gastric	37 (43.5%)	6	(16.2%)	

*****40% of samples the location of the tumor was not specified.

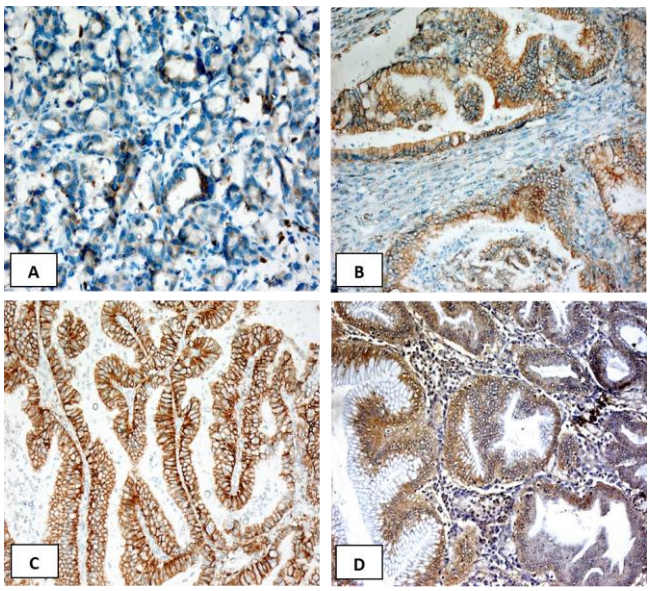

Figure 2: Immunohistochemistry for Her2/neu in gastric sections expressed as a brown membranous stain. A) case of moderately differentiated gastric carcinoma, malignant glands show visible weak Her2/neu protein expression at high power (Score 1+) (IHC, DAB, x 400); B) case of moderately differentiated gastric carcinoma, malignant glands show moderate membranous basolateral and complete Her2/neu protein expression (Score 2+) (IHC, DAB, x 200); C) case of moderately differentiated gastric carcinoma with papillary pattern shows strong membranous staining for Her2/neu (Score 3+) (x 200); D) Dysplastic acini with moderate Her2/neu expression (Score 2+) in a case of gastric carcinoma (IHC, DAB x 200)

The positive expression of Her2/neu (3+) was associated with moderately differentiated tumours, but this correlation is not statistically significant (P = 0.272)**.** Her2/neu positivity (3+) was found more in the tumours with T2 stage (28.6%).

Table 2: Immunohistochemistry - Fluorescence in-situ hybridization concordance in gastric carcinoma cases

IHC	FISH		Total
	Negative	Positive	
Negative	1 (50%)	1 (50%)	2 (100%)
Equivocal	17 (60.7)	11 (39.3%)	28 (100%)
Positive	0	10 (100%)	10 (100%)
Total	18 (45%)	22 (55%)	40 (100%)

Cross tables; Pearson Chi- Square test; p value = 0.004 (significant).

Her2/neu expression in gastric non-malignant lesions were nine cases of endoscopic biopsies diagnosed as gastritis, and lesions in adjacent non-tumorous mucosa in gastrectomy specimens for GC cases (four cases of intestinal metaplasia, ten cases of adenoma with low-grade dysplasia, and seven cases of adenoma with high-grade dysplasia). The results of these non-malignant gastric lesions were compared to the results of forty adenocarcinoma cases (intestinal type), selected from the 85 carcinoma cases.

None of gastritis, intestinal metaplasia or adenoma with low-grade dysplasia samples showed strong cellular membranous positivity of Her2/neu

protein (3+). The positivity of Her2/neu (3+) was associated with both adenocarcinoma cases (17.5%) and a case of high-grade dysplasia (14.3%), this correlation was highly significant (P < 0.002) (Table 3).

Table 3: Her2/neu protein positivity in cases of gastritis, intestinal metaplasia, both low and high-grade dysplasia and adenocarcinoma

Item	Negative	Her2/neu Equivocal	Positive	Total
Gastritis	9 (100%)	0 (0%)	0 (0%)	9
Intestinal Metaplasia	0 (0%)	4 (100%)	0 (0%)	4
Adenoma with low grade dysplasia	7 (70%)	3 (30%)	0 (0%)	10
Adenoma with high grade dysplasia	3 (42.85%)	3 (42.85%)	1 (14.3%)	7
Adenocarcinoma	19 (47.5%)	14 (35%)	7 (17.5%)	40
TOTAL	38 (54.3%)	24 (34.3%)	8 (11.4%)	70

Cross tables; Pearson Chi- Square test; p value = 0.002 (highly significant).

Her2/neu expression by FISH

Within the group of cases showed equivocal Her2/neu protein expression (2+), 17 cases did not display Her2/neu gene amplification, and 11 cases (39.3%) showed gene amplification. All cases with Her2/neu protein positive expression (3+) displayed gene amplification. A negative Her2/neu protein expression case (0/1+) showed gene amplification. The correlation was statistically significant (Fig. 3).

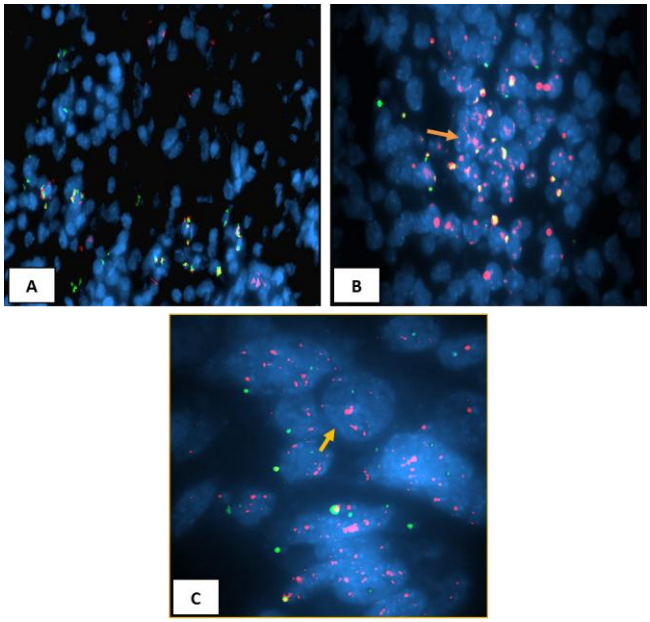

Figure 3: Fluorescent in situ Hybridization using probes directed against Her2/neu in gastric carcinoma (FISH, Her2/neu gene, x1000). A) case of moderately differentiated gastric carcinoma, negative for Her2/neu gene amplification, IHC score 2+, Red signals (Her2/neu gene), green signals [chromosome enumeration probe 17 (CEP17)], blue signals (nuclei stain with DAPI); B) case of moderately differentiated gastric carcinoma, positive for Her2/neu gene amplification, showing red clusters (orange arrow) and was, IHC score 2+; C) case of moderately differentiated gastric carcinoma, positive for Her2/neu gene amplification, showing red clusters (orange arrow), and was IHC score 3+

After IHC and FISH examination, cases with IHC score 3+ or IHC score 2+ with positive gene amplification by FISH positive were considered positive cases, while cases with IHC score 0, 1+ or 2+ without gene amplification were negative cases. Considering both IHC and FISH results, there were 23 of 85 cases included in the study positive for Her2/neu (27%), while 62 cases were negative (73%).

Discussion

Gastric cancer is not a single disease, but a conglomerate of histologically, biologically and genetically heterogeneous diseases, conditioned by the gradual accumulation of various genetic and epigenetic alterations leading to the activation of several molecular pathways resulting in markedly different responses to the same treatment. In the wake of increased molecular pathways underlying the breast cancer, more is actually known about the biological behaviour of gastric cancer and its intrinsic subtypes, particularly the identification of the Her2/neu amplified gastric cancer subtype [14]. The first randomised Phase III trial (ToGA) showed that trastuzumab in combination with conventional chemotherapy is superior to conventional chemotherapy alone in Her2/neu-positive advanced gastric cancer. Therefore, an accurate evaluation of Her2/neu status in gastric cancer has become increasingly important [15]. We found that intestinal-type adenocarcinoma accounts 63.5% of our cases, which is consistent with the reported data by Zeeneldin et al. [16], was 47.6%. Most of our cases belong to moderately differentiated tumours (58.9%), which is in contrast to the other study, that they reported 58.1% of cases belong to tumours with G-III / IV [16].

In our study, 14.2% of GC and GEJ carcinoma cases were Her2/neu-positive (score +3) by IHC. Her2/neu positivity after FISH examination (IHC +3 or IHC +2/FISH positive) was 27%. In the ToGA trial, the percentage of Her2/neu-positive (IHC 3+ or IHC 2+/FISH positive) GC or GEJ cancer patients was 22.1% overall and around 10.4% of IHC 3+ in resected samples [17], Jørgensen [8], found 19% Her2/neu positivity, Chen et al. (18) and Otsu et al. (19) reported a range of 5.1 % to 15.6% for Her2/neu overexpression.

In our research, 13% of intestinal type had an expression (3+). In mixed type, 20% were strongly positive at intestinal type foci, while only one case of diffuse type showed strong positivity [19-23], find a prevalence in intestinal type ranging from 6.1% to 28.57%. The percentage of poorly differentiated tumours with Her2/neu overexpression is about half the percentage of moderately differentiated tumours.

None of the well-differentiated tumours shows expression of Her2/neu, while Shan and his colleagues [24] found that Her2/neu overexpression in the Chinese patient was in 11.1%, 37.5% and 0% in well, moderately and poorly differentiated tumours, respectively. Similarly, Her2/neu overexpression was 16%, 20% and 6% of well, moderate and poor differentiation respectively in the Indian study [25]. Many types of research demonstrate the significant association between Her2/neu overexpression and tumour differentiation [18]. Others do not find this association [26]. These conflicting data may be due to different sample sizes and the low prevalence of Her2/neu in GC and GEJ adenocarcinoma.

In the present study, there is a higher Her2/neu overexpression in T2 category and N1 category with no statistically significant correlation between them this with an agreement with Kataoka et al. [27]- Kunz et al. [28]. In contrast to data reported by Liang et al. [29] that Her2/neu positivity significantly related to TNM stage of gastric cancer.

We found 39.3% of equivocal cases have Her2/neu gene amplification by FISH, while all studied cases score 3+ by IHC showed gene amplification. One case showed negative IHC expression for Her2/neu protein (Score 1+) revealed gene amplification by FISH. These are in agreement with Yan et al. [26] and Shan et al. [30]. In the current study, Her2/neu gene was amplified in all samples with oncoprotein overexpression at 3+ level, which is consistent with the reported data by Yan and his colleagues [26] and reported in 97.5%, 87.5% and 73.9 of the score +3 samples in studies conducted by Shan et al.[30], Gordon et al. [13] and He et al. [31] respectively.

Coping with our findings, many studies enrolled Her2/neu gene amplification in Her2/neu IHC-negative cases in 1.3%- 22.58% (Yan et al. [26] and Gordon et al. [13] and He et al. [31] and Shan et al.[30]. Discrepancy between protein expression and gene amplification indicates that overexpression of Her2/neu in GC, differently from breast cancer, may be regulated by several mechanisms, including transcriptional activation of other genes or post transcriptional events [14], these data highlights the need and importance of further clarifying the relationship between Her2/neu gene amplification and protein overexpression in GC.

We observed Her2/neu immunoreactivity in normal-appearing gastric acini adjacent to a tumour in about 13% of the studied gastrectomy specimens.This matches with the study done by Zhang et al. [32] in Chinese patients samples, which reported Her2/neu overexpression in adjacent non-tumorous tissues in 4 of the 72 gastrectomy specimens of GC. A significant difference for Her2/neu overexpression between the GC and the non-tumor group was observed (18.1 vs. 5.6%, $P<0.05$).Wang et al. [33] showed that patients with advanced gastric cancer had Her2/neu expression on the adjacent non-tumor stomach tissues.

We found a significant association between Her2/neu expression and high-grade dysplasia and adenocarcinoma. Thus, a stepwise increase in the expression of Her2/neu was seen in low-grade dysplasia, high-grade dysplasia and carcinoma. In our study, four cases of intestinal metaplasia (100%), three cases of adenoma with low-grade dysplasia (30%) and three cases of adenoma with high-grade dysplasia (42.85%) showed equivocal Her2/neu immunostaining (score 2+). Slesak et al. [34] reported a high frequency of expression of Her2/neu in the chronic gastritis area with intestinal metaplasia in the territory of gastric carcinoma.

Rossi et al. [35] and Hu et al. [36] and Fassan et al. [37] described the upregulation of Her2/neu in gastric dysplastic lesions. Her2/neu overexpression is not an event acquired at a later moment by malignant gastric cells, but represents the initial timing of this process probably occurring in early stages of cancerogenesis with subsequent increase of expression proportionally to disease progression, making possible the Her2/neu test on either the primary tumor or a metastatic tumor with similar results of Marano and Roviello [14], that is explain this finding.

In conclusion, Her2/neu expression in Egyptian patients was comparable to that in other populations; 27% of Egyptian patients with primary GC and GEJ adenocarcinoma were Her2/neu-positive on IHC and FISH. Her2/neu positivity (3+) was common in the mixed, intestinal type and moderately differentiated carcinoma. The results highlight the necessity of FISH test for further categorization when gastric cancer cases are equivocal (2+) by IHC to determine eligibility for the targeted therapy. Stepwise increase in the expression of Her2/neu was seen in low-grade dysplasia, high-grade dysplasia and carcinoma cases implying its role in cancer evolution.

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Basic Science

Detection of Cancer Stem Cells in Colorectal Cancer: Histopathological and Immunohistochemical Study

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Abstract

BACKGROUND: Growing evidence supports the notion that the onset of tumorigenesis could occur through cancer stem cells (CSCs). These tumour cells show low proliferative rates, high self-renewal capacity, propensity to differentiate into active proliferating tumour cells & resistance to chemoradiotherapy thus, possibly causing local recurrences & metastasis formation. CD44 has been used as a marker to isolate CSCs from colorectal carcinoma (CRC).

AIM: To investigate the immunohistochemical expression of cancer stem cells marker (CD44) in CRC and correlate its expression with the clinicopathological aspects, TNM staging and modified Dukes' classification.

MATERIALS AND METHODS: Tumour biopsies from colectomy specimens of 60 patients with CRC were stained with hematoxylin-eosin for histological evaluation then immunostained with monoclonal antibodies against CD44 which was detected in term of negative or positive expression.

RESULTS: CD44 was demonstrated in 58.3% (35/60) of cases and showed statistically significant correlation with tumour site and histological type (p-value < 0.05). However, CD44 showed statistically insignificant inverse correlation with tumour invasiveness (T), lymph node status (N), grade, TNM stage grouping and modified Dukes' classification, while it was directly correlated with distant metastasis (M) (p-value > 0.05). Chi-square /Fisher exact test proportion independence and the p-value are set significant at 0.05 level.

CONCLUSION: the CD44 rate of expression is higher in the colon than rectum and in adenocarcinoma than mucinous and undifferentiated carcinoma. CD44 showed statistically insignificant relation with T, N, M, grade, TNM stage grouping and modified Dukes' classification.

Introduction

Colorectal cancer (CRC) is the third most common type of cancer [1]. Although the median overall survival of patients with metastatic colorectal cancer has increased from 12 months to approximately 24 months over the past decade as a result of an improvement in systemic therapies including new chemotherapeutic agents, the 5-year survival is still pessimistic [2].

A growing body of evidence supports the notion that only a small subset of cells within a solid tumour has 'stem-like' characteristics. These tumor-initiating cells, or cancer stem cells (CSCs), distinct from non-malignant stem cells, show low proliferative rates, high self-renewal capacity, propensity to differentiate into active proliferating tumour cells, and resistance to chemotherapy or radiation [3]. Notably, owing to their high expression of DNA repair mechanisms, detoxifying enzymes, such as aldehyde dehydrogenase-1 (ALDH1), and molecular pumps, CSCs might survive radiochemotherapy; thus, possibly causing local recurrences and metastasis formation despite treatment [4].

Despite the potentially high clinical relevance of CSCs, little is known about the prognostic value of the expression of putative CSC markers in colorectal cancers. Contradictory findings have been reported about the association between the expression of CD44 and tumour progression [5].

Material and Methods

A total of 60 stored, formalin fixed, paraffin embedded tumour biopsies from colectomy specimens of patients with colorectal cancer were collected from Kasr El Aini Hospital and multiple private laboratories with the permission of the head of these labs, the specimens were anonymous for confidentiality and replaced by numbers.

The site of the tumour was classified into the right colon (cecum, ascending colon, hepatic flexure and transverse colon), left colon (splenic flexure, descending colon and sigmoid) and rectum, while the size of the tumour was calculated as the length of the largest diameter. Size and site, as well as age and sex, were obtained from the pathology reports of the patients. Undifferentiated carcinoma cases were documented immunohistochemically from where the cases were recruited.

The tumour extension into other organs, distant metastasis if present and the lymph node status were also obtained from the diagnosis present in the pathology reports (clinical data of distant metastasis in other organs were also obtained from the patient's sheet).

The paraffin blocks of the tumour were serially sectioned at 4 μm thickness. Afterwards, they were stained with routine hematoxylin-eosin stain for pathological examination and morphologic classification of the colorectal cancer according to the recommendations of the World Health Organization [6] including histological types, subtypes, tumor grade, depth of tumor invasion, perineural invasion and lymphovascular emboli while staging was performed using modified Dukes' classification of the disease [7], and TNM staging system [8] for each case.

Paraffin section from each case was processed for immunostaining using CD44 Std. / HCAM AB-4 (0.7 ml. of antibody prediluted 0.05 mol/L Tris-HCl, pH 7.6 containing stabilising protein and 0.015 mol/L sodium azide – Thermo Fisher Scientific. UK) and Econo Tek HRP Anti-Polyvalent (DAP) ready-to-use (Scy Tek Laboratories inc. USA) detection system.

CD44 stained sections were examined at high power for immunohistochemical expression and were divided into negative (no immunoreactivity in any cells) and positive (membrane and/or cytoplasm immunoreactivity present) [9].

The antibody labels approximately 90% of all lymphocytes, both T cells and B cells [10], were positively stained lymphocytes were used as an internal positive control.

In colorectal cancer, metastasis was almost exclusively a property of the CSCs that exhibited long-term self-renewing capacity [11]. So we used to divide the histological types, tumour grade and invasiveness (T) into metastatic and non-metastatic groups to correlate them with the rate of CD44 expression.

Statistical methods

SPSS version 18.0 was used for data management. Mean and standard deviation described quantitative data and non-parametric t-test compared two independent groups and non-parametric ANOVA compared more than two groups. Chi-square /Fisher exact test proportion independence and the p-value are set significant at 0.05 level.

Results

The characteristics of the study population are shown in Table 1. The subjects consisted of 29 males (48.3%) and 31 females (51.7%), with a mean age of 54.86 years (median, 56 years; range, 25–75 years) and a mean tumour size of 6.25 cm (median, 6 cm; range, 2–16 cm). Most of the cases were adenocarcinoma (75%) followed by mucinous carcinoma (21.7%) and undifferentiated carcinoma (3.3%). The tumour was distributed regarding the site; (75%) in the colon while (25%) of the cases were found in the rectum. The TNM staging is applied according to the American Joint Committee on Cancer (AJCC) and the International Union for Cancer Control (UICC) where it encodes the extent of the primary tumor (T), regional lymph nodes (N), and distant metastases (M) 3, 17, 31, and 9 patients had stage I–IV cancers, respectively [8]. According to Modified Dukes' classification [7] cases in this study are classified into 20 cases (33.3%) B, 31 cases (51.7%) stage C and 9 cases (15%) stage D with no cases of stage A.

Expression of CD44s in primary tumour was demonstrated in 58.3% (35/60). The relationships between tumour CD44s expression and the clinicopathological features of CRC are summarised in Table 1.

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Table 1: Description of set of patients and CD44 rate of expression in the study

		No. (%)	CD44 expression		P-value
			+ve no. (%)	-ve no. (%)	
Patients		60	25 (41.7%)	35 (58.3%)	
Gender:					
Male		29 (48.3%)	16 (55.2%)	13 (4.48%)	0.63
Female		31 (51.7%)	19 (61.3%)	12 (38.7%)	
Age:					
Range		25-75 yrs			0.07
Mean		54.867	57.086	51.760	
Std. deviation		11.0415	8.8695	13.0776	
Tumour site:					
Colon		45 (75%)	30 (66.7%)	15 (33.3%)	0.02*
Rectum		15 (25%)	5 (33.3%)	10 (66.7%)	
Tumour size:					
Range		2-16 cm.			0.29
Mean		6.250	5.929	6.700	
Std. deviation		2.5669	2.1287	3.0687	
Histological type:					
Adenocarcinoma		45 (75%)	31 (68.9%)	14 (31.1%)	0.01*
Mucinous		13 (21.7%)	4 (30.8%)	9 (69.2%)	
Undifferentiated		2 (3.3%)	0 (0%)	2 (100%)	
Adenocarcinoma: **		45 (75%)	31 (68.9%)	14 (31.1%)	
Metastatic		8	6 (75%)	2 (25%)	1.0
Non metastatic		37	25 (67.6%)	12 (32.4%)	
Mucinous: **		13 (21.7%)	4 (30.8%)	9 (69.2%)	
Metastatic		1	1 (100%)	0 (0%)	0.38
Non metastatic		12	3 (25%)	9 (75%)	
Tumor grade: **					
Grade 1		0 (0%)	0 (0%)	0 (0%)	0.10
Grade 2		53 (83.3%)	33 (62.3%)	20 (37.7%)	
Grade 3		7 (11.7%)	2 (28.6%)	5 (71.4%)	
Grade 2: **		53 (83.3%)	33 (62.3%)	20 (37.7%)	
Metastatic		8	6 (75%)	2 (25%)	0.7
Non metastatic		45	27 (60%)	18 (40%)	
Grade 3: **		7 (11.7%)	2 (28.6%)	5 (71.4%)	
Metastatic		1	1 (100%)	0 (0%)	0.29
Non metastatic		6	1 (16.7%)	5 (83.3%)	
Tumor extent (T):					
T1	T1+T2	1 (1.7%)	7 (77.8%)	2 (22.2%)	0.52
T2		8 (13.3%)			
T3	T3	45 (75%)	25 (55.6%)	20 (44.4%)	
T4a	T4a+T4b	3 (5%)	3 (50%)	3 (50%)	
T4b		3 (5%)			
T1+T2: **		9 (15%)	7 (77.8%)	2 (22.2%)	
Metastatic		1	1 (100%)	0 (0%)	1.0
Non metastatic		8	6 (75%)	2 (25%)	
T3: **		45 (75%)	25 (55.6%)	20 (44.4%)	
Metastatic		5	4 (80%)	1 (20%)	0.36
Non metastatic		40	21 (52.5%)	19 (47.5%)	
T4a+T4b: *		6 (10%)	3 (50%)	3 (50%)	
Metastatic		3	2 (66.7%)	1 (33.3%)	1.0
Non metastatic		3	1 (33.3%)	2 (66.7%)	
Lymph node status (N):					
N0	N0	22 (36.7%)	15 (68.2%)	7 (31.8%)	
N1a N1b	N1a+N1b	15 (25%) 5 (8.3%)	12 (60%)	8 (40%)	0.55
N2a N2b	N2a+N2b	11 (18.3 %) 7 (11.7%)	8 (44.4%)	10 (55.6%)	
Distant metastasis (M):					
M0		51 (85%)	29 (56.9%)	22 (43.1%)	0.72
M1a M1b		4 (6.7%) 5 (8.3%)	6 (75%)	3 (25%)	
Stage grouping:					
I		3 (5%)	2 (66.7%)	1 (33.3%)	
II		17 (28.4%)	11 (64.7%)	6 (35.3%)	0.78
III		31 (51.6%)	16 (51.6%)	15 (48.4%)	
IV		9 (15%)	6 (66.7%)	3 (33.3%)	
Modified Dukes':					
Stage A		0 (0%)	0 (0%)	0 (0%)	
Stage B		20 (33.3%)	13 (65%)	7 (35%)	0.55
Stage C		31 (51.7%)	16 (49.6%)	15 (50.4%)	
Stage D		9 (15%)	6 (66.7%)	3 (33.3%)	
Perineural invasion:					
Absent		52 (86.7%)			
Present		8 (13.3%)			
Lympho-vascular emboli:					
Absent		58 (96.7%)			
Present		2 (3.3%)			

(*) statistically significant p-value; (**) pathological parameters such as the grade, histological types and invasiveness (T) of the tumour were classified into metastatic and non-metastatic groups, the metastatic groups showed a higher rate of CD44 expression than non-metastatic ones.

According to Modified Dukes' classification [7] cases in this study are classified into 20 cases (33.3%) B, 31 cases (51.7%) stage C and 9 cases (15%) stage D with no cases of stage A.

Expression of CD44s in primary tumour was demonstrated in 58.3% (35/60). The relationship between tumour CD44s expression and the clinicopathological features of CRC is summarised in Table 1.

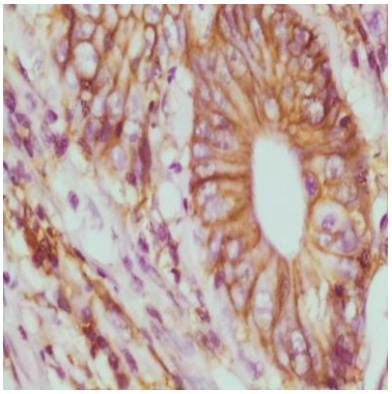

Figure 1: Adenocarcinoma GII with strong CD44 expression; membranous and focal cytoplasmic staining (x400)

CD44 showed a higher rate of expression in colon rather than rectal cases (p-value 0.02) and a higher rate of expression in adenocarcinoma cases than mucinous and undifferentiated carcinoma cases (p-value 0.01). The correlation was statistically significant by Chi-square /Fisher exact test proportion independence (p-value is set significant at 0.05 level).

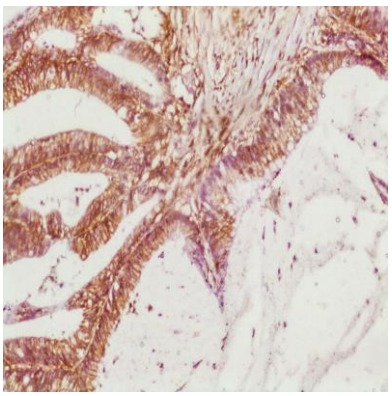

Figure 2: Mucinous carcinoma GII with strong CD44 expression and evident lymphocyte staining as internal control (x100)

The CD44 rate of expression showed statistically insignificant correlation with distant metastasis (M) and the metastatic group of all parameters including grade, histological types and invasiveness. Whereas it showed statistically insignificant inverse correlation with the grade, invasiveness (T), lymph node status (N), TNM stage grouping and modified Dukes' staging (except for stage IV and stage D respectively which represent the metastatic stage) (p-value > 0.05).

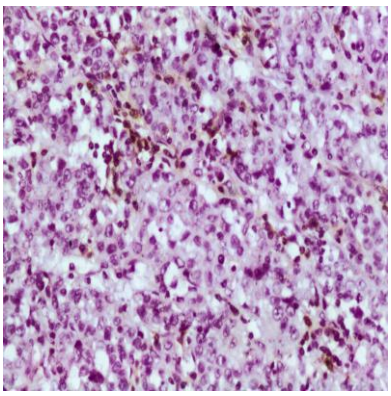

Figure 3: Undifferentiated carcinoma showing negative CD44 expression with focal staining of the lymphocytes which acts as internal control (x400)

No association was detected between CD44 expression and age, gender, tumour size, perineural invasion or lymphovascular invasion (p-value > 0.05).

Discussion

Cancer-related death from colorectal cancer is usually due to the development of distant metastasis. Approximately 70% of all patients diagnosed with CRC undergo potentially curative surgery, but half of those present with or develop advanced local disease or metastases [12]. Although several prognostic factors exist, including clinical staging classification, a more specific recognition marker for CRC with high metastatic potential would provide useful information for evaluating adjuvant therapies [13].

The concept of the contribution of colorectal cancer stem cells to tumour development is widely accepted, but the relation of individual CSC markers expression to disease prognosis is still not completely clear [14].

The relation between the site of the primary tumor and the rate of CD44 expression was statistically significant, where rectal tumors showed lower rate of CD44 expression (33.3%) than colonic tumors (66.7%) with slightly higher rate of expression in the right colon (70.8%) than in the left colon (61.9%) which is consistent with the results of [15] who found lower rate of CD44 expression in rectum (44.4%) than colon (64.4%). However, other studies showed higher rate of CD44 expression in rectum (48%) than colon (21.2%) [16], rectum (53.8%) and colon (30.8%) [13] and in rectum and left colon (53.8%) each then right colon (45.2%) (Al-Maghraby et al., 2012) [17].

The rate of CD44 expression varied according to histological type where there was statistically significant correlation between the rate of CD44 expression and the histological type. Adenocarcinoma cases showed the highest rate of CD44 expression (68.9%), followed by mucinous carcinoma (30.8%) then undifferentiated carcinoma which showed no CD44 expression. While other studies found a higher rate of CD44 expression in mucinous carcinoma (61.1%) than adenocarcinoma (49.5%) [18] and also higher in mucinous carcinoma (85.7%) than adenocarcinoma (55.9%) [15].

The role of CD44 as CSC marker itself is under debate, where Liu et al. (2014) [19] stated that CD44 is of functional importance for cancer initiation, progression, maintenance of the properties of CSC. While Pitule et al. (2014) [14] suggested that the use of CD44 as a single prognostic marker of CRC behaviour is impossible. Moreover Galizia et al. (2012) [20] results showed that evaluation of combined CD133/CD44 expression could be useful to identify putative colorectal CSCs than CD133 and CD44 individually which failed to identify colorectal CSCs.

The inconsistency and controversy of results from various studies correlating CD44 immunoexpression in relation to clinicopathological parameters may be related to technical factors, such as the type of antibody, differences in immunostaining method, type of tissue blocks, immunostaining scoring method and the cut-off for negative/positive or low/high [17].

We can also suggest another cause of controversy of results; which is the different functions of CD44 that may be presented in some tumours and absent in others. This suggestion is supported by Gunthert (1996) [21] who mentioned that during the malignant transformation process, and depending on the origin, the cell either gains or loses its ability to express some forms of this adhesion molecule. Thus, in human malignancies, it seems that the staining pattern for CD44 isoforms differs between the different tumour types, supporting the concept that the growth advantage for cancer cells contributed by CD44 depends on the cellular background.

A limitation of this study was the small scale of cases and unspecified selection of single parameter such as; studying CD44 expression in relation to recurrent cases, certain histological type, grade or stage (whether of invasiveness, lymph node status or metastasis) or even in relation to age, sex or site individually.

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Basic Science

Short-term Exposure to 50-Hz Electromagnetic Field and Alterations in *NQO1* and *NQO2* Expression in MCF-7 Cells

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Abbreviations: AMI – acute myocardial infarction, MI – myocardial infarction, BP – blood pressure, HF – heart failure, EF – ejection fraction, STEMI – ST-Segment Elevation Myocardial Infarction, AF – atrial fibrillation.

Abstract

AIM: Extremely low-frequency electromagnetic fields (ELF-EMFs) have some genotoxic effects and it may alter the mRNA levels of antioxidant genes. The NAD(P)H: quinone oxidoreductase-1 (*NQO1*) and *NQO2* are ubiquitously expressed. Considering that there is no published data on the effect(s) of ELF-EMF (50-Hz) exposure and expression levels of *NQO1* and *NQO2* in the human MCF-7 cells, the present study was carried out.

METHODS: The ELF-EMF (0.25 and 0.50 mT) exposure patterns were: 5 min field-on/5 min filed-off, 15 min field-on/15 min field-off, and 30 min field-on continuously. In all exposure conditions, total exposure time were 30 minutes. The RNA extraction was done at two times; immediately post exposure and two hours post exposure. The effect of ELF-EMF on gene expression was assessed by real-time PCR.

RESULTS: The *NQO1* mRNA level (at 0h) decreased in the cells exposed to 5 min field-on/5 min filed-off condition at 0.25 mT EMF when compared with the unexposed cells. The *NQO2* mRNA level (at 0h and 2h) increased in the cells exposed to 5 min field-on/5 min filed-off condition at 0.50 mT EMF when compared with the unexposed cells.

CONCLUSIONS: Alterations in the *NQO1* and *NQO2* mRNA levels seem at the "5 min field-on/5 min field-off" condition.

Introduction

Today electromagnetic fields (EMFs) are commonly present in our daily life. There are many reports in relation to effects of the extremely low-frequency electromagnetic fields (ELF-EMFs) on biological systems such as genotoxic effects [1-3] and alterations in expression levels of many genes [4, 5]. A relationship between elevation of reactive oxygen species (ROS) production and/or increasing the lifetime of ROS and the EMFs effects on biological systems has been reported [3]. Therefore, studies with respect to the alterations in mRNA levels of antioxidant genes are highly important to human public health. Very recently it has been reported that when the human breast cancer MCF-7 cells were exposed to the ELF-EMF, the mRNA levels of several antioxidant genes showed significant alterations [5].

NAD(P)H: quinone oxidoreductase-1 (NQO1; EC 1.6.5.2; OMIM: 125860) is a flavoprotein that catalyses the 2-electron reduction of various quinones and redox dyes, such as phylloquinone and the vitamin K menadione [6]. It has been reported that *Nqo1*-null mice exhibited increased toxicity when administered menadione compared with the wild-type mice, indicating a role for NQO1 in protection against quinone toxicity [7]. NAD(P)H: quinone oxidoreductase-2 (NQO2; EC 1.10.99.2; OMIM: 160998) is another member of NQOs and at the protein level, it shows 49% similarity with the liver cytosolic NQO1 protein. It has been stated that NQO1 and NQO2 are ubiquitously expressed [8, 9]. It has been reported that genetic polymorphisms in the NQO1 and NQO2 altered the risks of several complex multifactorial diseases which are associated with oxidative stress [10-14]. On the other hand, alterations of the mRNA levels of the NQO1 and NQO2 were observed in the SH-SY5H cells after exposure to

morphine and methadone [15, 16].

Considering that there is no published data on the effect(s) of ELF-EMF exposure and expression levels of *NQO1* and *NQO2* in the MCF-7 cells, the present study was carried out.

Materials and Methods

Cell culture

Human breast adenocarcinoma cell line MCF-7 was obtained from National Cell Bank of Iran (NCBI) (Pasteur Institute, Iran). Cells were seeded approximately 24h before EMF exposure at the density of 3×10^5 cells/ml in 100 mm surface treated Petri dishes (Jetbiofil).

Electromagnetic field exposure system and exposure conditions

Apparatus generated the 50-Hz EMF and exposure conditions were reported previously [5]. Three conditions of exposure were designed; two intermittent and one continuous. In all three exposure conditions, we tried to have a total exposure time of 30 minutes. The EMF exposure conditions were: 1) 5 min field-on/5 min filed-off, 2) 15 min field-on/15 min field-off, 3) 30 min field-on continuously. Control cultures were located in the exposure apparatus when the power was off. Control Petri dishes for each of three conditions were kept in disconnected solenoid for an equal time to EMF exposure. The RNA extractions were done at two times; immediately post exposure (0 h) and two hours post exposure (2 h).

RNA extraction, cDNA synthesis and Real-time RT-PCR

RNA extraction, cDNA synthesis and real-time PCR were performed as reported previously [5]. All samples had a high quality of RNA ($OD_{260/280}$ = 1.8-2.1). Primers specific for the studied genes and TATA box-binding protein (*TBP*, OMIM: 600075; used as a reference gene) were designed using Allele ID software (v.7.5, Premier Biosoft International, Palo Alto, CA, USA). The primer sequences are shown in Table 1. The primers were specific for mRNAs and could not amplify genomic DNA. Relative gene expression was calculated according to the $2^{-\Delta\Delta Ct}$ method based on the threshold cycle (Ct) values.

Table 1: The sequence of primers used in real-time PCR

Genes	Forward 5'---3'	Reverse 5'---3'	Product size (bp)
NQO1	ACTATGCCATGAAGGAGGCTG	CCTTCAGTTTACCTGTGATGTCC	129
NQO2	TTACGCTATGGCAGGTAAGAAAGT	CCTCGGCTCAAGGTTCATGG	157
TBP	CCCGAAACGCCGAATATAATC	TCTGGACTGTTCTTCACTCTTG	134

Statistical analysis

Data are shown as the mean ± SD of three independent experiments. Statistically, significant differences were assessed using one sample student *t*-test by SPSS software (SPSS Inc.). $P < 0.05$ was considered statistically significant compared to the controls.

Results

Table 2 showed the alterations of the *NQO1* and *NQO2* mRNA levels after the cultured MCF-7 cells were exposed to 0.25 and 0.50 mT 50-Hz EMF. The *NQO1* mRNA level (at 0 h) significantly decreased in the cells exposed to 5 min field-on/5 min filed-off condition at 0.25 mT EMF when compared with the unexposed cells. The *NQO2* mRNA level (at 0 h and 2 h) significantly increased in the cells exposed to 5 min field-on/5 min filed-off condition at 0.50 mT EMF when compared with the unexposed cells.

Table 2: mRNA levels (mean ± SD) of *NQO1* and *NQO2* genes in MCF-7 cells after exposure to 50-Hz electromagnetic field

Genes	Field intensity (mT)	Times after exposure	Exposure conditions		
			30 min cont.	15 On/15 Off	5 On/5 Off
NQO1	0.25	0h	0.78 ± 0.15	0.99 ± 0.05	0.91 ± 0.02*
	0.25	2h	0.98 ± 0.12	1.02 ± 0.07	0.90 ± 0.15
NQO1	0.50	0h	1.17 ± 0.11	1.06 ± 0.14	1.21 ± 0.21
	0.50	2h	1.16 ± 0.18	1.06 ± 0.09	1.01 ± 0.19
NQO2	0.25	0h	0.68 ± 0.14	0.91 ± 0.15	0.95 ± 0.07
	0.25	2h	1.06 ± 0.15	0.96 ± 0.08	0.91 ± 0.11
NQO2	0.50	0h	1.08 ± 0.07	1.09 ± 0.26	1.18 ± 0.06*
	0.50	2h	1.10 ± 0.06	1.07 ± 0.19	1.24 ± 0.08*

*P < 0.05 all values compared with untreated controls (=1) using one sample *t*-test.

Discussion

Previously we showed that the EMFs had some significant effects on the alterations of antioxidant genes and the alterations maximally seem at "the 15 min field-on/15 min field-off condition" [5]. The present data indicated that alterations in the *NQO1* and *NQO2* mRNA levels seem at "the 5 min field-on/5 min field-off" condition.

Some prodrugs used as antitumor agents could be activated by endogenous tumor-selective human enzymes such as NQO1 and NQO2 [8]. The elevated level of NQOs in some primary human tumours in comparison with normal surrounding cells and the fact that the NQO1 and NQO2 are ubiquitously expressed [8, 9] make them good target enzymes for bioreductive activation of prodrugs [8, 10]. Many efforts have been done on up-regulating these genes especially *NQO1* to increase the

effectiveness of cancer chemotherapy with prodrugs and also in order to decrease the side effects of chemotherapy.

β-lapachone is a σ-naphthoquinone originally extracted from the Brazilain lapacho tree. Its mechanism of action involved the reduction of the 1,2-carbonyl sites by NQO1. It has been shown that β-lapachone has great potential for the treatment of cancers with high levels of NQO1 [10].

To our knowledge, this study is the first to show the alteration of mRNA levels of the *NQOs* in human cultured cells due to ELF-EMFs. Further experiments should be done on other intensities of ELF-EMFs and conditions of exposure to find the most effective and long-lasting exposure on these genes and other oxidoreductases. Besides, the ELF-EMFs in combination with quinone-based antitumor prodrugs (such as β-lapachone) should be used to investigate the effect of these two treatments.

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Basic Science

Spectrum of Childhood and Adolescent Ovarian Tumors in India: 25 Years Experience at a Single Institution

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Keywords: paediatric population; ovary; malignancy; immature teratoma; incidence.

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Abstract

BACKGROUND: Ovarian tumour in children and adolescent girls form an uncommon but important part of gynaecological malignancies. They account for 1% of all the childhood malignancies and 8% of all abdominal tumours in children. Since the ovarian cysts are thought to arise from mature follicles, these tumours were considered to be infrequent in the paediatric population.

AIM: The rarity of this condition prompted us to conduct this study and share our experience on the incidence and clinicopathological features of different ovarian tumours in girls up to 20 years of age observed in last 25 years at a single tertiary care hospital.

MATERIAL AND METHODS: This was a retrospective study conducted in the Department of Pathology at a tertiary hospital, Delhi. All ovarian tumours up to the age of 20 years in the past 25 years (1990-2014) were included for the purpose of studying the clinicopathological aspects of ovarian tumours in this age group. Descriptive statistics for prevalence and age-wise prevalence was done. Chi-square test, to find an association between the age, laterality and size with malignancy was performed.

RESULTS: We received a total of 1102 cases of ovarian tumours over the period of 25 years (1990 to 2014), of which 112 (10%) cases were seen in girls up to 20 years of age. The mean age of the patients was 15.3 ± 4 years. The most common presenting complaint was pain abdomen (46.4 %) There was a statistically significant correlation found between size and malignancy status of tumours in our study (p = 0.00). Of 112 cases of ovarian tumours, 39/112 (34.8%) were malignant and 73/112 (65.2%) were benign. Mature cystic teratoma (27.6%) was the most common type of benign tumour in this age group and immature teratomas were the most common type of malignant ovarian neoplasms.

CONCLUSION: Premenarchal girls with ovarian masses may have varied presentations. Abdominal pain is the most common presenting complaint of young adolescent girls with adnexal masses. So the index of suspicion should be kept high and prompt investigations like ultrasound must be performed early to rule out such adnexal masses. Immature Teratoma was the most common malignant and mature cystic teratoma was the most common benign tumour in our study.

Introduction

Ovarian tumour in children and adolescent girls form an uncommon but important part of gynaecological malignancies. They account for 1% of all the childhood malignancies and 8% of all abdominal tumours in children. It is estimated that almost 10-30% of all the ovarian neoplasms occurring in girls up to 17 years of age are malignant [1, 2]. Tumours of the Ovary are divided into neoplastic and non-neoplastic processes. While the non-neoplastic conditions include corpus luteal cysts, follicular cysts, and endometriotic cysts, the neoplastic processes include both benign tumours like mature cystic teratomas and highly malignant tumours like yolk sac tumours. According to the literature, paediatric ovarian tumours mostly belong to the group of germ cell tumours in contrast to the tumours occurring in the adult females, which are mostly surface epithelial in origin [3]. Since the ovarian cysts are thought to arise from mature follicles, these tumours were considered to be infrequent in the paediatric population.

The rarity of this condition prompted us to conduct this study and share our experience on the incidence and clinicopathological features of different ovarian tumours in girls up to 20 years of age observed in last 25 years in our tertiary care hospital.

Material and Methods

The study was conducted in the department of pathology at a tertiary hospital, Delhi. All ovarian tumours up to the age of 20 years received in the Dept. of Pathology in the past 25 years (1990-2014) were included for the purpose of studying the clinicopathological aspects of ovarian tumours in this age group. Clinical features like age, laterality, size and stage of the tumour were recorded in the case record form from the pathology archives. For statistical purposes, we included the non-neoplastic cases comprising of corpus luteal cyst, follicular cyst and endometriotic cyst in benign cases. The borderline surface epithelial tumours are included in the malignant category for the same reason. Descriptive statistics for prevalence and age-wise prevalence was done. Chi-square test, to find an association between the age, laterality and size with malignancy was performed.

Results

We received a total of 1102 cases of ovarian tumours over the period of 25 years (1990 to 2014), of which 112 cases were seen in girls up to 20 years of age. This constituted 112/1102 (10%) of all the ovarian tumours received so far. The mean age of the patients was 15.3 ± 4 years (range 1.5 years – 20 years). Figure 1 shows the age wise distribution of benign and malignant tumours in our study. It was seen that while the incidence of benign and malignant tumours was similar to 14 years of age, it was much higher for both, in girls more than 15 years of age. However, there was no statistically significant association between age and malignancy status of the patients (p = 0.122) (Figure 1).

The most common presenting complaint was pain abdomen (46.4 %) followed by lump abdomen (24%) and abdominal distension (10.7%) (Figure 2). While a majority of the patients presented with vague abdominal pain there were 7 patients who presented as acute pain abdomen in the emergency outpatient ward. These patients were later found to have either twisted (5) or ruptured (2) ovarian cyst. On histopathological examination, it was seen that 4/5 mature cystic teratomas (mct) and 1 dysgerminoma presented as torsion while 1 mucinous cystadenoma and 1 mct presented with a ruptured cyst. While 4 cases of torsion occurred on the right side the remaining 3 occurred on the left side.

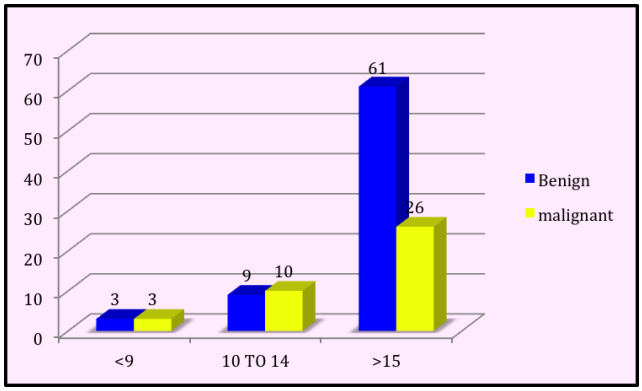

Figure 1: Frequency of benign and malignant tumours in different age group in our study

There were 4 patients who were incidentally diagnosed by the clinicians, 2 of these patients had torsion of the ovarian cyst but were clinically diagnosed as acute appendicitis with peri appendicitis by the department of surgery however later during the surgery they were found to have ovarian masses. The other 2 patients diagnosed incidentally were at gestational ages of 20 weeks and 25 weeks respectively and their ovarian masses were detected on prenatal sonography which was then later removed surgically. Seven of 39 patients with malignant neoplasm gave a history of anorexia and weight loss.

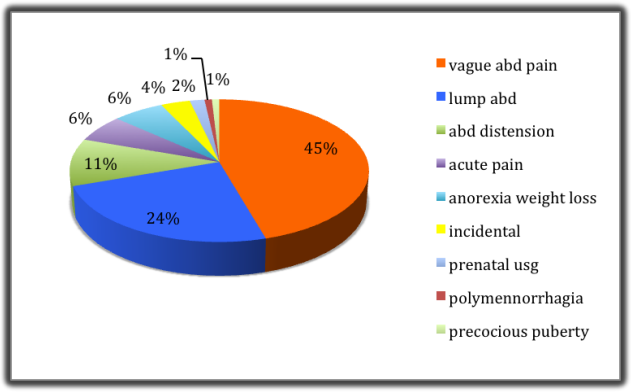

Figure 2: Frequency distribution of various presenting complaints in our study

In this study, 85/112 (75%) were Hindus, 23 Muslims and 4 others including Christians, Sikhs etc. There was no statistically significant correlation between the malignancy status and religion of the patient.

We categorised the gross findings into size laterality,and cut surface [solid/cystic (unilocular or multilocular)/solid and cystic] of the masses, as shown in Table 1. The size was further subdivided into 3 categories of less than 9 cm, 10-14 cm and >15 cms

(Table 1). It was noted that a majority of masses measuring less than 10 cm (48/112) were benign whereas those measuring 10-14 cm were predominantly malignant (23/112) cases. Surprisingly however masses measuring > 15 cm were almost equal (7) for benign and (9) malignant tumours. There was a statistically significant correlation found between size and malignancy status of tumours in our study (p = 0.00).

Laterality of each tumour was also recorded from the case record forms and it was seen that there was no statistically significant correlation found between laterality and malignancy status of tumours in our study (p = 0.71). Only one case of bilateral mature cystic teratoma was found in this study (Table 1).

Gross appearance of tumours was correlated with histopathological type in our study (Table 1). The majority of our cases were unilocular (62 cases i.e. %) which included mostly benign cystic teratoma, serous cystadenoma, mucinous cystadenoma, corpus luteal cyst, follicular cyst and endometriosis cyst. Multilocular cystic tumours were found in 7 cases of which most were benign cystic teratoma (4) followed by serous cystadenoma (2) and borderline mucinous tumours (1). Forty cases of solid or solid cystic / variegated tumours were found of which 37 cases were malignant and the remaining 3 were benign (fibroma).

Table 1: Frequency distribution of size, laterality, gross findings and treatment choices in our study

Size distribution of ovarian tumours			
Size		Benign	Malignant
<10		48	7
10 to 15		18	23
>15		7	9
Laterality distribution of ovarian tumours			
Laterality		Benign	Malignant
Right		34	17
Left		38	22
Bilateral		1	
Gross findings of ovarian tumours			
Gross		Benign	Malignant
Cystic	Unicystic	62	-
	Multicystic	5	2
Solid-cystic		6	37
Treatment choices of ovarian tumours			
Type of treatment		Benign	Malignant
Cystectomy		70	2
Unilateral salpingo-oophorectomy		20	15
Bilateral cystectomy		1	
Total abdominal hysterectomy with bilateral salpingo-oophorectomy			4

Table 2 shows the age wise histopathological distribution of 112 cases of ovarian tumours, of which 39/112 (34.8 %) were malignant and 73/112 (65.2%) were benign. Whereas the incidence of benign ovarian tumours (47.8%) was marginally lower to malignant ovarian tumours (52.1) in premenarchal girls up to 14 years of age, 69.6% of the tumours were benign and the remaining 30.3% were malignant in girls >15 years of age. 52.6% of all the ovarian tumours occurring in our study population were germ cell tumours. Malignant tumours in girl's up to 20 years of age constituted 3.5% (39/1102) of all the ovarian tumours received so far in our study. 71.1% of all the malignant tumours occurring in our study were germ cell tumours. It was seen that of the 73 benign neoplasms, mature cystic teratoma (27.6%) was the most common type of tumour in all age groups followed by serous cystadenoma and mucinous cystadenoma which were exclusively seen in girls >15 years of age in this study. It was further noted that up to 14 years of age, germ cell tumours were the most common type of ovarian tumours apart from a single case of granulosa cell tumour seen in 10-14 years of age. However, after 15 years of age, the incidence of surface epithelial tumours (34%) was only marginally higher as compared to the germ cell tumours (33%). All the cases of corpus luteal cysts, endometriotic cysts and follicular cysts were also seen in the age group of 15 to 20 years of age. 6 cases seen in the age group of 0 to nine years were all germ cell tumours.

Table 2: Age-wise distribution of ovarian tumours in our study

Histopathological type of tumour		<9	0-14	>15
Germ cell tumour				
Benign	Mature cystic teratoma	3	8	20
Malignant	Immature teratoma	1	3	5
	Yolk sac tumour	2		1
	Dysgerminoma		2	6
	Mixed germ cell tumour		3	5
Surface epithelial tumours				
Serous	Benign			21
	Borderline			2
	Malignant			-
Mucinous	Benign			11
	Borderline			2
	Malignant			1
Endometrioid adenocarcinoma				1
Sex cord stromal tumours				
Granulosa cell tumours			1	1
Sertoli-Leydig cell tumours				2
Sclerosing stromal tumour				1
Non-neoplastic lesions of ovary				
Corpus lutein cyst				2
Simple cyst				6
Endometriotic cyst				2

Amongst the 39 malignant cases, immature teratomas, constituting 8% of all ovarian masses, were the most common type of malignant ovarian neoplasms. Most of the malignant tumours (28/39; 71%) were seen beyond 15 years of age. While immature teratoma constituted nine cases (8%) and is observed in all age groups, dysgerminomas (8) were seen in girls more than 9 years of age. Immature teratoma was the most common GCT in our study There were 6 malignant cases of surface epithelial origin (4 cases of borderline serous/mucinous cystadenoma, 1 mucinous cystadenocarcinoma and 1 endometrioid adenocarcinoma) and all of them were seen in age group beyond 15 years. One case of Granulosa cell tumour was also seen in the latter age group.

Operations (Table 2) performed were unilateral salpingo-oophorectomy (n = 35), cystectomy (n = 72) and unilateral salpingo-oophorectomy with contralateral biopsy (n = 2). Two patients had an incidental appendicectomy. Total abdominal hysterectomy with bilateral salpingo-oophorectomy was done in 4 cases of stage 4 malignant cases including mixed germ cell tumour, mucinous

cystadenocarcinoma and yolk sac tumour. Patients with yolk sac tumors (n = 2), immature teratomas (n = 2) and mixed germ cell tumor had elevated alpha-fetoprotein (AFP) levels (from >350 ng/ml to 12340 ng/ml), and one patient with dysgerminoma had raised serum LDH levels (2203 IU/L).

Discussion

Ovarian tumours in young girls are uncommon, but when present they are a source of much anxiety to the patients and their families, as the management of these tumours directly affect the fertility of these girls. It is known that at puberty when menarche begins there are changes in the hormonal levels of the hypothalamus, pituitary gland, and ovaries, so these girls are predisposed to the development of various ovarian masses at puberty [4]. According to Harlan et al, menarche occurs at the age of 9.1 to 17.7 years with a mean age of 13 years [5]. In our study, patients ranged in age from 1.5 years to 20 years, with a mean age of 15.3±4 years at the time of presentation.

Adnexal masses in this age group may present in various ways like pain abdomen, lump abdomen or abdominal distention. According to the literature, abdominal pain is the most common symptom of premenarchal ovarian masses. In our study abdominal pain (45.5%) was the most common presenting symptom in concordance with the study by Ki EY et al where the incidence was an abdominal pain (47.7%), palpable abdominal masses (24.6%), incidental ultrasonographic findings (4.6%), and precocious puberty (6.2%). However, in our study one case of granulosa cell tumour presented with precocious puberty (0.89%) and in 4 patients (3.57%) it was incidental finding discovered on prenatal ultrasonography [4].

It is thought that paediatric patients are more prone to ovarian torsions due to a longer infundibulopelvic ligament [6]. Brandt ML reported that ovarian torsions are more common on the right side and are thus often misdiagnosed as acute appendicitis before surgery [7]. In our study, 6% of the patients presented with acute abdominal pain in the emergency OPD due to torsion of the ovarian cyst. Of these 2 were misdiagnosed as acute appendicitis before surgery. The appendicectomy specimens sent along with were histopathologically reported as a vermiform appendix in both the cases. The adnexal torsions on the left side are still under controversy for being less common than the right side. It is unclear whether the sigmoid colon helps prevent left adnexal torsion or whether these sided torsions are more commonly missed and managed non-surgically [8, 9]. However, in our study, we had 4 cases on the right side and 3 cases on the left side. Unlike Kandasamy Vijayalakshmi et al. [10] who reported mucinous cystadenoma to be the most common ovarian tumour undergoing torsion we found that mct was the most common tumour to undergo torsion in our study was mct.

Regarding gross features in our study cases, 111 cases were unilateral and only a single case of mature cystic teratoma was bilateral. Solid or solid-cystic ovarian masses are generally indicative of malignancy. In our study, we got 43 cases of solid/solid cystic cut surface, 37 of which were malignant. Five of the remaining 6 benign tumours were mature cystic teratoma and the other was an endometriotic cyst.

Ovarian malignancy accounts for 1% of all the childhood tumours in the previous studies but some studies like Oumachigui et al, 1991 and Sawai MM et al, 1973 found the incidence to be as high as six per cent and 11.2% respectively [2, 11, 12]. In the study conducted by Bren JL et al it was reported that 35% of all ovarian neoplasms in childhood and adolescent were malignant (Bren JL et al, 1977) [13]. In accordance with this study, we also found that 34.8% of all the ovarian tumours in our study were malignant. This was in contrast to Ehren et al. who found that 60 – 85% of all the ovarian neoplasm in Pediatric age group in their study were of germ-cell origin [2, 14]. Although Germ cell tumour was the most common malignant ovarian neoplasm in our study accounting up to 71.1% of all the malignant tumours of the ovary occurring in this age group. While immature teratoma was the most common malignant germ cell tumour in our study, dysgerminoma was most common in the studies conducted by Bhattacharyya, et al. and Mukhopadhyay et al [2,15] from India.

We found that though the incidence of surface epithelial tumours was almost equivalent to germ cell tumours in this study. In contrast to the study by Graspa D, et al, 2006 which reports the incidence of surface epithelial tumours in the prepubertal age group and adolescent age group is usually 15 - 20% we found this incidence to be 33.9% [16]. This finding could possibly be explained by the fact that our study population included girls up to 20 years of age when a lot of girls have achieved menarche and are already going through the various hormonal surges giving rise to these tumours.

Sex cord stromal tumours though uncommon can also develop in premenarcheal girls. They constitute 10%-25% of all paediatric ovarian neoplasms [4]. Granulosa cell tumours are the most common form of sex cord tumours and are frequently associated with hyperestrogenism or precocious puberty [17-19]. In our study, we had an equal incidence of granulosa cell tumour and Sertoli Leydig cell tumour (1.7%) but only 1 patient presented with precocious puberty.

Since this is a retrospective analysis, close follow-up for benign cases was not necessary where involvement was only unilateral. The only case of benign bilateral mature cystic teratoma had undergone bilateral cystectomy. But patients having malignant ovarian tumours needed follow-up regarding appropriate therapy and survival. However, since the population our hospital caters belong to far off rural destinations, follow up becomes difficult as most of these patients do not revert back for postsurgical treatment. So far, we could retrieve records for only 10 cases of malignant ovarian tumours who attended follow-ups. It was observed that six out of eight dysgerminoma cases of patients in stage I-A survived till date and other two cases lost to follow-up. One case of mixed germ cell tumour and mucinous cystadenocarcinoma who were stage 4 at presentation did not survive. Subjects having other mixed germ cell tumours were advised appropriate treatment, but unfortunately, most of them lost follow-up.

In conclusion, premenarchal girls with ovarian masses may have varied presentations. Abdominal pain is the most common presenting complaint of young adolescent girls with adnexal masses. So the index of suspicion should be kept high and prompt investigations like ultrasound must be performed to rule out such adnexal masses. Immature Teratoma was the most common malignant and mature cystic teratoma was the most common benign tumour in our study. Though germ cell tumours are the most common ovarian neoplasms in adolescent girls, the higher incidence of surface epithelial tumours in our study could not be disregarded. This was possibly because of our inclusion criteria of age up to 20 years when fair numbers of girls have already achieved menarche.

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Basic Science

Vascular Genetic Variants and Ischemic Stroke Susceptibility in Albanians from the Republic of Macedonia

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Keywords: ischemic stroke; vascular genetic variations; association; Albanians; Republic of Macedonia.

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Abstract

BACKGROUND: Acute first-ever ischemic stroke (FIS) is a heterogeneous, polygenic disorder. The contribution of vascular genetic variants as inherited causes of ischemic stroke has remained controversial.

AIM: To examine the association of genetic variants in vascular factors with the occurrence of FIS.

MATERIAL AND METHODS: The current research was performed in a group of 39 patients with FIS (study group) and 102 healthy volunteers (control group). We analyzed the prevalence of vascular genetic variants in following genes: *factor V, prothrombin, methylenetetrahydrofolate reductase (MTHFR), factor XIII, plasminogen activator 1, endothelial protein C receptor (EPCR), apolipoprotein B, apolipoprotein E, β-fibrinogen, human platelet antigen 1, angiotensin-converting enzyme (ACE), endothelial nitric oxide synthase (eNOS)* and *lymphotoxin alpha.*

RESULTS: It was found that heterozygous *LTA 804C>A* and *FXIII V34L Leu/Leu* were significantly more frequent in patients with FIS than in control group ($p = 0.036$ and $p = 0.017$, respectively). The frequency of *FXIII V34L Val/Val* was significantly lower in patients with FIS than in control group ($p = 0.020$). Other frequencies of vascular gene variants in patients with FIS and in control group were not significantly different.

CONCLUSIONS: This is the first comprehensive study to present data indicating that polymorphism of vascular genes in the prevalence of acute FIS exists in the Albanian population from the Republic of Macedonia. Variations in these genes have been detected in patients with acute FIS, suggesting that their combination might act in a susceptible or protective manner in this Albanian population.

Introduction

Ischemic stroke is a major public health problem with a heritable component to the risk of disease [1-3]. The last decade has been marked by initiatives aimed at raising awareness of the inherited predisposition to ischemic stroke. There has been an increase in studies that analysed polymorphic genes responsible for arterial and venous ischemic events. An increased understanding of genetics and molecular biology has fuelled this research and shed light on several genetic risk factors that may help to predict arterial events.

Most of the studies related to arterial thrombosis have actually involved patients with coronary syndromes [4], myocardial infarction [5] and events of the arterial circulatory system [6, 7], and they produced conflicting results. To date, most studies on the genetics of ischemic stroke have focused on candidate genes in a control group and unrelated cases. The causes of the majority of ischemic strokes include atherosclerotic, cardiogenic and lacunar (penetrating vessel) mechanisms [8]. Several animal and clinical studies clearly defined genetic predisposition to hereditary thrombophilia or atherosclerosis, in the case of atherosclerosis, and particularly, to the clinical expression of ischemic stroke [9]. Arteriosclerosis of the large vessels is the cause of cerebral infarction in almost half of all

ischemic events [10]. Arteriosclerosis involves numerous genes and some pathways thought to be involved in the development and rupture of atherosclerotic lesions [11].

Based on published data, the most common vascular genetic variations have been extensively studied, particularly in the following genes: *factor V (FV), prothrombin (factor II; PTH), methylenetetrahydrofolate reductase (MTHFR), factor XIII (FXIII)* [12, 13], *plasminogen activator 1 (PAI-1)* [14-18], *endothelial protein C receptor (EPCR), apolipoprotein B (APOB)* [19], *apolipoprotein E (APOE)* [20], *β-fibrinogen (β-FG, FGB)* [21], *human platelet antigen 1 (HPA-1)* [22, 23], *angiotensin-converting enzyme (ACE)* [24, 25], *endothelial nitric oxide synthase (eNOS)* [26] and *lymphotoxin alpha* [27], which have been identified as risk factors for several diseases, such as arterial and/or venous thrombosis. However, with regard to testing for an association with susceptibility to ischemic stroke, study results are conflicting and the association is not always detected. Consequently, routine testing for polymorphisms associated with ischemic stroke is not part of the diagnostic protocol for elderly individuals, but the results are still debatable.

This study is the first comprehensive study of the vascular gene variations associated with first-ever ischemic stroke in the Albanian population from the Republic of Macedonia. The purpose of this research was to examine the association of vascular gene variations in 13 genes and 17 genetic variants in patients with acute FIS and compare the frequencies in the patient and control groups.

Material and Methods

Sampling

This study was a case-control study (comparative analytical study) carried out from September 2008 to August 2010. In total, 231 patients with FIS as a study group and 194 healthy volunteers as a control group were enrolled in the initial study. The inclusion criteria were consecutive patients admitted to the Neurology Department with confirmed diagnosis acute FIS, age ranging from 18 to 90 years, an absence of a history of stroke and completion of interview and analysis. All patients gave informed consent prior to participation in this study. The exclusion criteria were patients younger than 18 years, patients, who had experienced an acute hemorrhagic stroke, a known malignancy, acute or chronic renal or failure or acute myocardial infarction and patients who had recent inflammatory or immunologic disease. Acute FIS was defined as a clinical syndrome on the basis of clinical symptoms and (a) recent infarct(s) in the clinically relevant area of the brain, using brain computed tomography (CT) and/or magnetic resonance imaging (MRI).

Healthy controls that were free from cerebrovascular diseases were selected from among local residents, using the same exclusion criterion as for patients. Informed consent was obtained from all participants prior to conducting the study. The protocol of the study was reviewed and approved by the professional college at Clinical Hospital Tetovo (Nr.02-1280/2, 02.07.2008) [28].

Within a study of the genetics of the Macedonian population, a detailed description of the study subjects and study design were performed as previously described [29, 30]. Briefly, after signed consent was given to obtain and store a DNA sample, 212 subjects were recruited in total (stratified into 58 healthy controls and 12 patients of Macedonian origin and 103 healthy controls and 39 patients of Albanian origin) all residents of Tetovo and its neighbouring region who met the study inclusion criteria to engage in the proposed national project over a 2-year period (2010–2012), examining gene variations associated with diseases of blood vessels in the Republic of Macedonia, which was originally designed as a prospective, nationwide, multicenter case-control study (ID 13-3589/1, 26.07.2010).

Among others, the objectives were to investigate the distribution of candidate genes in healthy Macedonian and Albanian populations and the association of candidate genes with stroke in Macedonians and Albanians from the Republic of Macedonia. Of these, genetic analyses were completed for 141 (66.51%) participants, all of whom were Albanians, while others are in progress. The present study comprises 39 patients with acute FIS treated at the Clinical Hospital Tetovo, Department of Neurology as a study group and 102 healthy volunteers as a control group. Each individual was interviewed on a one-to-one basis, his/her genealogy was recorded for the past three generations, and their Albanian origin was ascertained by making enquiries as far back as their four grandparents. Admixture, if any, was recorded for each individual. Individuals with only one Albanian parent were excluded from the study.

Blood sampling and processing

A sample of peripheral vein whole blood (10 mL) was collected by venipuncture in standard collection tubes containing anticoagulant ethylenediaminetetraacetic acid (EDTA) in the first 24 h of admission and then dispensed into two tubes (one for complete blood picture and the other for extraction of DNA). The first tube was transferred to the biochemical laboratory for routine analysis and the second tube was separated and referred for genetic analysis.

DNA extraction, molecular genetic screening and storage

At the Institute of Immunobiology and Human Genetics, part of the Faculty of Medicine, Ss Cyril and Methodius University of Skopje, Skopje, Republic of Macedonia [28-30], genomic DNA was isolated from peripheral blood leukocytes by the phenol-chloroform extraction method [31] or with BioRobot EZ1 workstation (QIAGEN) [32].

Genetic testing of the candidate genes was based on the polymerase chain reaction and reverse hybridization principle using the CVD SripAssay [ViennaLab (Laboradiagnostica GmbH, Austria)]. The DNA samples were subsequently stored in the anthropology project field of the Macedonian Human DNA Bank (hDNAMKD) [33].

Statistical analysis

The population genetics analysis package, PyPop, developed by the Biostatistics Core for the Workshop [34-36] was used for analysis of the genetic data for this report. All analyses were based on available data. The results were collected, tabulated and statistically analysed by personal computer and the commercially available statistical software package SPSS version 17 (SPSS Inc. Chicago, Illinois, USA). The distribution of gene polymorphism frequency was not perfectly normal.

Descriptive statistics were used for the prevalence of genetic polymorphisms. Between-group differences in genotype frequencies were compared using a two-by-two contingency table and analysed by chi-square analysis with Yates' correction test. Pearson P values, crude odds ratios (OR), and Wald's 95% confidence intervals (CI) were calculated to test the associations between mutations/polymorphisms and FIS with free statistical calculators (http://www.quantitativeskills.com/sisa/statistics/two2hlp.htm). The level of statistical significance was set at $p = 0.05$.

Results

We analysed genetic tests for a total of 141 participants, divided into two different groups: data for 39 patients with FIS (mean age: 62.56 years; range: 44–82 years), and the results of 102 healthy subjects (mean age: 48.86 years; range: 27–71 years).

The baseline and demographic characteristics of the study participants are presented in Table 1.

The mean age at first onset FIS was significantly higher in the patient groups older than 60 years than in the age-matched control group, and the difference was statistically significant (t = 7.836; $p = 0.000$).

Table 1: Participants included in the study classified by age group

	Patients		Controls	
Age Group	N	%	N	%
18–49	2	5.13	44	43.14
50–59	12	30.77	46	45.10
60–69	16	41.03	11	10.79
>70	9	23.08	1	0.98
Total	39	100	102	100
Mean ± SD (years)	62.56 ± 8.73		48.86 ± 10.60	
Range	44–82		27–71	

t = 7.836; $p = 0.000$

Genotype frequencies were compared separately for all vascular gene variations between cohorts of patients and controls. The genotype distributions in the study and control groups are provided in Table 2.

When genotype frequencies were stratified into distribution frequencies, we observed that in most cases, higher frequencies of gene mutations were detected in the control group than in the patient group. The *APOE E3/E3* polymorphism was present in only one patient, and the *fibrinogen beta (-455G>A)* mutation was not present in any patient, whereas the frequencies of heterozygote carriers of f*actor V (Leiden-G1691A; R2-H1299R), PTH, MTHFR-677, MTHFR-1298, FXIII, LTA, HPA 1, ACE, PAI-1, eNOS-T786C* and *eNOS-G894T* were lower in patients than in control subjects.

Heterozygotes for the *factor V Leiden* mutation were more frequent in control healthy subjects (8.82%) than in the group of patients with FIS (3 or 7.69%).

The prevalence of heterozygote variants in *PTH 20210G>A* was higher among controls than among patients with FIS (6.86% vs. 2.56%, respectively).

Analysis of the study group for the *MTHFR C677T* polymorphism indicated that 15 (38.46%) patients were homozygous for the wild-type (*CC*), 21 (53.84%) were heterozygous (*CT*), and the remaining 3 (7.69%) were homozygous for the mutant allele (*TT*). In the control group of 102 analyzed subjects, the following results were obtained: 27 persons (26.47%) were homozygous for the wild-type (*CC*), 55 individuals (53.92%) were heterozygous for the polymorphism (*CT*), and 20 individuals (19.61%) were homozygous for the variant type (*TT*).

The prevalence of wild-type homozygotes and heterozygotes for the *FXIII V34L Leu34* polymorphism differed significantly between patients with acute FIS and their respective controls.

In the *EPCR* gene, we observed a significant difference in the distribution of the *CC* and *CG/GG* genotype groups between the control and patient groups. The carriers of the *EPCR 4600AG* genotype

were more frequent in patients with FIS (10 or 25.64%) than in control healthy subjects (20 or 19.61%). In contrast, carriers of the *EPCR 4678CG* genotype were more frequent in control healthy subjects (50 or 49.02%) than in patients with FIS (18 or 46.15%), except for carriers of the *EPCR 4678GG* genotype, which were more frequent in patients with FIS (16 or 41.03%) than in healthy subjects (24 or 23.53%).

Table 2: Genotype frequencies, Pearson`s *P*-values, odds ratios and Wald's 95% confidence intervals in patients with FIS and healthy Albanian controls

Gene and genetic variant	Control group (n = 102) (%)	Study group (n = 39) (%)	Statistical analysis	
			OR (95% CI)	Pearson's *P*-values
FV 1691G>A (Leiden)				
N	93 (91.18)	36 (92.31)	0.988 (0.886–1.101)	0.829
HET	9 (8.82)	3 (7.69)	1.147 (0.328–4.018)	0.829
HOM	0 (0)	0 (0)	*	*
FV H1299R				
N	77 (75.49)	32 (82.05)	0.674 (0.265–1.714)	0.405
HET	25 (24.51)	7 (17.95)	1.484 (0.583–3.777)	0.405
HOM	0 (0)	0 (0)	*	*
PTH 20210G>A				
N	95 (93.14)	38 (97.44)	0.357 (0.042–3.002)	0.324
HET	7 (6.86)	1 (2.56)	2.800 (0.333–23.533)	0.324
HOM	0 (0)	0 (0)	*	*
MTHFR 677C>T				
CC	27 (26.47)	15 (38.46)	0.567 (0.264–1.258)	0.164
CT	55 (53.92)	21 (53.84)	0.687 (0.307–1.54)	0.361
TT	20 (19.61)	3 (7.69)	0.270 (0.069–1.06)	0.051
MTHFR 1298A>C				
AA	54 (52.94)	18 (46.15)	1.313 (0.626–2.751)	0.471
AC	44 (43.13)	20 (51.28)	1.388 (0.622–2.909)	0.385
CC	4 (3.92)	1 (2.56)	0.750 (0.079–7.154)	0.802
FXIII V34L				
Val/Val	67 (67.68)	17 (45.94)	2.463 (1.139–5.329)	0.020
Val/Leu	25 (25.25)	13 (35.14)	2.049 (0.871–4.823)	0.097
Leu/Leu	7 (7.07)	7 (18.92)	3.941 (1.217–12.763)	0.017
EPCR 4600A>G				
AA	80 (78.43)	29 (74.36)	1.254 (0.531–2.963)	0.606
AG	20 (19.61)	10 (25.64)	0.798 (0.338–1.884)	0.606
GG	2 (1.96)	0 (0)	*	0.265
EPCR 4678G>C				
CC	28 (27.45)	5 (12.82)	2.573 (0.914–7.24)	0.066
CG	50 (49.02)	18 (46.15)	2.016 (0.675–6.017)	0.203
GG	24 (23.53)	16 (41.03)	3.373 (1.191–11.704)	0.019
eNOS 786T>C				
N	35 (34.31)	11 (28.21)	0.133 (0.592–2.984)	0.489
HET	54 (52.94)	20 (51.28)	1.178 (0.504–2.757)	0.705
HOM	13 (12.75)	8 (20.51)	1.958 (0.645–5.948)	0.232
eNOS 894G>T				
N	41 (40.20)	14 (35.90)	1.200 (0.559–2.578)	0.639
HET	52 (50.98)	24 (61.54)	1.352 (0.626–2.937)	0.446
HOM	9 (8.82)	1 (2.56)	0.325 (0.038–2.803)	0.286
LTA 804C>A				
N	82 (80.39)	22 (56.41)	2.423 (0.144–5.623)	0.036
HET	20 (19.61)	13 (33.33)	0.413 (0.178–0.958)	0.036
HOM	0 (0)	4 (10.26)	*	*
HPA-1 a/b				
1a/1a	75 (73.53)	27 (69.23)	1.235 (0.549–2.775)	0.609
1a/1b	24 (23.53)	11 (28.21)	1.273 (0.551–2.944)	0.572
1b/1b	3 (2.94)	1 (2.56)	0.926 (0.092–9.287)	1.000
ACE I/D				
I/I	0 (0)	1 (2.94)	*	0.079
I/D	49 (61.25)	23 (67.65)	*	0.098
D/D	31 (38.75)	10 (29.41)	*	0.057
APO E ε2/ε3/ε4				
E2/E3	1 (1.25)	0 (0)	*	0.856
E3/E3	60 (75.00)	1	*	0.447
E3/E4	15 (0)	0 (0)	*	0.504
E4/E4	4 (5.0)	0 (0)	*	0.690
PAI-1				
4G/4G	25 (24.51)	11 (28.21)	0.826 (0.36–1.897)	0.653
4G/5G	53 (51.96)	19 (48.72)	0.815 (0.337–1.968)	0.649
5G/5G	24 (23.53)	9 (23.08)	0.852 (0.3–2.421)	0.764

n = absolute number; % = frequency; OR = Odds ratio; CI = Confidence Interval; *, cannot be calculated because expected < 5, χ^2 test.

The *eNOS* mutation frequencies were higher in the patient group. There were no significant differences in genotype distribution between patient and control groups.

Heterozygotes for the *LTA C804A* polymorphism were more frequent in patients with FIS (13 or 33.33%) than in control healthy subjects (20 or 19.61%).

HPA-1 1a/1b and *ACE I/D* mutation frequencies were higher in the control than in the study group, but the polymorphism frequency was not significantly different between the study and control groups.

No homozygous or heterozygous mutants for *APO B* were detected in any of the groups (in all patients and controls, the *APO-B* genotype distribution was normal; these data are not shown in Table 2).

PAI-1, 4G/5G and *5G/5G* mutation frequencies were higher in the control than in the study group, but the polymorphism frequency was not significantly different between the study and control groups.

The prevalence of all investigated gene variations did not differ significantly between the studied groups (Pearson's P value > 0.05), except for the prevalence of the homozygous wild-type and heterozygous genotypes, *FXIII V34L Leu34* carriers and homozygotes (*GG*) for the factor *EPCR G4678C* polymorphism. There were also no significant differences with regard to ethnicity (all participants were Albanians from the Republic of Macedonia). The frequency of *ACE I/D* carriers was 67.65%, that of *eNOS-G894T* heterozygotes was 61.54% and the *MTHFR C677T CT* genotype was detected in 53.84% of patients.

The most frequent *ACE* genotype in patients with FIS was *I/D*, with an observed frequency of 67.65%; a lower frequency was found for the *D/D* genotype (29.41%) and the lowest frequency was found for the *I/I* genotype (2.94%).

The most frequent *PAI-1* genotype in patients with FIS was *4G/5G*, with an observed frequency of 48.72%; a lower frequency was found for the *4G/4G* genotype (28.21%) and the lowest frequency was found for the *5G/5G* genotype (23.08%). The frequencies of the *PAI-1 4G/4G* and *4G/5G* genotypes were slightly increased in healthy individuals (24.52% and 51.96%, respectively), but that of *PAI-1 5G/5G* was decreased (23.53%).

Discussion

Traditionally, the greatest clinical challenge in the prevention of ischemic stroke is focused on the modification of the main risk factors for atherosclerosis, including symptomatic treatment of hypertension, diabetes, hypercholesterolemia and smoking [10-12].

For the first time in the Republic of

Macedonia, the determination of a heritable component to the risk of acute FIS was carried out in unrelated cohorts from an Albanian population living in the Republic of Macedonia. We performed a genetic analysis of patients presenting with ischemic stroke that clinically was their first stroke episode and community controls who were subsequently included in a national project [28-30]. The purpose of this study was to investigate the role of specific genes and to obtain data that would support an association of gene polymorphisms regarding the aetiology of ischemic stroke.

Excluding *FXIII Leu34* carriers and homozygotes (*GG*) for the factor *EPCR G4678C* polymorphism, these preliminary results of genetic analysis in the present study did not find any significant positive (susceptible) association between the investigated gene polymorphisms and FIS (Pearson's P value greater than 0.05) in this cohort of Albanians from the Republic of Macedonia. These results are of interest because they differ from those of other studies.

Many of the traditional risk factors that increase the risk of ischemic strokes, such as hypertension, atrial fibrillation, diabetes mellitus and cigarette smoking, are well known and are modifiable or avoidable [37]. Genetic risk factors are often considered not to be modifiable; however, knowledge of genetic risk factors can provide insights into pathophysiological pathways and targets for drug therapy [10-12]. In the general population and in patient cohorts, risk factor profiles change with increasing age [38, 39]. Ischemic stroke is the most common cerebrovascular disease, and it is often attributable to the inherited predisposition, especially when apparent at a relatively early age. Approximately 10% of ischemic strokes occur at ages under 45 years [40]. Ischemic stroke is more frequent in women aged 20–30 years and in men older than 35 years [41].

Although many of our patients were middle-aged or elderly (see Table 1), it is clear that in our patient group, the number of elderly participants exceeded the number of middle-aged and younger patients. However, further research would provide clear evidence of the impact of gene polymorphisms and their role in the aetiology of ischemic stroke as the combined effects of multiple susceptibility genes. Heritability seems to have a major role in younger stroke patients and patients with a family history of stroke [42-48].

Inconsistent results obtained from various authors highlight the genetic role among different ethnic groups [49, 50]. In Macedonia [51], *FV Leiden* is more prevalent in Macedonians (6.9%) than in Albanians (2.9%), whereas the prevalence of *FV Leiden* was higher in Kosovo (3.4%) [52] than in Albania (1.8%) [53].

The present study shows that the *PTH G20210A* polymorphism is not important in the case of ischemic stroke, similarly to Erten et al. [54].

Two meta-analyses, one from individuals of European descent [55] and another from those of non-European descent [48] report an association with a modest effect for common variants in the genes for coagulation *factor V, MTHFR, PTH* and *ACE*.

The *MTHFR C677T* mutation has been linked to an increased risk for ischemic medical events in a recent population study [56]. Studies of some populations with certain ethnic backgrounds from Asian countries, North America and Europe [57-60] and from the Eastern Mediterranean region found that the prevalence of the *MTHFR C677T* polymorphism in its homozygous state is variable [61-63].

An elevated total homocysteine (tHcy) level in plasma (hyperhomocysteinemia) is the consequence of decreased activity of *MTHFR* and is associated with a slight increase in the risk of thrombosis, whereas the common *C677T* polymorphism in *MTHFR* has been associated with an increased risk of the development of different cardiovascular diseases [64], including ischemic stroke.

The population frequencies of *MTHFR C677T* homozygotes vary in the United States from 44.6% in Caucasians to 42% of Hispanics and 25.6% African-Americans [65]. The frequency of the *MTHFR C677T* genotype is often reported to be high in European, Asian and Central and South American (10%–32%) populations and low in different African populations (0–3%) and also to show geographical gradients among Chinese Han populations [66]. The frequency of the *MTHFR C677T* genotype is variable in different geographic and ethnic groups: 28%–32.8% in Indian [67-69] and 12.3% in Chinese populations [70].

The prevalence of a homozygous gene mutation in *MTHFR C677T* was high in Turkish stroke patients [71]. In a study in Tamilians [72] the *CT* genotype was seen in 18.1% and the *TT* genotype in 1.38%, whereas the *MTHFR A1298C* polymorphism was more prevalent (47.2%) in a Hungarian study [73].

Previously published studies failed to find an association between *MTHFR* (*MTHFR-677* and *MTHFR-1298*) genotypes and haplotypes and plasma homocysteine levels in patients with occlusive artery disease and deep venous thrombosis [74] or between *MTHFR* (*MTHFR-677* and *MTHFR-1298*) [75] and *PAI-1* polymorphisms [76] and occlusive artery disease or deep venous thrombosis in Macedonians.

Previous studies in Albanians with ischemic stroke also found that elevated plasma homocysteine levels (hyperhomocysteinaemia) were associated with the presence of *MTHFR* (*MTHFR-677* and *MTHFR-1298*) gene polymorphisms [28, 30]. In addition, meat with a high fat content and hyperhomocysteinemia are not the only causes of acute FIS in the Republic of

Macedonia [77]. Moreover, *MTHFR-677* and *MTHFR-1298* gene polymorphisms were not associated with occurrence rates of FIS [28, 30], but there is no data on the Albanian population regarding the PAI-1 gene and other susceptibility genes that are involved in the development and progression of ischemic stroke and their possible association with ischemic stroke.

Pezzini et al. [78] showed that the risk of ischemic stroke increased 3-fold with the presence of two or more mutations. Co-occurrence of homozygous *MTHFR TT* and *ACE D/D* genotypes yielded a highly significant moderate risk of leukoaraiosis (ischemic white matter demyelination). A synergistic effect of the *MTHFR TT* and *ACE D/D* genotypes and drinking or smoking has been found [79].

Our results demonstrate that the *Factor XIII Val34Leu* polymorphism influences the occurrence of acute FIS, in agreement with one study [12] and in contrast to another [13]. In the recent study was concluded that significant association of *Factor V Leiden (G1691A), Factor R2 (A4070G),* and *Prothrombin (G20210A)* genetic polymorphism with occlusive artery disease or deep venous thrombosis in Macedonians was not found [80].

Although previous studies demonstrated that the *PAI-1 4G/4G* polymorphism might protect against ischemic stroke [14-18], our results show that the *4G/4G* genotype of the *PAI-1* gene is less frequent in patients with FIS than in controls, similarly to Endler et al. [17].

We observed a difference in the frequency of the *ACE I/I, I/D* and *D/D* polymorphisms between stroke patients and controls, confirming that homozygosity for the *ACE D/D* polymorphism does not confer a higher risk of ischemic stroke [54] than the *I/D* and *I/I* genotypes. Conversely, other studies reported that homozygosity for the *ACE D/D* polymorphism is a strong risk factor for ischemic stroke [24, 25]. Several studies have reported that the *HPA-1b* polymorphism is associated with ischemic stroke [22, 23]; however, *GP 1a (HPA5)* is correlated weakly with ischemic stroke [43]. In the present study, the frequencies of the *1a/1a, 1b/b* and *1a/1b* genotypes differed between stroke patients and controls, indicating that the *1b/b* genotype is not a risk factor for ischemic stroke.

Results of the present study demonstrated that the frequency of the *APOE, ε2/ε3/ε43* polymorphisms were not significantly different between stroke patients and controls and support the notion that *APOE, E3/E3* polymorphisms are not associated with ischemic stroke [20, 54]. Excluding *FXIII Leu34* carriers and homozygotes (*GG*) for the factor *EPCR G4678C* polymorphism, the preliminary results of genetic analysis in the present study did not find any significant positive (susceptible) association between the investigated gene polymorphisms and FIS (Pearson's P value > 0.05) in this cohort of Albanians from Macedonia.

The small number of participants in each group limited our power of analysis. Consequently, the genotype distribution of the investigated single-susceptibility polymorphisms was under-represented or not present, particularly in the patient group. On the other hand, the very weak statistical findings may suggest that if we had a large number in each group, we could find much stronger associations and differences that we missed in this small study population. However, in the control group, the single susceptibility polymorphisms were present, which means that differences between the tested groups are probably due to ethnic differences rather than recruitment bias. There are possibilities that some negative findings might be a consequence of low statistical power. Such results have previously been reported in studies with certain patient groups that were also without genotype distribution of the investigated single- susceptibility polymorphisms. Larger samples may be needed in order to resolve these limitations, validate our results and confirm our conclusions, which are our next goals. In this case-control cohort with FIS, in which many of our patients were middle-aged or elderly, the single susceptibility polymorphisms with FIS had the lowest prevalence. These data emphasise the need for primary and secondary prevention measures in middle-aged and elderly populations targeting modifiable lifestyle vascular risk factors.

Although previous studies have identified susceptibility genes that are involved in the development and progression of ischemic stroke [6, 41, 48, 55, 78], the present study showed that the examination of individual variants of *FXIII Leu34* and homozygotes (*GG*) for the factor *EPCR G4678C* polymorphism could affect the occurrence of ischemic stroke. Other analysed single gene polymorphisms in this study do not represent risk factors in the aetiology of ischemic stroke in patients. However, some results indicate that the *ACE I/D, eNOS-G894T* heterozygous and *MTHFR C677T CT* genotype combination may result in a significantly higher risk of FIS in this Albanian population from Macedonia.

This was the first comprehensive study in an Albanian population from the Republic of Macedonia to indicate polymorphic variation in the prevalence of acute FIS. Mutations in these genes in the percentage of patients who have acute FIS have been observed, suggesting that their combination might act in a synergistic and cumulative manner in this Albanian population, the study of which is our next goal and should be tested in groups of different ethnic origin.

In summary, the results of mutant genotype frequency analysis in a sample of Albanians from the Tetovo region can be used for characterization of the current genetic profile of Albanians, anthropological comparisons, and association studies with different diseases.

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Basic Science

Effect of Pseudocereal-Based Breakfast Meals on the First and Second Meal Glucose Tolerance in Healthy and Diabetic Subjects

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Keywords: Quinoa; buckwheat; white wheat bread (WWB); incremental area under the curve (IAUC); plasma glucose response; glycemic index (GI).

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Abstract

BACKGROUND: Many studies have indicated that the incidence of serious diabetic complications may be reduced through strict glycemic control. A low glycemic index diet is one tool to improve insulin resistance and improve glycemic control in type 2 diabetes mellitus (T2DM).

AIM: The objective was to study the effect of pseudocereals-based breakfasts (quinoa and buckwheat) on glucose variations at first meal (breakfast) and second meal (standardised lunch) in healthy and diabetic subjects.

SUBJECTS AND METHODS: Twelve healthy subjects and 12 patients with Type 2 DM (not- insulin dependent) were recruited in the study. Subjects were provided with quinoa and buckwheat breakfast meals. A standardised lunch was provided 4 h after breakfast. Postprandial blood glucose response after breakfast and the second meal effect was measured in healthy and diabetic subjects. Incremental area under the curve (IAUC) values for glucose was measured in response to the breakfast and lunch. The glycemic index of the 2 pseudocereals-based test breakfasts was determined. A white wheat bread (WWB) was served as a reference breakfast meal.

RESULTS: In post-breakfast analyses, healthy subjects showed that buckwheat meal had significantly lower IAUC values for blood glucose compared to WWB reference meal ($P < 0.001$) while quinoa meal showed no significance. In diabetic subjects, buckwheat and quinoa meals had significantly lower IAUC values for blood glucose compared to WWB reference meal ($P < 0.001$ and $P < 0.05$ respectively). Blood glucose concentrations started to decline gradually for the quinoa and buckwheat but not for WWB in all healthy and diabetic subjects and returned to near-fasting baseline levels by 210 min. Post-lunch analyses indicated higher IAUC for the two breakfast types in healthy and diabetic subjects. In addition, the quinoa and buckwheat breakfast meals were followed by a significantly flatter blood glucose response to the second meal for the period between 270 and 330 min. At the end of the second meal period, values were below or near-fasting baseline levels in the breakfast period. The blood glucose concentration after consuming quinoa meal showed a high peak at 30 min similar to that of WWB reference meal. This peak resulted in a high glycemic index (GI) for quinoa (89.4). The GI of buckwheat recorded a low value (26.8).

CONCLUSION: The two studied pseudocereals; quinoa and buckwheat have high potential to improve glucose tolerance at the first and second meal (lunch) and are recommended to be introduced in our daily diet for healthy and diabetic subjects.

Introduction

Diabetes mellitus is a progressive chronic disease associated with microvascular and macrovascular complications [1]. Postprandial hyperglycemia is a major risk factor for cardiovascular disease and increased cardiovascular morbidity and mortality in diabetic subjects [2]. Many studies have stated the incidence of serious diabetic complications may be reduced through strict glycemic control [3]. A low glycemic index diet is one tool to improve insulin resistance and improve glycemic control in type 2 diabetes mellitus (T2DM) [4].

Although the ability of certain pseudocereals to lower postprandial glycemia is reported [5], no studies have been conducted on the effect of pseudocereals on the subsequent meal. The second meal effect is the ability of grains to reduce

postprandial glycemia not only after a meal at which they were taken but also at a subsequent meal in the day. This effect is beneficial for blood glucose control in diabetic patients and also causes an unanticipated decrease in insulin demands at the subsequent meal [6].

The second meal effect occurs when the glycemic index (GI) of one meal influences the glycemic response to a second meal [7]. A low glycemic index breakfast has been shown to lower the postprandial glucose response to lunch [8]. The second meal effect is important for individuals with type 2 diabetes and for healthy individuals trying to control their glucose excursions as well. Jenkins et al. [9] determined that the Staub-Traugott effect (the second meal effect), lasted from breakfast to lunch (a 4 hour period) by showing that slow digesting carbohydrate breakfast meals improved glucose tolerance during a subsequent lunch. Nowadays, the second meal effect is beginning to be taken into consideration.

Quinoa and Buckwheat are grains which belong to the “pseudocereals”category. They are characterised by an excellent nutrient profile [10] and absence of gluten proteins (prolamines) found in commonly used cereal grains such as wheat, barley, and oats [11]. Pseudocereals are essentially starchy crops, rich in essential amino acids, fatty acids, minerals, and vitamins that make them an alternative to traditional nutrient starch based gluten-free foods. Pseudocereals are starchy seeds as cereals but they are not members of the grass family.

Buckwheat (*Fagopyrum esculentum Moench*) is denominated as ‘king of the healing grains'. It is a rich source of essential amino acids, fibres, minerals, antioxidants, folate, omega-3 fatty acids, potassium, B vitamins [12]. Buckwheat has also been suggested as a low GI grain ingredient in some designed foods [13, 14, 15].

Buckwheat grains contain numerous nutraceutical compounds as catechins, rutin, and many other polyphenols [16]. These functional components are shown to influence positively on the variations in blood glucose levels, on hypertension, and dyslipidemia [17]. Moreover, buckwheat grains are a rich source of soluble and total dietary fibre dietary fibre and are useful in lowering the incidence of obesity and diabetes [18].

Quinoa (*Chenopodium quinoa Willd*) is an ancient seed cultivated in the Andean region [19]. It is characterised by its high nutritional properties [20]. Quinoa seeds are rich in proteins having a well-balanced content of essential amino acids when compared to those of common cereals [21]. Quinoa contains significant amounts of phytochemicals including saponins, flavonoids, phytosterols, phenolic acids, fat-soluble vitamins, fatty acids, trace elements and minerals which have many advantageous biochemical effects [22]. Studies on the hypoglycemic effects of Quinoa in vivo are rare. A previous study recommended quinoa to be used as an alternative to commonly used grains in the production of cereal-based gluten-free products with a low GI [23].

The aim of this study was to determine the postprandial glycemic response after consuming breakfast meal containing quinoa or buckwheat as compared to the white wheat bread reference meal in healthy and diabetic subjects. In addition, the effect of these breakfast meals on postprandial glycemic response after a second meal (lunch) was studied and glycemic indexes of the previously mentioned grains were calculated.

Subjects and Methods

Subjects

During this randomised prospective study, healthy and diabetic subjects consumed three breakfast and lunch meal combinations for a period of three weeks. Twelve healthy subjects and 12 patients with Type 2 DM (non-insulin dependent) were recruited in the study. The 12 healthy subjects were six women and six men with a mean age of 30 years (range, 22–40) and normal body mass index (mean BMI of 21.6 ± 1.5 kg/m^2). The 12 patients with Type 2 DM were seven women and five men with a mean age of 55 years (range, 40–68) and body mass index of 24 ± 1.6 kg/m^2. Their mean duration of diabetes was 10 years. Before the start of the experiment, fasting blood glucose of diabetic subjects was monitored for a minimum period of 6 months. The average value of fasting blood glucose was 126 mg/dL. Patients controlled their diabetes by taking oral hypoglycemic agents beside their normal daily diet; they have not been diagnosed with other chronic diseases and were taking breakfast regularly. On the day of the experiment, patients consumed tested and reference breakfasts and lunch with no oral hypoglycemic agents. The volunteers were enrolled for this study in the National Research Centre (NRC), Cairo, Egypt, after a written informed consent was obtained from each of them. Participants were non-smokers and were not exercising on a regular basis.

Methods

The effect of two pseudocereals-based test breakfasts and white wheat bread (WWB) served as the reference meal was evaluated. Blood glucose response after breakfast and after the second meal was measured in healthy and diabetic subjects.

Subjects were provided with the test breakfast meals. Two of the meals were composed primarily of

two pseudocereals either quinoa or buckwheat; the third meal was the reference white wheat bread WWB. The food items contained in the breakfast control and test meals are provided in Table 1. Quinoa seeds were bought from our local market; while buckwheat seeds imported from Russia, were purchased from Dubai market, United Arab of Emirates. The two tested boiled pseudocereals (quinoa; buckwheat) and the WWB reference breakfast meals were served with butter and cheese to balance the fat and protein contents of the meals. In addition, 100 mL milk (3% fat), 150 mL water and 150 mL tea were provided with each meal. The size of all meals corresponded to 50 g available carbohydrates, 20 g protein, and 9.6 g fat and provided 1533 kJ.

Table 1: Composition of the breakfast meals

Breakfast	WWB	Quinoa	Buckwheat	Cottage cheese	Butter	Milk	Water	Tea
	g	*g*	*g*	*g*	*g*	*mL*	*mL*	*mL*
WWB meal	125	-	-	40	7	100	150	150
Quinoa meal	-	80	-	50	1.5	100	150	150
Buckwheatmeal	-	-	67	75	5	100	150	150

WWB, white wheat bread (reference meal).

Blood glucose concentration readings were taken every 30 min using Accu-Check Active meter (Roche Diagnostics GmbH, Germany) [24].

White wheat bread WWB

WWB made from 100% refined wheat flour (extraction 72%) was used as the reference food. The bread was made from 300 g white-wheat flour, 200 ml. water, 3 g dry yeast and 3 g salt. Each dough was proofed for 60 minutes to allow fermentation, then flattened and cut into round pieces of 15 cm diameter and 0.2 cm thickness, and a second proofing was made for 30 minutes. WWB was baked in an oven at 250° C. WWB was served as the reference meal.

Second-meal study

Four hours after consuming the test and reference breakfast meals, healthy and diabetic subjects were served a second standardised high-GI meal. This meal consisted of 100 g deep- fried meatballs, 250 g mashed potatoes, and 60 g canned sweet corn (El Tahya, Cairo, Egypt). Lunch meal provided 2384.88 kJ. In addition, 250 mL water was served with each meal. Both breakfast and lunch meals provided approximately 45% of a total daily energy intake.

Estimation of blood glucose response after breakfast and second-meal

Subjects were requested to fast overnight for 10–12 hours. Capillary blood sample was obtained by a finger prick using a monoejector Lancet device (Accu-ChekSoftclix). Blood samples were taken before breakfast meals to determine fasting blood glucose concentrations (0 min). Postprandial blood samples were taken at 30, 60, 90, 120, 150, 180 and 210 min after the breakfast. In addition, blood samples were taken for glucose determination immediately before the second meal (240 min, ie, 4 h after breakfast considered as 0 times for the second meal) and at 270, 300, 330, 360 and 390 min post-lunch. The first drop of blood was placed onto the strip and readings were taken (within 5-10 sec) and recorded.

Calculation of Glycemic index (GI)

The GIs of quinoa and buckwheat were determined after the test meals had been served to healthy subjects. The WWB reference meal and the test meals were taken by the subjects in random order after an overnight fast and were separated by a washout period for the duration of one week. The meals were served at the same time in the morning and subjects were asked to consume all meals within 10-12 min.

The Incremental areas under curves (IAUC) of test meals and reference meal of each individual were calculated. The GI for the test meals was then calculated by dividing the value of glucose IAUC of the test meal by that of the reference meal for the same individual ignoring any area beneath the baseline [25] multiplied by 100 as shown in the following equation:

$$\text{Glycemic index} = \frac{\text{IAUC of test meal}}{\text{IAUC of reference meal}} \times 100$$

Statistical analysis

Differences between fasting and postprandial glucose concentrations were determined and incremental area under the curve (IAUC) calculations were completed using the AutoCAD program. The IAUC for blood glucose was calculated between 0–60, 0–120, 0–180 and 0–210 minutes postprandial for all participants for breakfast, and 240-300, 240-360, 240–390 min for lunch. Paired t- test were used to identify differences between quinoa, buckwheat, and the WWB reference meal. *P*-Value < 0.05 indicated a statistically significant difference for all tests. All continuous variable data are reported as the mean ± standard error. Statistical Package for the Social Sciences software (SPSS) for windows (SPSS Inc., Chicago, IL, version 17.0) was used for the statistical analysis.

Results

First meal responses in healthy and diabetic subjects

Glucose tolerance curves:

The blood glucose tolerance curves are shown in (Fig. 1 & 2) and (Fig. 3 & 4) for healthy and diabetic subjects after consumption of three breakfast meals. By comparison with the WWB reference meal, the blood glucose response curves and the peak rise of blood glucose were significantly reduced after consumption of buckwheat meal ($p < 0.001$). No significant differences were observed in the peak rise of blood glucose after consumption of quinoa meal as compared to the WWB reference meal. In healthy subjects, there was a quick rise in blood glucose concentrations (mean ± SD) and peaked at 30 min for the WWB reference, quinoa and buckwheat meals (152 ± 18.5 mg/dL, 153 ± 16.6 mg/dL, 124 ± 13.1 mg/dL respectively).

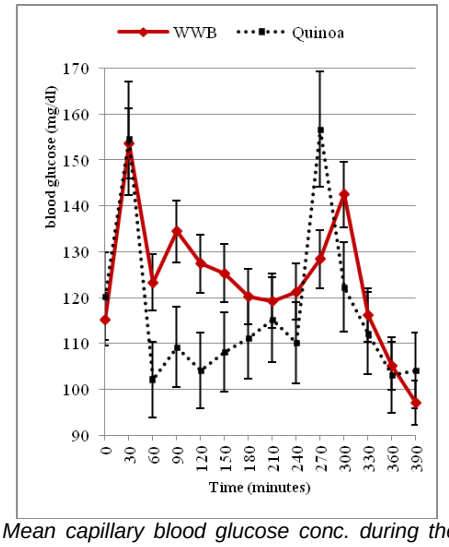

Figure 1: Mean capillary blood glucose conc. during the first and second meal following ingestion of WWB or Quinoa breakfast in healthy subjects

Otherwise, in diabetic subjects, blood glucose concentration peaked at 90 min (263 ± 14.8 mg/dL) for the WWB reference meal, while that of quinoa and buckwheat meal peaked at 60 min (264 ± 24.6 mg/dL) and at 120 min (174 ± 13.5 mg/dL) respectively. Blood glucose concentrations started to decline gradually in all healthy and diabetic subjects and returned to near-fasting baseline levels by 210 min (Fig. 1 & 2, 3 & 4). In contrast, diabetic subjects consuming the WWB reference meal had higher blood glucose concentrations at 210 min (174 ± 9.8 mg/dL) than that of the baseline level (112 ± 6.3 mg/dL).

Glucose incremental area under the curve (IAUC)

The first and second meal glucose IAUC of the reference and the two test meals are shown in Table 2.The percent change is given by the following formula:

$$\text{Percent change} = (\text{Reference mean} - \text{Test mean}) \div \text{Reference meal} \times 100$$

In healthy subjects, the greatest reduction in the breakfast periods glucose IAUC at 0–60 min, 0–120 min, 0–180 min and 0–210 min was achieved with buckwheat meal which recorded 80.8%, 73.2%, 74.5% and 76.2% respectively lower values than that after the WWB reference meal ($p<0.001$).

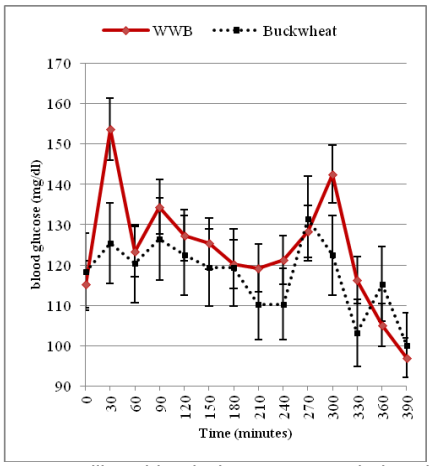

Figure 2: Mean capillary blood glucose conc. during the first and second meal following ingestion of WWB or Buckwheat breakfast in healthy subjects

The breakfast glucose IAUC of quinoa meal was not significantly different from the WWB reference meal, although it was reduced at 0–120 min, 0–180 min and 0–210 min by 9.9%, 14.0% and 5.4% respectively. However, at 0–60 min, the breakfast glucose IAUC of quinoa meal was 29.3% greater than that produced by the WWB reference meal and the difference was statistically significant ($p < 0.05$).

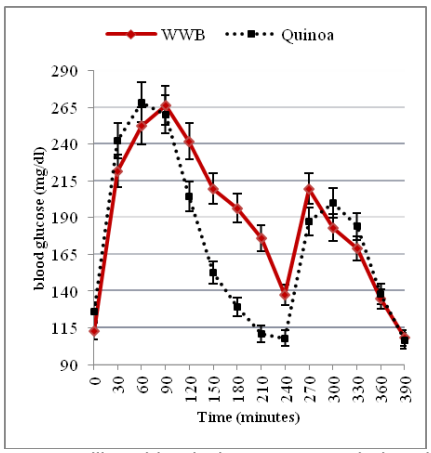

Figure 3: Mean capillary blood glucose conc. during the first and second meal following ingestion of WWB or Quinoa breakfast in diabetic subjects

In diabetic subjects, the greatest reduction in the breakfast periods glucose IAUC at 0–60 min, 0–

120 min, 0–180 min and 0–210 min was achieved with buckwheat meal which recorded 46.2 %, 48.6%, 50.8% and 53.4% respectively lower values than that after the WWB reference meal ($p < 0.001$).

No significant differences were observed in the breakfast glucose IAUC of quinoa meal at 0–60 and at 0–120 min that was reduced by 1% and 5.3%, while at 0–180 min and 0–210 min, reduction were 25.0% and 31.7% respectively lower values than that after the WWB reference meal ($p < 0.05$).

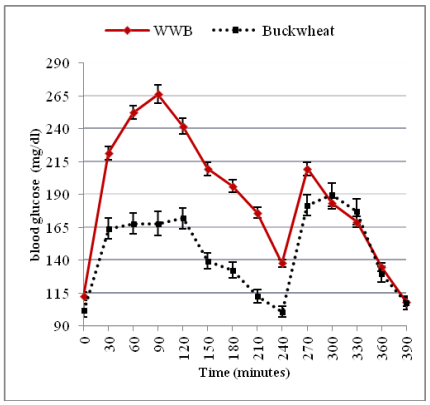

Figure 4: Mean capillary blood glucose concentration during the first and second meal following ingestion of WWB or Buckwheat breakfast in diabetic subjects

Second meal responses in healthy and diabetic subjects

Glucose tolerance curves:

Blood glucose concentrations determined immediately before and after the second meal are shown in (Fig.1 & 2) and in (Fig. 3 & 4) for healthy and diabetic subjects. From these figures, it is shown that the blood glucose concentrations just before the second meal (at 240 min) were significantly lower after the quinoa and buckwheat breakfast meals than after the WWB reference meal in healthy and diabetic subjects. These concentrations were below the breakfast fasting baseline values.

There was a quick rise in blood glucose concentrations that peaked at 270 min for the quinoa and buckwheat meals (155 ± 18.7 mg/dL and 130 ± 9.5 mg/dL) in healthy subjects after lunch. Otherwise, in diabetic subjects, blood glucose concentrations peaked at 300 min (198 ± 16.8 mg/dL and 192 ± 14.9 mg/dL). Glucose values during the second meal period were fairly flat for quinoa and buckwheat breakfast meals between 270 and 330 min (ie. 30-90 min. after consumption of the lunch).

At the end of the second meal period, values were below or near-fasting baseline levels in the breakfast period (Fig. 1-4) in healthy and diabetic subjects.

Glucose incremental area under the curve (IAUC)

The second meal IAUC calculations began at 240 min. In healthy subjects, the second meal glucose IAUC at 240–300 min, 240–360 min and 240–390 min that followed the buckwheat breakfast meal recorded higher values than that after the WWB reference meal by 53.5% ($p < 0.05$) for the first period of time, while it showed no significant difference for the other periods of time (17.5% and 18.7% respectively). High significant differences were observed in the second-meal glucose IAUC of quinoa breakfast meal at 240–300 min, 240–360 min, 240–390 min, and values were 197.2%, 126.0% and 127.9% respectively greater than that after the WWB reference meal ($p < 0.001$) (Table 2).

Table 2: Postprandial blood glucose incremental area under the curve (IAUCs) values after the reference white wheat bread, quinoa and buckwheat breakfast meals and the following second meal in healthy and diabetic subjects (n = 12)

		WWB	QUINOA	BUCKWHEAT		
		Mean ± SE	Mean ± SE	Mean ± SE	*P - value	
	Time	I	II	III	a	b
HEALTHY	0-60 min	1257 ± 14	1625 ± 28	241 ± 5	<0.05	<0.001
	0-120 min	2123 ± 37	1912 ± 43	569 ± 10	NS	<0.001
	0-180 min	2681 ± 46	2307 ± 39	683 ± 12	NS	<0.001
	0-210 min	2857 ± 50	2703 ± 47	679 ± 15	NS	<0.001
	240-300 min	527 ± 12	1566 ± 35	809 ± 14	<0.001	<0.05
	240-360 min	789 ± 14	1783 ± 40	927 ± 21	<0.001	NS
	240-390 min	791 ± 25	1803 ± 31	939 ± 16	<0.001	NS
DIABETICS	0-60 min	5373 ± 157	5423 ± 93	2891 ± 49	NS	<0.001
	0-120 min	13613 ± 176	12892 ± 225	7000 ± 157	NS	<0.001
	0-180 min	19864 ± 208	14889 ± 333	9764 ± 171	<0.05	<0.001
	0-210 min	22021 ± 230	15041 ± 263	10262 ± 175	<0.05	<0.001
	240-300 min	2801 ± 29	3715 ± 63	3857 ± 67	<0.05	<0.05
	240-360 min	4363 ± 46	7978 ± 178	7445 ± 127	<0.05	<0.05
	240-390 min	4363 ± 46	8519 ± 149	8023 ± 179	<0.05	<0.05

A = I vsII, b = I vs III. *P < 0.05 (significant), P < 0.001 (highly significant), p > 0.05 (not significant).

On the other hand, in diabetic subjects, the second meal glucose IAUC values at 240–300 min, 240–360 min, 240–390 min after the buckwheat and quinoa breakfast meal produced both a significantly higher glucose area than that after the WWB reference meal (by 37.7%, 70.6% and 83.9% for the former; and 32.6%, 82.9% and 95.3% for the later ($p < 0.05$) (Table 2).

Glycemic indexes of the test breakfast meals

The GIs of all the test breakfast meals are given in Table 3. The GI of the buckwheat meal (GI: 26.8) was significantly lower than that of the WWB reference meal (GI:100) ($p < 0.001$). The GI for the quinoa meal (GI:89.4) was not significantly different from that of the WWB meal (GI:100).

Table 3: Glycemic Index of the reference and test meals (per 50 g carbohydrates portion)

Breakfast meals	Glycemic Index (Mean ± SE)
WWB	100.0^{a} ± 2.0
Quinoa	89.4^{b} ± 1.9
Buckwheat	26.8^{c} ± 1.5

Values in the same column with different superscript letters are significantly different ($P < 0.05$).

Discussion

In this study, the effect of quinoa and buckwheat meals on glycemic response of healthy and diabetic subjects has been investigated. This study was designed to test the first meal response of a pseudocereal breakfast meal containing either quinoa or buckwheat and to determine their residual effects after the second meal of high glycemic index lunch. The goal of this study was to maintain blood glucose levels close to normal baseline as possible without risking hypoglycemia.

After the first meal, postprandial glucose excursions and IAUC values were lower for the quinoa meal compared to the control WWB reference meal, while the lowest postprandial glucose variations and IAUC were observed for the buckwheat meal. Previous studies have similarly reported that buckwheat significantly lowered plasma glucose responses as compared with the refined wheat flour bread [26].

For diabetics, the blood glucose excursions' recovery periods returning to the baseline level are longer than those for healthy subjects [27]. In other words, diabetic subjects had a slower rate of glucose clearance in the blood, while healthy subjects had better blood glucose clearance following ingestion of meals. This concept is consistent with the present results showing that concentrations of blood glucose after consuming quinoa and buckwheat meal breakfasts steadily began to decline to below baseline levels by 120 min in healthy and by 210 min in diabetic subjects (Fig. 1-3). Meanwhile, concentrations of blood glucose after consuming WWB reference meal began to decline, and returned to a value slightly higher than baseline level in healthy subjects, while in diabetic subjects, a high blood glucose response was recorded and blood glucose failed to return to the baseline value by 210 min (Fig. 1-3). Postprandial glycemia for type 2 diabetes vary widely depending on the type and the amount of carbohydrate consumed [28].

In this study, peak blood glucose response after consuming buckwheat meal was lower than that of quinoa and WWB reference meal in both healthy and diabetic subjects (Fig. 2-4). It must be noted that in diabetic subjects, blood glucose peak was observed at 60 min (264 mg/dl) and at 90 min (263 mg/dl) after the consumption of quinoa and WWB reference meal respectively. The buckwheat meal resulted in a lower blood glucose peak at 120 min (174 mg/dl) than did the quinoa, or WWB reference meal. These results may be attributed to the variability in two polysaccharides: amylose and amylopectin of quinoa, wheat and buckwheat. Information in the literature suggests that considerable variability exists in the amylose content of quinoa (3% - 20%) [29] which is considered to be lower than in cereals as wheat (20%-30%) [30]. Furthermore, amylose content of buckwheat starch granules recorded the highest values and fluctuates between 15% and 52% and has higher resistant starch content [31].

Several studies have shown that the amylose content was negatively correlated with the onset of gelatinization [32]; the degree of gelatinization is greater when the amylose content is low and vice-versa. This phenomenon leads to that amylose will be more susceptible to be hydrolyzed by alpha-amylase (starch digestive enzymes) and become glucose resulting in an increase in blood sugar levels [33]. In other words, starches with low amylose level have higher glycemic indexes [34]. Inversely, starches with a higher amylose content, as in buckwheat, will be less susceptible to gelatinization, amylose retrogrades more rapidly than amylopectin after cooling, and forms an amylase-resistant crystal structure (resistant starch) leading to reduction in the rate of digestion [35], that is slowing the breaking down into glucose, which makes the low glycemic index. Such previous studies are in accordance with our results as quinoa recorded an early postprandial blood glucose peak (60 min) and a high glycemic index 89, while buckwheat recorded postprandial blood glucose peak later (120 min) and a low glycemic index 26.8. In an *In vitro* study by Wolter et al [36], it was reported that quinoa bread showed highest predicted GI (95) as compared to WWB (GI = 100). Other studies made in individuals with celiac disease showed that quinoa has a glycemic index slightly lower than that of gluten-free bread and pasta [37]. All these results were found to be similar to the present study.

Despite the high GI of quinoa and early high blood glucose peak (peak at 30 min in healthy and at 60 min in diabetic subjects) [38], concentrations of blood glucose after consuming quinoa meal breakfasts steadily began to decline and returned to below baseline levels. This could be explained by the fact that quinoa contains considerably a high content of health-beneficial phytochemicals including vitamin E, iron, zinc and magnesium contents, as well as saponins, phenolics and phytosterols [39]. These bioactive compounds may attenuate carbohydrate metabolism and hyperglycemia; improve pancreatic β-cell function and insulin release [40]. Abugoch found antioxidants capacity compounds such as polyphenols, phytosterols, and flavonoids in grains of quinoa [41]. These compounds may be related to the effects of the reduction in postprandial glucose levels in the individuals tested, suggesting an improvement in insulin action and pancreatic function.

A second-meal effect in the form of a reduced glucose response to a high GI lunch was also shown after quinoa and buckwheat breakfast meal.

The results of the present study add to evidence that quinoa and buckwheat breakfast meals improve glucose tolerance at a subsequent high glycemic second meal as compared to WWB

reference meal in healthy and diabetic subjects. Before lunch, at 240 min, blood glucose concentrations for quinoa and buckwheat were below fasting baseline values than in breakfast period. In contrast, blood glucose concentrations in subjects consuming WWB reference meal recorded a value higher than the fasting baseline values in breakfast period.

Furthermore, in diabetic subjects it is clear that quinoa and buckwheat breakfast meal improved postprandial glycemia resulting in a flattened postprandial curve (270; 300; 330 min), indicating modulation in insulin response to the glucose released into blood, and prevention of rebound hypoglycemia. Consumption of quinoa meal has been shown to improve glucose tolerance at the subsequent meal. This effect may be attributed to the fact that quinoa contains Alpha-glucosidase inhibitors which act at the small intestine's brush border, inactivating the enzyme responsible for breaking down complex carbohydrates (slowing digestion and absorption of high GI meal lunch), prolonging glucose absorption, and flattening the postprandial glycemic curve [42] (low glucose peak 198, plateau during postprandial glucose at 270; 300; 330 min).

Results of the present study confirmed that buckwheat breakfast meal lowered first and second blood glucose response in healthy and diabetic subjects. In healthy subjects, buckwheat starch was so slowly digested that it failed to increase postprandial blood glucose concentrations to a measurable extent. In diabetic subjects, a relevant control of the glucose release in the first two hours was observed, resulting in a flattening of the glucose curve followed by a slow decrease of blood glucose concentration then returning to the pre-prandial level. Previous studies suggested that the delay of the absorption rate of the carbohydrate component is possibly due to the high viscous fibre content and the high ratio of amylose starch to amylopectin that induced reductions in both postprandial glucose and insulin responses [43].The high amount of resistant starch content in buckwheat has been identified as a key contributing factor to its low GI characteristics. The amylase enzyme is unable to break down this form of starch, therefore it passes undigested throughout the body and may be used as a nutrient source for gut microflora and providing a mechanism for blood glucose control [44].

Another proposed mechanism involved in buckwheat's glucose lowering abilities is its high content of D-chiro-inositol known as fagopyritols (D-CI is a rare isomer of vitamin B8 that is naturally found in many grains). Buckwheat containing the second high concentrations next to mung beans [45]. Fagopyritols are structurally similar to a galactosamine derivative of D-chiro-inositol, a putative insulin mediator that facilitates a decrease in blood glucose concentrations [46]. Furthermore, buckwheat grain contains D-fagomine, a natural glucose analogue that is not digestible. This substance is a simple soluble fibre that controls the postprandial glycemic response by delaying the absorption of carbohydrates in the small intestine delivering these nutrients to the large intestine, improving the assimilation of the nutrients and also maintaining a healthy digestive gut system. These findings support a mechanism related to a slow rate of glucose delivery to the blood. In addition to a prolonged digestive phase is a prolonged suppression of plasma fatty acids, which has been shown to be associated with improved response to insulin action [47].

At the end of the second meal period, values were near or below fasting baseline levels as in the first meal period for all breakfast types.

This study showed that despite quinoa has a high glycemic index, blood glucose after consuming quinoa meal breakfasts steadily began to decline and return to below baseline levels. Quinoa meal also improves glucose tolerance after the second meal. The low-glycemic index breakfast meal containing buckwheat has a remarkable impact on glycemic control as it improves glucose tolerance after the first and second meal in healthy and diabetic subjects. Both pseudocereals quinoa and buckwheat improve glycemic control after the second meal presented by the flattened glucose curve (270; 300; 330 min), and glucose levels returned to near or below fasting baseline levels as in the first meal period preventing rebound hypoglycemia.

In summary, quinoa and buckwheat pseudocereals could be considered as a new source of specific foods with potential health benefits to improve first and second-meal glucose excursions in healthy and diabetic subjects. In addition, it should be noted that despite the fact that quinoa can help to improve the management of Type 2 DM as it shows sustainable blood glucose response after the first and second meal, it must be consumed in a measured amount as blood glucose level peaked early at 60 min in diabetic subjects. This phenomenon must be considered and needs more investigations.

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Assessment of the *−174G/C* (rs1800795) and *−572G/C* (rs1800796) *Interleukin 6* Gene Polymorphisms in Egyptian Patients with Rheumatoid Arthritis

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Keywords: Rheumatoid arthritis; Interleukin 6; *IL6–174G/C*, *IL6–572G/C*; gene polymorphism.

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Abstract

AIM: This study aimed to investigate genotype and allele frequencies of −174 (rs1800795) and *−572* (rs1800796) *IL-6* promoter gene polymorphisms in Egyptian patients with rheumatoid arthritis (RA) in comparison to control group.

METHODS: The study was conducted on 198 Egyptian subjects (99 RA patients and 99 healthy control).The promoter region of *the IL-6* gene was amplified by PCR using DNAs from patients and the controls, and their PCR products were digested by suitable enzymes.

RESULTS: No statistical differences were found in *−572G/C* genotype (P = 0.177) or allele (P = 0.147) frequencies between RA patients and controls. Significant differences were observed in *−174G/C* genotype (P < 0.001) and allele (P < 0.001) frequencies between RA patients and controls.

CONCLUSION: A significant association of *IL-6 −174G/C* gene polymorphism and RA in Egyptian population was found with significantly higher frequencies of *GC* and *CC* genotypes and *C* allele in RA patients compared to controls. No association was found between *IL-6 −572G/C* gene polymorphism and RA.

Introduction

Rheumatoid arthritis (RA) is a multisystemic autoimmune inflammatory disease which has the prevalence of approximately 1% worldwide [1]. The interaction of genetic and environmental factors results in a cascade of immune reactions, which contributed to the pathogenesis of RA. The common manifestations of RA include synovitis, joint damage, and structural cartilage and bone damage. Also, uncontrolled active RA causes disability, diminished quality of life and number of extra-articular manifestations and comorbidities especially cardiovascular diseases which result in increased mortality [1, 2]. The aetiology and pathogenesis of RA are not clearly defined. However, some genetic factors were found to be contributed to RA susceptibility. *HLA-DR* loci were the most associated gene with RA susceptibility, although *HLA-DR* accounts only for approximately one-third of the genetic predisposition to RA [3]. Other genes also contributed to the genetic susceptibility to RA are cytokine genes such as TNFα, IL-1β and IL-6 genes which account for the relatively small additional role in RA genetic predisposition [3].

Interleukin 6 (IL-6) is one of the most studied pro-inflammatory cytokines that have the important role in the pathogenesis of RA. Serum and synovial fluid levels of IL-6 and soluble IL-6 receptors (sIL6R) were found to be elevated in patients with RA [4]. IL-6 promotes the production of autoantibodies such as Rheumatoid Factor (RF) and Anti-Citrullinated Peptide Antibody (ACPA). In addition, it triggers the imbalance between Th17 cells and regulatory T cells (Treg). It also has a role in promoting synovial inflammation and cartilage and bone destruction as well as extra-articular manifestation including cardiovascular, psychological and skeletal disorders [5]. Blockade of IL-6 activity with a soluble anti-IL-6 molecule tocilizumab has been found to decrease the disease activity as well as the radiological progression of RA [6].

The human *IL-6* gene is located on chromosome 7p21. Among the polymorphic sites described in the *IL-6* gene promoter, there are two biallelic polymorphisms that may be associated with differences in cytokine production: *−174G/C* (rs1800795) and *−572G/C* (rs1800796). These polymorphisms consist of a single nucleotide change from guanine (G) to cytosine (C) at positions −174 and −572 in the promoter region, respectively [7]. *IL-6* promoter polymorphisms have been associated with susceptibility to RA, however, conflicting results were observed in different populations [8, 9].

In the present study, we aimed to investigate genotype and allele frequencies of − 174 and −572 IL-6 promoter gene polymorphisms in Egyptian patients with rheumatoid arthritis in comparison to control group.

Subjects and Methods

Study design

This study was conducted on 99 Egyptian rheumatoid arthritis patients recruited from Internal Medicine and Rheumatology Clinic and Department of National Research Centre. The control group included 99 healthy Egyptian subjects with no family histories of any autoimmune diseases. The RA patients were diagnosed according to American College of Rheumatology (ACR) criteria [10]. Full medical history was taken and thorough clinical examinations were performed for all patients. Laboratory investigations including erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), rheumatoid factor (RF) and anti-cyclic citrullinated peptide (anti-CCP) were performed. Also, Disease Activity Score (DAS) and Larsen score were calculated [11, 12]. Written informed consents were taken from patients and controls, and the study was approved by the National Research Centre ethics committee.

Methods

Blood collection and molecular analyses

Venous blood samples were collected in EDTA tubes and stored at −80 °C, till DNA extraction. Genomic DNA extraction from white blood cells was carried out by QIA gene extraction Kit.

Determination of IL-6 gene polymorphisms

The final volume of PCR reaction mixture was 25µl containing 40 ng genomic DNA, 10 picomoles each of forward and reverse primers at the concentration of 1X, 1X PCR master mixture.

To identify (−174) G/C polymorphism, the forward and the reverse primers were (5′-TTGTCAAGACATGCCAAGTGCT-3′) and (5′-GCCTCAGAGACATCTCCAGTCC-3′) and for (−572) G/C polymorphism, the forward and the reverse primers (5′-GGAGACGCCTTGAAGTAACTGC-3′) and (5′-GAGTTTCCTCTGACTCCATCGCAG-3′) were used. PCR amplification was carried out at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at (57°C and 55°C for 30 sec for −174G/C & −572G/C respectively), extension at 72°C for 30 sec, and final extension at 72°C for 7 min. The PCR product of −174G/C was digested by Fast digestion of 5 units of *Nla III* enzyme at 37°C yielded G allele (244 + 133 + 11) bp and C allele (133 + 111 + 56) bp products. For −572 G/C polymorphism, *MbiI* fast digestion restriction enzyme at 37°C was used yielded G allele (102, 61) bp and C allele (163) bp products. The obtained fragments' sizes were analysed on a 2% agarose gel [13].

Statistical Analysis

Data was analysed using the Statistical Program for Social Science (SPSS) for Windows, Version 16.0 Chicago, SPSS Inc., 2007. The statistical data are reported as the mean ± SE, frequencies and percentages when appropriate. Comparison between two means and more than two means was done using student t-test and ANOVA test respectively. Chi-square and Fisher's exact test were used to examine the relationship between different variables. A statistical significance was considered when $P \leq 0.05$ and Logistic regression was used for calculating the odds ratio.

Results

Comparison between RA patients and controls in selected characteristics are shown in Table 1. The female percent in RA patient was significantly higher than that in controls. The mean serum level of Anti- CCP of RA patients was significantly elevated to reach 25-fold that of controls. Also, the mean serum level of CRP of RA patients was significantly increased by 70% compared with controls. In addition, the mean serum levels of RF-IgA, IgG, and IgM were significantly elevated to reach (17-, 28-, and 34- fold, respectively) those of controls.

Table 2 show genotype and allele frequencies of *−572G/C* gene polymorphism in RA patients and controls. No statistical differences in genotype or allele frequencies between RA patients and controls were found.

Table 1: Comparison between RA patients and controls in selected characteristics

Variables	RA patients N = 99	Controls N = 99	P value
Female (%)	90 (90.9%)	58 (58.59%)	< 0.001
Anti- CCP, Mean ± SE	160.4 ± 16.47	6.52 ± 0.56	<0.001
CRP (mg/L), Mean ± SE	24.39 ± 3.31	14.17 ± 3.15	<0.05
RF IgA (IU/L), Mean ± SE	157.2 ± 29.15	9.37 ± 1.16	<0.001
RF-IgG (IU/L), Mean ± SE	354.8 ± 66.78	12.56 ± 1.45	<0.001
RF IgM (IU/L), Mean ± SE	175.6 ± 35.66	5.15 ± 0.93	<0.001

Table 3 show genotype and allele frequencies of *−174G/C* gene polymorphism in RA patients and controls. Regarding GC genotype, RA patients group showed higher frequency compared to control group (46.5% and 23.2% respectively) (P < 0.001, OR = 3.409).

Table 2: The genotype and allele frequencies of *−572G/C* polymorphism in RA patients group and controls

Genotype	RA patient N = 99 n (%)	Control N = 99 n (%)	P value	Odd ratio (95% CI)
GG	26 (26.3)	36 (36.4)		1.000 (Reference)
GC	64 (64.6)	58 (58.6)	0.177	1.528 (0.824-2.832)
CC	9 (9.1)	5 (5.1)	0.130	2.492 (0.748-8.308)
Allele				
G	116 (58.6)	130 (65.7)	0.147	1.351 (0.899-2.031)
C	82 (41.4)	68 (34.3)		

Also, there was higher frequency of CC genotype in RA group (9.1%) compared to control group (1%) (P = 0.001, OR = 15.341). In accordance to allele frequencies, C allele was found in higher frequency in RA patients group (32.3%) compared with controls (12.6%) (P < 0.001, OR = 3.305).

Table 3: The genotype and allele frequencies of *−174G/C* polymorphism in RA patients group and controls

Genotype	RA patient N = 99 n (%)	Control N = 99 n (%)	P value	Odd ratio (95% CI)
GG	44 (44.4)	75 (75.8)		1.000 (Reference)
GC	46 (46.5)	23 (23.2)	0.000	3.409(1.827-6.361)
CC	9 (9.1)	1 (1)	0.001	15.341(1.88-125.181)
Allele				
G	134 (67.7)	173 (87.4)	0.000	3.305(1.976-5.528)
C	64 (32.3)	25 (12.6)		

Discussion

Rheumatoid arthritis is a chronic inflammatory disease resulting from a cascade of immunological reactions. IL-6 is an acute phase cytokine contributes to host defence against infectious agents and tissue injuries by inducing immunological and hematopoietic responses. However, uncontrolled persistent production of IL-6 may lead to the development of several immune-mediated diseases. Thus, dysregulated persistent production of IL-6 has a crucial role in the pathogenesis of RA [14].

Regarding *IL-6 −572G/C* gene polymorphism, our study identified that the *GC* genotype is the most frequently genotype observed in both RA patients and controls and no significant differences were observed in the genotypes and alleles frequencies between RA patients group and controls (Table 2). Our results were in agreement with Zavaleta-Muñiz et al., 2013 who found no significant differences in allele or genotype frequencies of this polymorphism between RA and controls. But in contrast to our results, they found that the *GG* genotype was the most frequently observed genotype in the Mexican mestizo population among patients with RA and healthy controls (54% and 60.8% respectively) [7]. Also, Li et al., 2014a observed no association for *IL-6 −572G/C* gene polymorphism and RA in Chines Han population. And in agreement with our results, they observed that GC genotype was the most frequent genotype found in both RA patients and controls (48.8% and 45.9% respectively) [9]. Our findings also were in agreement with other studies in which they found no relationship between *IL-6 −572G/C* gene polymorphism and RA in different populations such as Taiwan and Turkish population [8, 15]. However, in contrast to our results Lo et al., 2008 found that the most frequent genotype in RA patients was *CC* genotype (60.8%) which was similar to the genotype distribution of the controls [15].

Regarding *IL-6 −174G/C* gene polymorphism, our results found that both *GC* and *CC* genotypes were associated with RA risk, and *C* allele increased the susceptibility of RA in the studied Egyptian population. Our results came in agreement with Li et al., 2014a who observed a statistically significant association for *IL-6 −174G/C* gene polymorphism and RA. They identified higher frequency of *C* allele in RA patients compared to controls (2.1% versus 0.5%) OR = 4.823 and P = 0.016 [9].

Moreover, in agreement with our findings, Li et al., 2014b identified that the frequency of *CC* genotype and *C* allele were significantly higher in RA patients compared to controls (2% versus 0.25% and 10.24% versus 0.88% respectively) (P < 0.001). Their results also showed a significantly increased risk of RA for the *CC* genotype and the *C* allele of *IL-6 −174G/C* gene polymorphism in Chines population [16].

On the other hand, our results were against different studies in which they found no differences in allele or genotype frequencies of *IL-6 −174G/C* gene polymorphism between RA and controls in a different population such as Mexican and Turkish populations [7, 8]. The controversy of results may be due to different origin or ethnicity.

IL-6 is a pro-inflammatory cytokine that has different pleiotropic activities including induction of acute phase proteins and stimulation of T and B cells, synoviocytes and osteoclasts. These inflammatory reactions result in cartilage and bone damage as well as other systemic manifestations [17]. Previous study

observed that polymorphisms in the promoter region of the *IL-6* gene may be responsible for changes in the transcriptional activity and the expression of IL-6 in serum and synovial tissue, which could, in turn, lead to greater inflammation and thus affect the clinical status of RA patients [18]. In other study, patients with the *−174G* allele showed higher rates of progression of erosive damage, although it was not statistically significant even in the presence of longer disease duration at baseline [19].

In conclusion, this study revealed a significant association of *IL-6 −174G/C* gene polymorphism and RA in Egyptian population with significantly higher frequencies of *GC* and *CC* genotypes and *C* allele in RA patients compared to controls. No association between *IL-6 −572G/C* gene polymorphism and RA was found in our cohort RA patients. Also, no correlations between different genotypes and all measured biomarkers were observed.

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Fibrosis in Chronic Hepatitis C: Correlation between Immunohistochemically-Assessed Virus Load with Steatosis and Cellular Iron Content

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Keywords: Fibrosis; HCV; HCC; NS3/NS4; VEGF; VEGFR2; FISH.

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Abstract

AIM: We aimed study impact of hepatocytic viral load, steatosis, and iron load on fibrosis in chronic hepatitis C and role of VEGF and VEGFR overexpression in cirrhotic cases in evolving HCC.

MATERIAL AND METHODS: Total of 120 cases were included from TBRI and Beaujon Hospital as chronic hepatitis C (CHC), post-hepatitis C cirrhosis, and HCC. Cases of CHC were stained for Sirius red, Prussian blue and immunohistochemically (IHC) for HCV-NS3/NS4. HCC were stained IHC for VEGF and by FISH.

RESULTS: Stage of fibrosis was significantly correlated with inflammation in CHC ($P < 0.01$). Noticed iron load did not correlate with fibrosis. Steatosis was associated with higher inflammation and fibrosis. The cellular viral load did not correlate with inflammation, steatosis or fibrosis. VEGF by IHC was significantly higher in cases of HCC when compared to cirrhotic group ($P < 0.001$). Amplification of VEGFR2 was confirmed in 40% of cases of HCC. Scoring of VEGF by IHC was the good indicator of VEGFR2 amplification by FISH ($P < 0.005$).

CONCLUSION: Grade of inflammation is the factor affecting fibrosis in CHC. The degree of liver damage is not related to cellular viral load or iron load. Steatosis is associated with higher inflammation and fibrosis. VEGF by IHC is correlated with overexpression of VEGFR2 by FISH.

Introduction

Hepatitis C virus (HCV) is a global epidemic affecting approximately 3% of the world's population. Egypt has the highest prevalence of hepatitis C virus (HCV) in the world, estimated nationally at 14.7%. Our study's objective was to delineate the evidence on the epidemiology of HCV infection among the different population groups in Egypt and to draw analytical inferences about the nature of HCV transmission in this country [1]. In the 15–59 year age groups, the prevalence of HCV antibody was found to be 10.0% (95% CI = 9.5 –10.5) and that of HCV RNA to be 7.0% (95% CI = 6.6–7.4). In children, 1–14 years old, the prevalence of HCV antibody and HCV RNA were 0.4% (95% CI = 0.3–0.5) and 0.2% (95% CI = 0.1–0.3) respectively. Approximately, 3.7 million persons have chronic HCV infection in the age group 15–59 in 2015. An estimated 29% reduction in HCV RNA prevalence has been seen since 2008, which is largely attributable to the ageing of the group infected 40–50 years ago during the mass schistosomiasis treatment campaigns. Prevention efforts may have also contributed to this decline, with an estimated 75% (95% CI = 6–45) decrease in HCV incidence in the 0–19 year age groups over the past 20 years [2].

Steatosis is a complication of HCV infection and the mechanisms of its development are complex, involving viral and host factors. Steatosis that is

prevalently viral is associated with HCV genotype 3, and steatosis that is prevalently metabolic is associated with non-3 genotypes. Viral steatosis is correlated with the level of HCV replication, whereas metabolic steatosis is related to insulin resistance. The two types of steatosis have a different impact on HCV disease and may have an additive effect [3].

It has been generally accepted that either steatosis by itself aggravates fibrosis or the factors that are causing steatosis may be aggravating fibrosis. Overall, steatosis, whether metabolic or HCV induced, worsens the sequence of events leading to advanced fibrosis in patients with HCV and needs to be addressed when managing patients with HCV [4].

Syed and Sadiq [5] have found a positive association between HCV-NS3 immunopositivity and the histological parameters of grading and staging, suggesting that greater amounts of virus are present in more advanced chronic liver disease.

Iron overload in the liver induces oxidative stress leading to cell membrane damage, DNA instability and mutagenesis [6]. Due to these effects, iron can be considered a proinflammatory, profibrogenic factor and a potential carcinogen. Since the implementation of serological diagnostic tests for HCV identification, elevated serum iron-overload indices or appearance of iron deposits in liver cells have been observed in 10-40% of patients with chronic hepatitis C and 50% of patients suffering from both chronic hepatitis C (CHC) and HCC [7].

There is evidence that iron overload leads to an increased risk of HCC, failure of antiviral treatment and significantly worsens clinical outcomes in patients suffering from chronic HCV infection [8]. Clinically, HCC tends to be hypervascular and, for that reason, transarterial chemoembolization has proven to be effective in managing many patients with localised disease. More recently, angiogenesis has been targeted effectively with pharmacologic strategies, including monoclonal antibodies against vascular endothelial growth factor (VEGF) and VEGF receptors, as well as small-molecule kinase inhibitors of the VEGF receptor [9].

The aim of this paper was to study the impact of the hepatocytic viral load, hepatocytic steatosis, and hepatic iron and copper load on fibrosis in a sample of chronic hepatitis C and to study the role of VEGF and VEGFR overexpression in a sample of cirrhotic cases in evolving HCC.

Material and Methods

The material of this study is retrieved from the archives of Pathology Departments of Theodor Bilharz Research Institute – Giza, Egypt and Beaujon Hospital – Clichy, France during the years 2013 and 2014. For each case, personal, medical and clinical data were obtained from the records of Pathology Departments of both centers and cases that match the following criteria were included: Positive HCV mRNA by PCR, no history of treatment or previous transplantation, no evidence of concomitant hepatitis B virus (HBV) infection, no evidence of parasitic liver disease, no diabetes mellitus and no malignancy other than HCC.

Ninety-four cases met the selection criteria. For each case:

A. Four-µm sections were cut from each paraffin block and stained with Hematoxylin and Eosin (H&E), Sirius red stain and Perl's Prussian blue stain.

B. Detailed histopathological assessment was done regarding:

1) the grade of inflammation and stage of fibrosis according to METAVIR scoring system [10];
2) the degree of steatosis according to the SAF algorithm [11]; and
3) the presence of ferric iron as blue or purple deposits [12] in Perl's Prussian blue-stained sections. We expressed the results of an iron load as positive or negative.

Immunohistochemical procedure

Immunohistochemistry for NS3/NS4 was performed on sections cut from the paraffin blocks with a commercially available mouse monoclonal Anti-Hepatitis C Virus NS3/NS4 antibody (ab113612, Abcam, USA). Briefly, 4 µm-thick sections are put onto positively charged slides (Superfrost plus, Menzel-Glaser, Germany) and the slides were stained by an automated platform—the Dako autostainer Link 48. Heat-induced antigen retrieval was used for 30 min at 97°C in the manufacturer's high-PH EnVision™ FLEX Target Retrieval Solution and the primary antibody was used at a dilution of 1 in 50. Sections from wedge liver biopsy obtained during open cholecystectomy operation performed to a patient with no serological evidence of HCV infection were added in each run, and the antibody was omitted to serve as negative control.

Interpretation of IHC

Immunohistochemical assessment of hepatocyte viral content was performed according to Rullier et al. (2001) [13]. Cells with powder-like brown cytoplasmic granules were considered positive and were semi-quantitated: Score 0: 0% positive cells; +1: <10% positive cells; +2: 10%-50 % positive cells; +3: >50% positive cells.

Photomicrography

Photomicrographs included in this study were captured for significant results and data by AxioCam MRc5 camera mounted to Zeiss Scope A1 Microscope (Hamburg, Germany).

FISH procedure

VEGFR2 FISH probe labelled in Orange 5-TAMRA dUTP was ordered from Empire Genomics (Buffalo, New York, USA) and preparation of slides was carried out according to their manual; *Probe details:* Gene: VEGFR2 (Orange 5-TAMRA dUTP), Loci: 4q12 and we use Spotlight FISH Tissue implementation kit (ZytoVision GmbH, Bremerhaven, Germany).

Slides were deparaffinized in Xylene for 15 min and repeated 1 more time. Then dehydrated in 100%, 100%, 90%, +80% ethanol each for 5 min. Washed 2 times, each 2 min in deionized water, Pretreatment by incubating slides with heat pretreatment solution for 15 min at 98°C, transfer slides immediately to deionized water for 2 min two times. Apply pepsin solution and incubate for 15min at 37°C. Then wash for 5 min in wash buffer SSC and 1 min in deionized water then dehydrate in 70%, 90% and 100% ethanol, each for 1 min. Air dry slides. Denaturation and Hybridization by pipetting 15 µl of VEGF probe each onto individual samples. Denature the slides at 75°C for 10min (on the hot plate), transfer the slides to a humidity chamber and hybridise overnight at 37°C in a hybridization oven. Post-hybridization and detection, remove the rubber cement by submerging in wash buffer for 3 min, and then incubate the slides in 70%, 90% and 100%ethanol. Each for1 min. Air dry the samples while protected from light. Pipette 40 µl DAPI /DuraTect Solution on to the slides.

Interpretation of FISH

We used fluorescence microscope (Olympus X51) [using 2 filters red (FITC) and DAPI] using a × 100 oil immersion objective lens; the microscope is attached to high-resolution video camera (Jale) and monitor. We captured and interpreted photos using hardware (Cytovision 2.3, USA).

Statistical analysis

SPSS software version 18 was used for data management and analysis. Quantitative data were presented as mean ± SD. Qualitative data were presented as frequencies and percentages. To study the relationship between variables, Spearman's correlation coefficient was calculated. Tests were considered statistically significant when $P< 0.05$

Results

VEGF by immunohistochemistry in HCC and Cirrhotic groups

Cases of HCC (20 cases) were tested for expression of VEGF by immunohistochemistry, against cirrhotic cases (6 cases). Immunohistochemical expression of VEGF was found in 26%-50% of malignant hepatocytes (score 2) in 11 cases of HCC, while 5 cirrhotic cases showed the immunohistochemical reaction in 51%-75% of hepatocytes (score 3).

Table 1: Activity, iron load, steatosis and HCV-NS3/NS4 IHC expression in different studied groups

	Group 1 Number (%)	Group 2 Number (%)	Group 3 Number (%)	Group 4 Number (%)	Total Number (%)	P value
Activity						
A0	2 (4.4%)	0 (0%)	0 (0%)	0 (0%)	2 (2.1%)	
A1	32 (71.1%)	7 (28%)	13 (72.2%)	2 (33.3%)	54 (57.4%)	0.7
A2	11 (24.4%)	17 (68%)	5 (27.8%)	4 (66.7%)	37 (39.4%)	
A3	0 (0%)	1 (4%)	0 (0%)	0 (0%)	1 (1.1%)	
Total	45 (100%)	25 (100%)	18 (100%)	6 (100%)	94 (100%)	
Iron						
Positive	8 (17.8%)	3 (12%)	2 (11.1%)	0 (0%)	13 (13.8%)	
Negative	37 (82.2%)	22 (88%)	16 (88.9%)	6 (100%)	81 (86.2%)	0.6
Total	45 (100%)	25 (100%)	18 (100%)	6 (100%)	94 (100%)	
Steatosis						
S0	28 (62.2%)	8 (32%)	10 (55.6%)	2 (33.3%)	48 (51.1%)	
S1	9 (20%)	11 (44%)	6 (33.3%)	2 (33.3%)	28 (29.8%)	
S2	5 (11.1%)	2 (8%)	1 (5.6%)	2 (33.3%)	10 (10.6%)	0.2
S3	3 (6.7%)	4 (16%)	1 (5.6%)	0 (0%)	8 (8.5%)	
Total	45 (100%)	(100%)	18 (100%)	6 (100%)	94 (100%)	
HCV-NS3/NS4						
Negative (0)	13 (28.9%)	9 (36%)	8 (44.4%)	1 (16.7%)	31 (33%)	
Score +1	15 (33.3%)	11 (44%)	8 (44.4%)	3 (50%)	37 (39.4%)	
Score +2	14 (31.1%)	4 (16%)	2 (11.1%)	2 (33.3%)	22 (23.4%)	0.6
Score +3	3 (6.7%)	1 (4%)	0 (0%)	0 (0%)	4 (4.3%)	
Total	45 (100%)	25 (100%)	18 (100%)	6 (100%)	94 (100%)	

However, no expression in more than 75% (score 4) of hepatocytes was found in cirrhotic cases, in contrast to 6 cases (30%) of HCC with score 4 expressions (Table 1-2, Figures 1-2).

Table 2: VEGF IHC overexpression in different grades of HCC

VEGF- IHC Score	HCC Grade Grade I Number (%)	Grade II Number (%)	Grade III Number (%)	Total	P value
VEGF (IHC)					
0 (< 5%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
1(6% - 25%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
2(26% -50%)	8 (40%)	3 (15%)	0 (0%)	11 (55%)	<0.005
3 (51% - 75%)	0 (0%)	2 (10%)	1 (5%)	3 (15%)	
4 (> 75%)	0 (0%)	1 (5%)	5 (25%)	6 (30%)	
Total	8 (100%)	6 (100%)	6 (100%)	20 (100%)	
VEGFR2 (FISH)					
Normal	8 (40%)	4 (20%)	0 (0%)	12 (60%)	<0.001
Overexpression	0 (0%)	2 (10%)	6 (30%)	8 (40%)	
Total	8 (100%)	6 (100%)	6 (100%)	20 (100%)	

Our study showed that the immunoreactivity to VEGF in HCC is correlated with the degree of tumour differentiation. All poorly differentiated HCCs showed VEGF expression in more than 50% of hepatocytes while all well-differentiated tumours showed expression in less than 50% of hepatocytes.

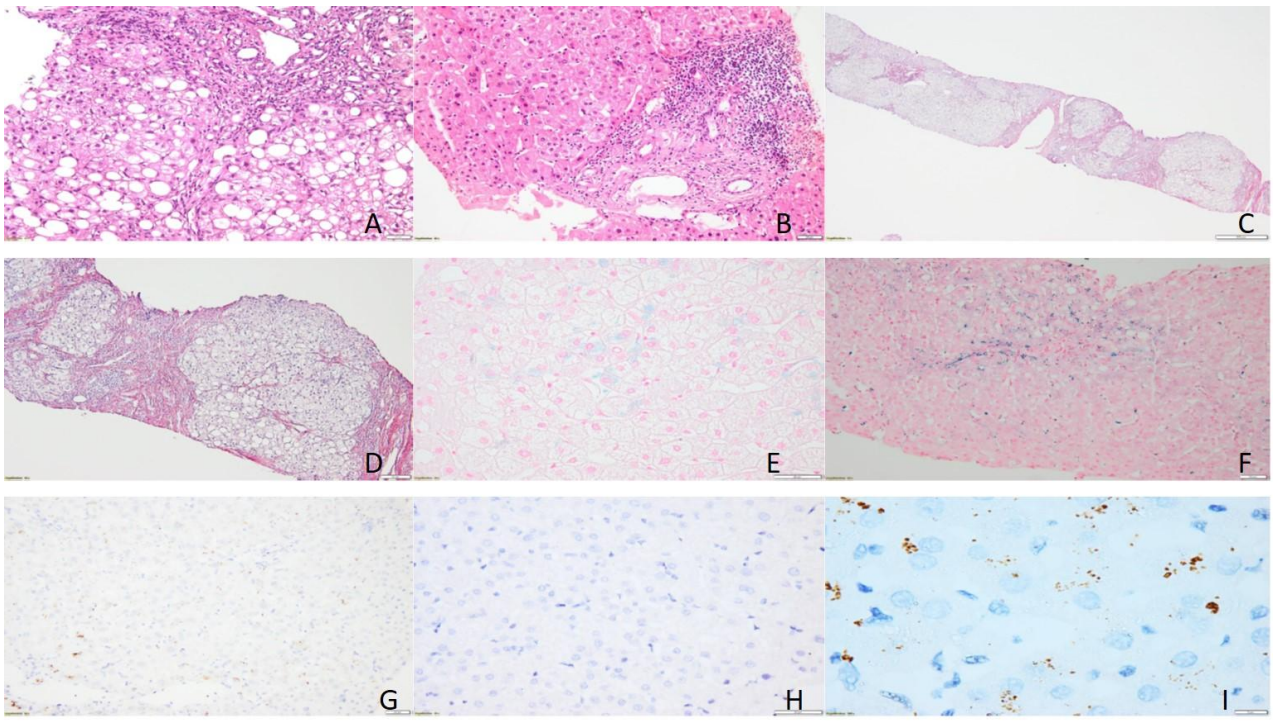

Figure 1: Liver sections: A and B showing marked steatosis and moderate portal inflammation respectively (HxE, 200x and 100x), C and D showing complete liver cirrhosis (Sirius red, 50x and 100x), E and F showing mild and moderate iron deposits (Prussian blue, 200x and 100x), G, H and I showing Negative, score 1 and score 3 viral load (IHC, NS3/NS4, 50x, 100x and 1000x)

VEGFR2 by FISH in different grades of HCC

Overexpression of VEGFR2 correlated with the degree of differentiation in cases of HCC. Well-differentiated HCCs showed normal expression of VEGFR2, while poorly differentiated tumours showed overexpression of the gene (Table 3; Figure 3). Our study has detected a strong correlation between the degree of immunoreactivity to VEGF and the amplification of VEGFR2 recorded by FISH technique.

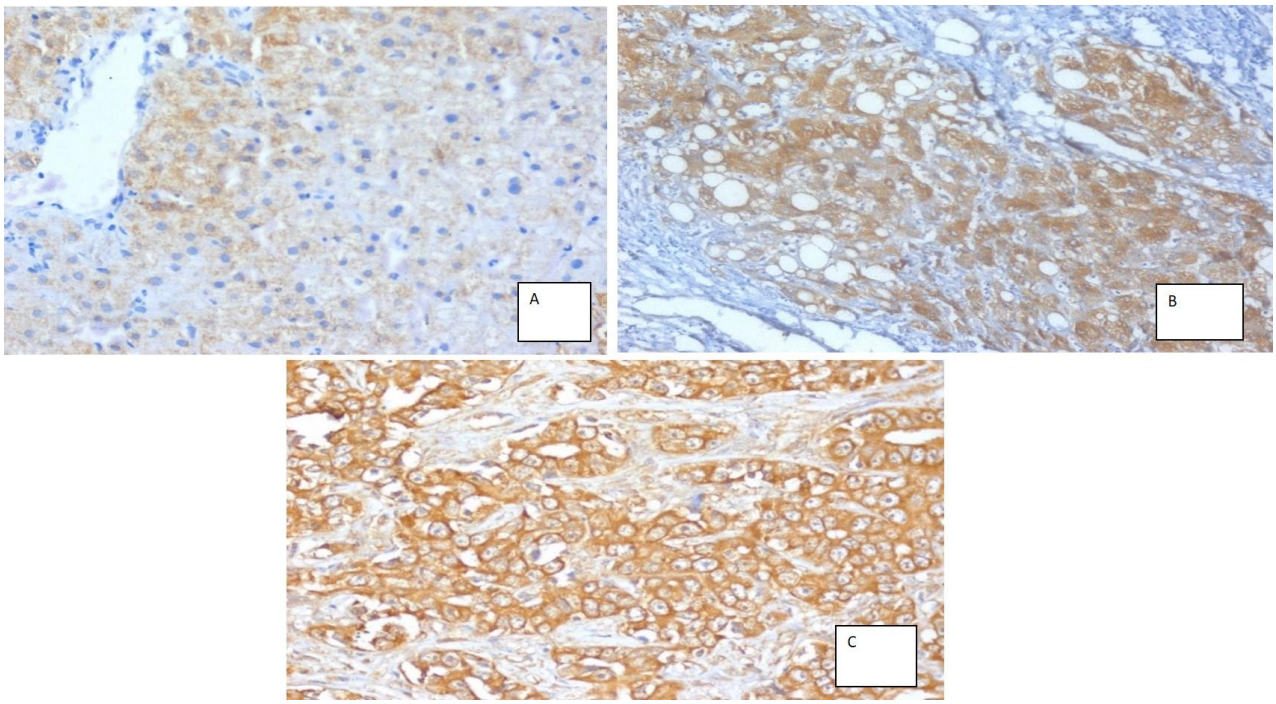

Figure 2: A) Case of CHC showing immunoreactivity to VEGF ±10%, IHC 100x. B) Liver biopsy from a cirrhotic case showing immunoreactivity to VEGF in about 60% of hepatocytes (Score 3); IHC 400x. C) Case of HCC showing immunoreactivity to VEGF in >75% of malignant hepatocytes (Score 4); IHC 400x

Discussion

In the current study, a correlation between the stage of fibrosis and the grade of inflammation in cases of CHC was found ($P < 0.01$). These data are supported by the results of a study which included 3068 patients with histologically confirmed CHC obtained from 10 clinical centres in Italy, Switzerland, France, Australia, and the United States. Leandro et al. [14] concluded that hepatic fibrosis, when considered as the dependent variable, was associated with a greater histologic activity, male sex, the presence of steatosis and older age. Also, in a study that included 346 CHC patients**,** Cua et al. [15] have linked hepatic fibrosis to the grade of portal/periportal inflammation and male gender. However, our study did not link male gender to higher grades of fibrosis ($P = 0.3$).

Table 3: VEGFR2 FISH amplification in different cases of HCC

	VEGFR2- FISH						
	VEGF- IHC Score	Normal Number (%)		Amplified Number (%)		Total Number (%)		P value
0	(< 5%)	0	(0%)	0	(0%)	0	(0%)	
1	(6% - 25%)	0	(0%)	0	(0%)	0	(0%)	
2	(26%-50%)	11	(91.7%)	0	(0%)	11	(55%)	< 0.001
3	(51%- 75%)	1	(8.3%)	2	(25%)	3	(15%)	
4	(> 75%)	0	(0%)	6	(75%)	6	(30%)	
	Total	12	(100%)	8	(100%)	20	(100%)	

Iron overload in our study was present in 13.8% of CHC liver biopsies, and it was not correlated with the stage of fibrosis ($P = 0.6$) or the grade of inflammation ($P = 0.9$). These data are consistent with Lin et al. [16]; where they have identified iron in 12.5% of studied liver biopsies and they have concluded that both serum iron and hepatic iron correlate with serum indices of chronic liver disease but are not related to grade and stage of liver histology. However, Missiha et al. [17] have found that iron overload has been associated with accelerated fibrosis. A study conducted on 58 Egyptian CHC patients has found that hepatic iron density is an independent predictor of advanced fibrosis [18].

In the current study, the immunoreactivity against HCV-NS3/NS4 was found in 67% of CHC cases. However, this finding did not correlate with the stage of inflammation ($P = 0.4$) or the grade of fibrosis ($P = 0.6$) in cases of CHC. In accordance, Liao et al [19], after studying 214 retrospectively collected cases, stated that hepatocyte expression of HCV - NS3 was not correlated with the serum viral load, a severity of the hepatic injury, or treatment response. On the other hand, in a study including 50 cases, Syed and Sadiq [5] have found a positive association between HCV-NS3 immunopositivity and the histological parameters of grading and staging. This discrepancy can be attributed to the variation in a number of studied cases as well as different HCV genotypes.

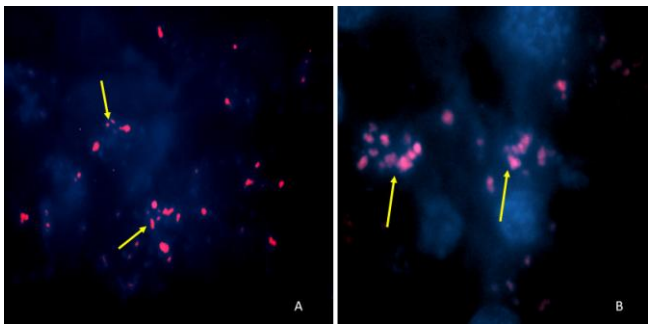

Figure 3: A) Case of HCC, G III, showing amplification of VEGFR2; more than two red signals in the nucleus; FISH X1000 B) from HCC grade II case showed high expression of VEGFR2 more than two red signals in the nucleus (FISH, magnification 1,000×)

Steatosis in our study was present in 48.9% of cases with varying degrees. This percentage is slightly lower than that of another study (54%) including 92 CHC Egyptian patients [20]. It was also consistent with the percentage reported by Wyatt et al. [21] who studied 233 cases, 50.2% of which showed steatosis.

Although steatosis was more prominent in groups with advanced fibrosis, no statistical correlation could be found between the steatosis and the stage of fibrosis in CHC patients ($P = 0.2$) in our study. This is consistent with a retrospective study performed on liver biopsies from 494 CHC patients by Perumalswami and colleagues [22]. They found that steatosis was associated with older age, higher BMI, and higher serum ALT levels but not with the presence of or subsequent progression of fibrosis. Our data are also consistent with Khokhar et al. [23]; in a study including 109 CHC patients, with no significant correlation could be found between steatosis and fibrosis.

However, our data contradict what Leandro et al. [24] have found in large and geographically different group of CHC patients; whereas steatosis was confirmed as significantly and independently associated with fibrosis in CHC. Ahmed et al., [12] also have found that fibrosis was associated with high AST level, age ≥40 years, and steatosis. Gordon et al. [24] conducted a study including 74 CHC patients and reported that steatosis grade appears to relate to hepatic fibrosis progression rate in chronic hepatitis C genotype non-3.

We have observed that the grade of inflammation increases with increasing steatosis. This observation, which couldn't be proven statistically (P =0.2), is consistent with data from studying 221 liver

biopsy-proven CHC patients by Adinolfi et al. [3]; steatosis is an important cofactor in increasing liver necroinflammatory activity. Morosan et al. [25] have studied liver biopsies from 1206 patients (including 1021 CHC patients) and have concluded that steatotic lesions associated with liver pathology increase the severity of the disease, regardless of the type of virus involved. Leandro et al. [14] have also found that steatosis was associated with higher BMI, older age and histologic activity.

Most CHC cases with detectable iron, showed positive immunoreactivity to HCV-NS3/NS4 (11 out of 13 cases), but no correlation could be found between the cellular viral load and the cellular iron content (P = 0.1). Also, no significant correlation could be found between the cellular viral load and the degree of steatosis in CHC patients of this study (P = 0.7). Unfortunately, we did not record studies correlating the hepatic viral load (as detected by immunohistochemistry) with steatosis or hepatic iron content.

We found that the expression of VEGF by IHC is more in HCC cases when compared to cirrhotic cases (P <0.001). This is compatible with a study including 90 Egyptian patients with CHC and HCC which has demonstrated that the highest value of VEGF expression by IHC was mostly encountered among HCC patients [26]. Another study that involved 16 patients with HCC, has reported that VEGF protein expression was higher in HCC as compared to the surrounding liver tissue [27]. Iavarone and colleagues [28] have measured the serum levels of VEGF in patients with CHC (with or without cirrhosis) and HCC. They have reported that serum levels of VEGF protein were significantly higher in HCC patients than in cirrhotic patients. Our study has also found a strong correlation between the expression of VEGF and the degree of HCC differentiation (P <0.001). This is consistent with Mi et al. [29]. They have found a positive relationship between the expression of VEGF in HCC tissue and the pathological differentiation and invasion of the tumour. Another study performed on 54 Egyptian HCC patients has found a statistically significant correlation between expression of VEGF and histopathological grading of HCC [30].

We conclude that the grade of inflammation is a factor affecting the stage of fibrosis in CHC. The degree of liver damage is not related to cellular viral load, iron load or copper load. Steatosis is associated with higher grades of inflammation and stages of fibrosis. VEGF by immunohistochemistry is correlated with the overexpression of VEGFR2 by FISH technique.

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Basic Science

Microbiological Assessment of *Moringa Oleifera* Extracts and Its Incorporation in Novel Dental Remedies against Some Oral Pathogens

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Abstract

AIM: To assess the antibacterial and antifungal potentials of different parts of *Moringa oleifera* plant using different extraction methods in attempts to formulate natural dental remedies from this plant.

MATERIAL AND METHODS: Three solvents extracts (Ethanol, acetone, and ethyl acetate) of different parts of Egyptian Moringa tree were prepared and tested against oral pathogens: *Staphylococcus aureus, Streptococcus mutans*, and *Candida albicans* using disc diffusion method; As well as to incorporate the plant extract to formulate experimental toothpaste and mouthwash. The two dental remedies were assessed against the same microbial strains. Statistical analysis was performed using One-Way ANOVA test to compare the inhibition zone diameter and t-test.

RESULTS: Ethanol extracts as well as leaves extracts demonstrated the highest significant mean inhibition zone values ($P \leq 0.05$) against *Staphylococcus aureus* and *Streptococcus mutans* growth. However, all extracts revealed no inhibition zone against *Candida albicans*. For dental remedies, experimental toothpaste exhibited higher mean inhibition than the mouthwash against *Staphylococcus aureus, Streptococcus mutans* and only the toothpaste revealed antifungal effect against *Candida albicans*.

CONCLUSION: The different extracts of different parts of Moringa showed an antibacterial effect against *Staphylococcus aureus* and *Streptococcus mutans* growth. The novel toothpaste of ethanolic leaves extract has antimicrobial and antifungal potential effects all selected strains.

Introduction

Although antibiotics and the chemotherapeutic agents have been used to fight many infectious diseases; none of the available drugs is free from unfavourable effects and limitations. In addition to the development of multi-drug resistant strains of bacteria which is also another growing problem [1-3]. All necessitates the search for alternate therapies to fight infections [4, 5].

Medicinal plants are among the raising therapeutic options since they contained various chemical compounds with antimicrobial effects [6-9]. In developing countries, due to the expensive cost of antibiotics, the use of medicinal plants for treatment of infection is considered the affordable and safer natural treatment modality [10-13].

Moringa oleifera is among the most valuable world's trees, as almost every part of the Moringa tree can be used for food or has curative characteristics [14, 15]. Remarkably, it was discovered that Moringa oil was used for skin care since ancient Egyptian time's [16].

Considerable studies have been conducted to evaluate the biologically active substances of varies part of this plants [17-20]. For its great antimicrobial effects, Moringa lately, has been used in different health care products [16]. The extracts of different parts of the tree with different solvents are expected to produce active substances with various antimicrobial effects. Hence, it was interesting to assess the antibacterial and antifungal potentials of different parts of Moringa oleifera plant using different extraction methods in attempts to formulate natural dental remedies from this plant.

Materials and Methods

Collection and extraction of plant parts:

The fresh leaves, roots, and seeds of Moringa Oleifera were supplied by the Egyptian Scientific Society of Moringa farm and identified by Prof. Aboelfetoh Mohamed Abdella, National research centre, Giza, Egypt. The different parts of the plant were washed with distilled water and air-dried at room temperature separately. Then each plant part was cut into small pieces (1-2 cm) to make it suitable for grinding. Fifty grammes of each part of Moringa powder (leaves, roots, seeds, and a mixture of all these parts) were kept soaked for 72 hours in 500 ml of ethanol 95%, acetone and ethyl acetate solvents at room temperature. The prepared mixtures were then magnetically stirred for 24 hours to obtain a homogenous mix and stored for 48 hours at 4°C to allow for extraction of active ingredients. Each of the resultant extracts was filtered using Whatman No. 1 filter paper and concentrated by a rotary vacuum evaporator (Rotary Evaporator HS2001N, ARC Distributors, India) at 40°C to evaporate the solvents used for extraction. The ethanol extraction yields (w/w); (19.38 %, 7.34%, 6.40%, and 12.36%) for leaves, roots, seeds and their mixture respectively, while acetone extraction yields; (15.20%, 5.38%, 4.30%, and 9.32%), and for ethyl acetate extraction yields; ((12.50%, 3.90%, 3.32% and 7.45%).

Microorganisms

Three microorganisms were used to test the antimicrobial potential of the prepared Moringa plant extracts, they were: *Staphylococcus aureus* (*S. aureus*) and *Streptococcus mutans* (*S. mutans*) *and Candida albicans* (*C. albicans*). *S. aureus* was isolated and serologically identified by dairy microbiological Lab., National Research Center, Giza, Egypt. While *S. mutans* were isolated from the dental plaque of high caries index patients by swabbing method from the oral cavity of the patients according to Sanchez et al., 2004 [21]. Selective media Mitis salivarius-bacitracin agar (MSB; BD Difco, Paris) was used to isolate and grow *S. mutans*. Both pathogenic bacteria were routinely transferred into Tryptone Soya broth (TSB; Difco laboratories, Detroit, MI, USA) and incubated at 37°C for 24 hours. *C. albicans* (ATCC 10231) was provided by the Department of Microbiology, Cairo University and maintained on Sabouraud Liquid Medium (1% peptone, 4% glucose and adjusted to pH 5.8) to grow at a temperature of 35-37°C for 48 hours.

Assessment of antimicrobial activity

A disc diffusion method was used to test the antimicrobial activity of ethanol, acetone, and ethyl acetate extracts of the different plant parts and their mixture on the profile growth of *S. aureus* and *S. mutans* and *C. albicans.* A total of 324 samples were divided into four main groups (n = 81) according to the plant parts (leaves, roots, seeds, and mixture) being tested. Each group was further subdivided into three subgroups according to the type of solvent used (ethanol, acetone, and ethyl acetate). In addition, each subgroup was divided into three smaller groups according to the type of pathogenic bacteria testing (*S. aureus*, *S. mutans* and *C. albicans*).

Two grammes of each prepared extract were dissolved in 5.0 ml of Dimethyl sulfoxide (DMSO) to get 400 mg/ml. A 0.1ml (approximately 10^9 cells/ml) of each pathogenic bacterium was dispersed on the surface of Tryptone soya agar. While 50 µl of the broth medium containing *C. albicans* was inoculated in a selective culture medium Sabouraud's Dextrose Agar (SDA) with the aseptically addition of Streptomycin. Sterile filter paper discs 4 mm in diameter were impregnated with 20 µl of each extract and placed on surface agar inoculated with pathogenic bacteria and fungus (triplicate for each tested microorganisms). Then, the plates were incubated at 35-37^{o}C for 24 hours. At the end of incubation period, the antimicrobial activity of different plant parts extracts was evaluated by determining the diameter of inhibition zones (mm) around each disc of extract solution (Fig.1a, Fig.1b).

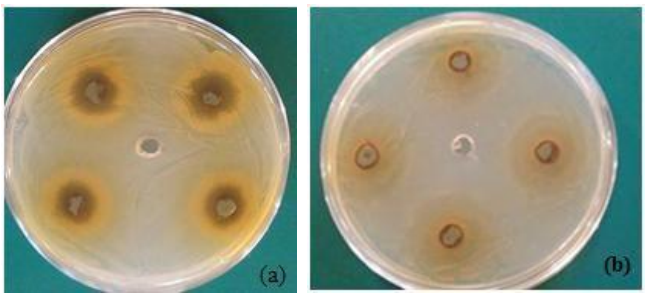

Figure 1: A ntibacterial activity of ethanolic extract of Moringa Oleifera leaves against the profile growth of (a) staphylococcus aureus and (b) Streptococcus mutans

Incorporation of Moringa Oleifera leaves into the experimental prepared toothpaste and mouthwash

The ethanol extract of *Moringa Oleifera* leaves was incorporated in specially prepared experimental Passive toothpaste and mouthwash base formulas with appropriate concentration for each remedy (patent under registration no 2015/2069, Ministry of scientific research, Academy of scientific research & technology, Egypt).

Assessment of antimicrobial Activity of the experimental prepared toothpaste and mouthwash

The antimicrobial activity of experimental toothpaste was screened by modified agar well diffusion method while the mouthwash was determined by disc diffusion method. A total of (144) samples were divided into four main groups (n = 36) according to the active additives incorporated in each dental base formula: A1: The experimental toothpaste base (control), A2: toothpaste with the active additive ethanolic leaves extract, A3: experimental mouths wash base formula, and A4: mouthwash with active ethanolic leaves extract. Each group was further subdivided into three subgroups (C) (n = 12) according to the type of pathogenic bacteria and fungus being tested.

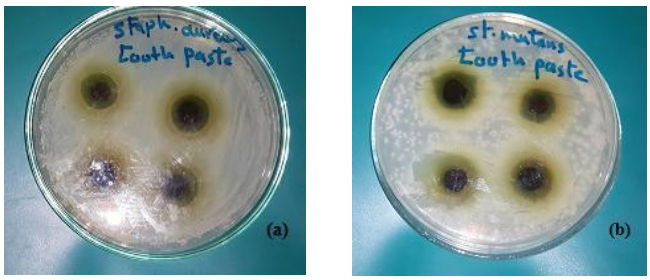

Figure 2: Antibacterial activity of the experimental toothpaste with the active additive Moringa leaves ethanolic extract on the profile growth of (a) staphylococcus aureus and (b) Streptococcus mutans

In the well diffusion method, a 50µl of diluted *S. mutans* and *S. aureus* were spread on the surface of Tryptone Soya agar plate, as well as 50 µl of the broth medium containing *C. albicans* was inoculated in a selective culture medium Sabouraud's Dextrose Agar. A sterile 5 mm cork- borer was used to cut four wells at equidistance in the plate. Similar calculated amount of toothpaste (250 mg) was introduced into the four wells. In disc diffusion method 20µl of mouthwash solution was placed on surface agar plate inoculated with the pathogenic bacteria and fungus (triplicate for each tested microorganisms). The tested plates were incubated at 35-37^{o}C for 24 hours. At the end of incubation period, the antimicrobial activity of experimental toothpaste and mouthwash were evaluated by measuring the mean diameter of the zone of inhibitions in millimetre (Fig. 2a, Fig. 2b, Fig. 3c).

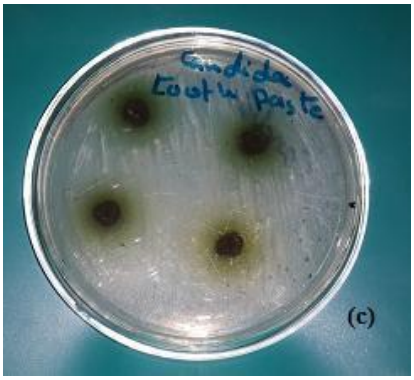

Figure 3: Antifungal activity of experimental toothpaste with the active additive Moringa leaves ethanolic extract on the profile growth of (c) Candida albicans

Statistical analysis

Data were analysed using IBM® SPSS® (SPSS Inc., IBM Corporation, NY, USA) Statistics Version 23 for Windows and then checked for normality using Kolmogorov - Smirnov test. One Way ANOVA test was used to compare between different extracts of Moringa Oleifera plant parts followed by Tukey's posthoc test for pairwise comparison. Independent t-test was used to compare between experimental toothpaste and mouthwash against selected bacterial and fungal strains. The significance level was set at P ≤ 0.05.

Results

The different extraction methods (ethanol, acetone and ethyl acetate) of different parts of M Oleifera plant were assessed against *S aureus*, *S mutans* and *C. albicans*. The means and standard deviations (SD) values for the Inhibition Zones (mm) were displayed in Tables 1 & 2 and Figures 4 & 5. For experimental mouthwash and toothpaste dental remedies; means and SDs of the Inhibition Zones (mm) against the same microbial strains are represented in Table 3 and Fig. 6.

Table 1: Means and Standard deviations (SDs) for the Inhibition Zones (mm) for different parts of *Moringa Oleifera* plant using different extraction methods

		Methods of extractions						P-value
		Ethanolic		Acetone		Ethyl acetate		
		Mean	SD	Mean	SD	Mean	SD	
Staphylococcus aureus	Leaves Extract	19.25 aA	0.96	11.25 aB	0.96	9.00 aC	0.82	≤0.001*
	Roots Extract	9.25 aA	0.50	5.50 cB	0.58	4.50 cC	0.58	≤0.001*
	Seeds Extract	3.25 c	0.96	2.50 d	0.58	2.75 d	0.50	0.354
	Mix Extract (1:1:1)	10.00 A	0.82	6.75 bB	0.50	5.75 bB	0.96	≤0.001*
p-value		≤0.001*		≤0.001*		≤0.001*		

Means with the same lower Case letter within each row are not significantly different at ≥0.05. Means with the same Upper Case letter within each Column are not significantly different at p≤0.05. *= Significant, NS= Non-significant.

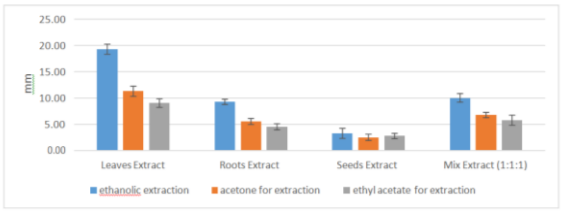

Figure 4: Mean Inhibition Zones (mm) for different parts of Moringa Oleifera plant against S. aureus growth using different extraction methods

Comparison of the mean inhibition zones against *S. aureus* growth; revealed a statistical significance difference regarding the extraction methods ($p \leq 0.05$). Where all ethanol extracts demonstrated highest mean inhibition values followed by acetone except for seeds extract and lowest values were for acetyl acetate extracts. The later seeds extract showed slightly higher mean value than acetone extract, however, it was statistically insignificant ($P \geq 0.05$) Table 1 and Figure 4.

Regarding plant parts, leaves extracts had highest mean inhibition values against *S. aureus* growth, followed by roots, plant parts mix and finally seeds extracts. Ethanol leaves extract recorded inhibition zone diameter (19.25 ± 0.96) followed by acetone (11.25 ± 0.96) and the lowest value was (9 ± 0.82) for acetyl acetate, respectively. Roots extracts mean values were: (9.25 ± 0.50), (5.50 ± 0.58) and (4.50 ± 0.58) for ethanol, acetone and ethyl acetate extracts respectively. While the mix extracts values were: (10 ± 0.82), (6.75 ± 0.50) and (5.75 ± 0.96) for ethanol, acetone and acetyl acetate respectively. All extracts showed a significant difference ($p \leq 0.05$) except seeds extracts; where all seeds extracts showed insignificant difference despite the differences in extraction method ($P \geq 0.05$) Table 1 and Figure 4.

Table 2: Means and Standard deviations (SDs) for the Inhibition Zones (mm) for different parts of *Moringa Oleifera* plant using different extraction methods

		Methods of extraction						p-value
		Ethanolic		Acetone		Ethyl acetate		
		Mean	SD	Mean	SD	Mean	SD	
Streptococcus mutans	Leaves Extract	aA 13.00	1.15	aB 8.25	0.50	aB 7.25	0.96	≤0.001*
	Roots Extract	bA 10.50	0.58	bB 5.25	0.50	cC 4.00	0.82	≤0.001*
	Seeds Extract	dA 4.75	0.96	cB 3.25	0.96	dB 2.25	0.50	0.007*
	Mix Extract (1:1:1)	cA 8.00	0.82	bB 5.75	0.50	bB 5.25	0.50	≤0.001*
p-value		≤0.001*		≤0.001*		≤0.001*		

Means with the same lower Case letter within each row are not significantly different at p≥0.05. Means with the same Upper Case letter within each Column are not significantly different at p≤0.05. *= Significant, NS= Non-significant.

Regarding the mean inhibition zones against *S. mutans* growth; statistical analysis revealed significance differences concerning the extraction methods and the plant parts evaluated ($p \leq 0.05$). These results are comparable to that obtained against *S. aureus*. Where ethanol extracts demonstrated highest significant mean inhibition values followed by acetone extracts and the lowest values were for acetyl acetate extracts ($P \leq 0.05$) Table 2 and Figure 5.

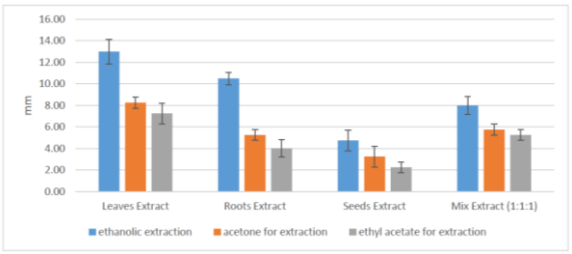

Figure 5: Mean Inhibition Zones (mm) for different parts of Moringa Oleifera plant against S. mutans growth using different extraction methods

Again, leaves extract had the highest mean inhibition zone values followed by the roots, mix and at last seeds extracts exhibited the lowest mean value and these results were statistically significant ($p \leq 0.05$) Table 2 and Figure 5. On contrary, no inhibition zone was detected against *C. albicans* fungus for the different plant part extracts using the three extraction methods.

Table 3: Means and Standard deviations (SD) of the Inhibition Zones (mm) for mouth wash and tooth paste

	Dental remedies				p-value
	Mouth Wash		Tooth Paste		
	Mean	SD	Mean	SD	
Staphylococcus aureus	11.75	1.26	17.75	0.96	≤0.001*
Streptococcus mutans	9.25	0.96	13.75	0.96	0.001*
Candidia albicans	0.00	0.00	13.25	1.50	≤0.001*

Means with the same lower Case letter within each row are not significantly different at p≥0.05. Means with the same Upper Case letter within each Column are not significantly different at p≤0.05. *= Significant, NS= Non-significant.

When comparing the mean inhibition zone values for toothpaste and mouth wash remedies, the toothpaste showed the higher mean inhibition zone values than the mouthwash against *S. aureus, S. mutans* and *C. albicans* respectively and it statistically significant ($P \leq 0.05$) Table 3 and Figure 6.

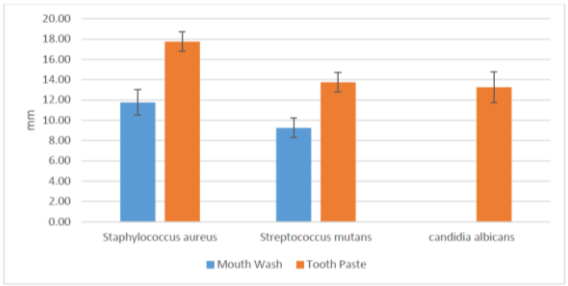

Figure 6: Mean Inhibition Zones (mm) for experimental mouthwash and tooth paste

The toothpaste exhibited mean inhibition values (17.75 ± 0.96), (13.75 ± 0.96) and (13.25 ± 1.5) against *S aureus*, *S. mutans* and *C. albicans* respectively. While mouth values were: (11.25 ±1.26), (9.25 ± 0.96) against *S. aureus* and *S. mutans*

respectively. The absence of inhibition zones against C albicans. Hence, only the experimental toothpaste had inhibition effect against *C. albicans* growth.

Discussion

In the present study, the antimicrobial and antifungal activities of *Moringa oleifera* plant parts (leaves, roots, seeds and their mixture) were tested against some oral pathogens as *S. aureus*, *S. mutans* and *C. albicans.* These microorganisms are associated with respiratory infection [22], dental caries [23] and oral candidiasis [24].

As it was reported in the literature that aqueous extracts of plants were generally exhibited little or no antimicrobial activities [25-27]. Therefore, in this study the three organic solvents; ethanol, acetone and acetyl acetate were used to prepare the *M. oleifera* plant extracts to explore the one with best antimicrobial activities. The results of this study revealed that all extracts of *Moringa oleifera* parts (leaves, roots, seeds and their mixture) have antimicrobial potentials (inhibition zones) against *S. aureus* and *S. mutans* with varying effectiveness. Differences in antimicrobial potency were found to vary with the type of organic solvent used to extract the active components and to plant part being evaluated (Leaves, seeds, roots, and their mixture).

On contrary, none of the extracts showed any antifungal activity against *C. albicans*, this is in agreement with two previous studies conducted by (Masika et al, 2012, Patel et al, 2014) [27, 28] who demonstrated that ethanolic leaf extract has no antifungal effect against *C. albicans.* The findings of this study, however, are in contrast to (Mahdi et al, 2014) [29] who reported that ethanol and acetone leaf extracts showed antifungal activity against *C. albicans*. The difference could be attributed to variation in the environment where the plant was collected, the season and the physiological stage of the plant when leaves were harvested [30].

While ethanolic extract, demonstrated the most effective antimicrobial activity against the *S. aureus* and *S. mutans* compared to acetone and acetyl acetate the extracts. Leaves extract also, showed greatest antimicrobial potential compared to other plant parts (seeds, roots, and the mixture). These latter results are in agreement with (Rahman et al, 2013 and Devedra et al, 2011) [31, 32] who mentioned that Moringa oleifera leaves contain bio-active substances with antibacterial potentials against a wide range of microorganisms. The leaves of *M. oleifera* plant are known to contain phytochemicals compounds as flavonoids, saponins, tannins and other phenolic compounds that have antimicrobial activities [33-35]. This would explain the antimicrobial activities observed in this study could be attributed to such compounds.

According to the results of this study, the plant leaf extract can be the added as a natural antimicrobial constituent in dental products to control oral pathogens. Such products will be supposed to have a preventive/therapeutic effect thus helping to remove dental plaque, the most important factor in oral infectious diseases such as dental caries and periodontal disease. Ethanol leaf extract of Moringa plant was used as an active constituent to formulate an experimental toothpaste and mouthwash. Other natural additives were used as a negative additive to configure dental products. The antimicrobial characteristics of dental products were examined against selected bacterial and fungal strains. The results showed that experimental toothpaste exhibited effective antimicrobial and antifungal activities against the all tested microorganisms. While, the experimental mouthwash revealed lesser antimicrobial potentials compared to toothpaste.

The previous observations could be attributed to several reasons as the difference in antibacterial activities is due to dissimilarities in the composition of both toothpaste and mouthwash base ingredients; variation in the proportion of added leaves extract for each remedy. Another explanation is that the ingredients of experimental toothpaste base formula do not suppress the antimicrobial activity of leaves extract [36]. Unexpectedly, the antifungal activity of the experimental toothpaste was found which could be attributed to the possible synergistic effect between leaf extract and the ingredients of toothpaste base formula. The results of the current study supported the antibacterial potential *M. oleifera* plant and could be the basis for promising applications of medicinal plant in the pharmaceutical industry to control oral pathogens.

In conclusions, within the limitations of present *in vitro* study: (1) the present study confirmed the antibacterial characteristics of *Moringa oleifera* extracts; (2) the plant extracts can be used to formulate new dental products to control oral pathogens owing to their antimicrobial potentials. However, further studies are required to clarify the optimal concentration, cell toxicity and physical stability before its clinical application

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Basic Science

The Hidden Function of Vitamin D

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Abstract

AIM: There are no reports regarding the influence of vitamin D on thymosin ß4 and the cluster of differentiation CD4 levels which are important for maintaining a healthy immune system. Consequently, we aimed to explore this relationship through a study.

MATERIAL AND METHODS: The study was carried out on 35 subjects, screened for 25-hydroxy vitamin D[25 (OH) D] using ELISA method and they were divided into two groups: Group 1 consists of 10 healthy subjects with sufficient vit. D level > 24.8 ng/ml. Group 2 consists of 25 subjects suffering, severely, from vitamin D deficiency at level < 11.325 ng/ml. Also, Thymosin ß4, CD4 and zinc levels were performed.

RESULTS: There were significant differences between the two groups in the concentration levels of thymosin β4, as the group 1 has shown higher levels (P = 0.005). Whereas, CD4 and zinc levels didn't show any significant difference between the two groups. At the same time, a significant positive correlation has been observed between vitamin D, thymosin β4, and CD4 at (r = 0.719; P = 0.001), and (r = 0.559, P = 0.001) respectively.

CONCLUSION: We concluded that vitamin D may be an essential factor that influence or determine the level of thymosin β4. This study is the first that focused on demonstrating that sufficient level of vitamin D may have the ability to influence the thymic hormone thymosin β4 levels. Further studies on large scale of subjects are needed to explore the positive correlation we had found between vitamin D and thymosin β4 and CD4.

Introduction

The impact of Vitamin D has been observed in many aspects [1]. Regarding immune system and human health lower levels of vitamin D may cause of immune dysregulation [2]**.** In addition**,** Wei and Christakos in 2015 revealed that vitamin D may have immunomodulatory properties [3]**.** Vitamin D influences human health because vitamin D receptor and its activating enzyme 1-α-hydroxylase (CYP27B1) are expressed in activated lymphocytes, macrophages and dendritic cells [1]**.** This suggests an important impact of vitamin D especially in the field of human immunology [4-6]**.** Vitamin D intake is highly dependent on nutritional habits where 47% of vitamin D source come from food supplements [7]**.**

Thymosin ß-4 is one of the thymic hormones [8], it is abundant in human cells and tissues, representing 70–80% of the total thymosin content [9] [10] it is an active peptide with 43 amino acids [8] it is omnipresent as intracellular protein, bind to and sequester G-actin to modulate cell migration [11]**.** Several physiological properties of Tβ4 have been reported; [12] repairing and remodeling of skin, neural system and heart tissues following injury [13]**,** assisting in the development of B cells to plasma cells to produce antibodies [14] implicated in lymphocyte maturation and differentiation [9, 15]**,** controlling cell morphogenesis and motility [16] preventing fibrosis [17]**,** acting as a modulator of wound healing and inflammation [18] and regulating immunity [19]**.** Tβ4 is the major actin-sequestering molecule in all eukaryotic cells [20]**.** (Tβ4) is considered to play a significant role in the cellular metabolism due to its actin-sequestering properties [12]**.**

A cluster of differentiation cells- often referred to as CD4 cells- are glycoprotein located on the surface of various types of immune cells restricted to T helper lymphocytes. It has an important function such as signal amplification and T- cell activation [21]**.**

CD4 is a co-receptor that assists the T cell receptor (TCR) in communicating with an antigen-presenting cell [22].

Zinc was found to be necessary for a normal functioning of the immune system [23]**,** altered zinc levels disturb the functions of innate immunity [24]**,** and mild zinc deficiency depresses immunity [25]**.**

This is the first investigation to describe the relationship between vitamin D deficiency, thymosin ß4, and CD4 levels.

Subjects and Methods

Throughout the period from January to March 2015 subjects were recruited from the outpatient clinic of the Centre of medical excellence at the NRC, where 40 subjects were screened by full medical history, thorough clinical examination, nutritional questionnaire, and anthropometric measurement. Screened subjects were enrolled into the study according to the following inclusion /exclusion criteria:

Inclusion: Age ranging from 18-40 years, both sexes, suffering from easy fatigability and lethargy and marked Vit D deficiency < 12 ng/ml.

Exclusion: Systemic diseases (cardiac, hepatic, renal, pulmonary, etc) malignancy of whatever nature, any autoimmune disease.

Thus, 25 subjects (males, females) suffering from easy fatiguability and lethargy, of average age of 27.28 ± 1.5 were enrolled into the study as group), in addition to 10 age and sex matched healthy subjects having normal levels of Vit D > 30 ng/ml as a control group (group 2) .

Methods

Blood samples were drawn from all subjects to estimate complete blood picture. Plasma was separated for determination of vitamin D, thymosin β4, CD4 and trace element zinc .and stored at -80°C until used.

Plasma 25(OH)D, thymosin β4, CD4 levels were determined using a commercial enzyme immunoassay according to the manufacturer instructions (Glory Science Co., Ltd, 2400 veterans Blvd. Suite 16-101,Del Rio, TX78840,USA), performed at National Research Centre medical physiology department serum vitamin D sufficiency was defined as > 20 ng/ml and severely deficient < 12 ng/ml according to the committee to review dietary reference intakes for vitamin D and calcium [26]**.** Zinc levels were estimated by spectrophotometric method (Salucea, Haansberg 19, 4874 NJ EttenLeur, Netherlands).

Statistical analysis

Independent sample student's t-test (two tails)was used to determine the significant difference between the two groups and expressed as mean±SE Pearson correlations were used to analyse the association between 25(OH)D with CD4 and thymosin ß4, chi-square test for non-parametric data was performed to examine the relation between the number of subjects with energy and those lacking energy as a symptom of vitamin D deficiency.

Results

The total number of Group 1 was 10 (8 females and 2 males) and the total number of Group 2 was 25 (21 female and 4 males) as shown in Table 1.

Table 1: Gender distribution

Gender	Group 1 (n = 10)	Group 2 (n = 25)
Females	8 (80%)	21 (84%)
Males	2 (20%)	4 (16%)

Age and BMI are shown in Table 2.

Table 2: Age and BMI of subjects

Parameters	Mean ± SD Group 1 N = 10	Mean ± SD Group 2 N = 25	Significance p
Age years	28.5 ± 4.64	28 ± 7.51	P = 0.636 NS
BMI Kg/m^2	26.81 ± 3.89	27.41 ± 5.52	P = 0.717 NS

BMI:(body mass index); NS : Non-significant.

As shown in Table 3, group 1 had significantly higher 25 (OH) D levels in comparison to group 2 at ($p < 0.001$) the majority were deficient with 25 (OH) D levels. In addition there was a significant difference between thymosin beta 4 in group 1 where it has shown remarkable increase in comparison to group 2, in addition, an increased level of CD4 has been observed in group 1 in comparison to group 2 but this increase wasn't statistically significant, zinc levels didn't show any significant difference between the two groups.

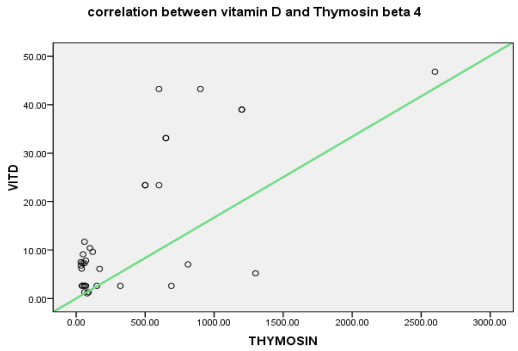
Table 3: Comparison between Group I and II regarding vitamin D, CD4, Thymosin ß4, and Zinc

Parameters	Mean ± SE Group 1 N = 10	Mean ± SE Group 2 N = 25	Significance p
Vitamin D µg/l	34.77 ± 2.82	5.16 ± 0.63	P = 0.001
CD4 pg/ml	2.32 ± 0.88	0. 737 ± 0.05	P = 0.108NS
Thymosinß4 ng/ml	$9.400E_2$ ± 202	$1.844E_2$ ± 60.65	P = 0.005
Zinc µg/dl	$1.800E_2$ ± 9.35	$2.06E_2$ ± 17.63	P = 0.201 NS

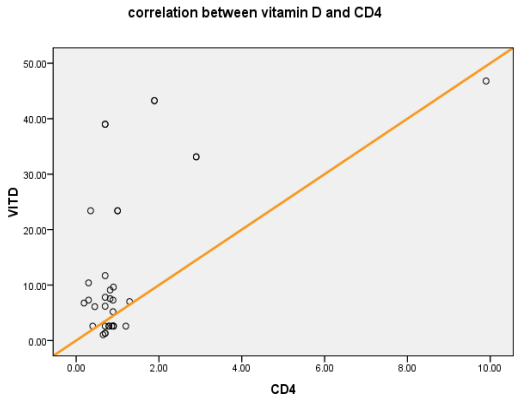
p < 0.001 = very highly significant difference; CD4: Cluster of differentiation 4.

Figure 1 demonstrate the significant positive correlations of this short study where Figure 1A and

Figure 1B represent the positive correlation between vitamin D, thymosin ß-4 and CD4 (r = 0.719, p = 0.001, r = 0.559, p = 0.001 respectively). Where Figure 1C has shown a significant positive correlation between thymosinß-4 and CD4 at r = 0.755, p = 0.001.



A)



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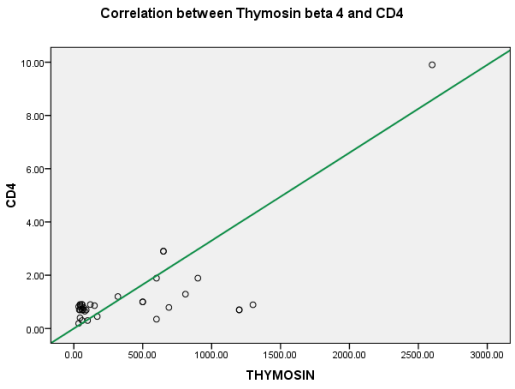

C)

Figure 1: A) positive correlation between vitamin D and thymosin ß-4 at r=0.719, p= 0.001; B) positive correlation between vitamin D and CD4 at r=0. 559, p= 0.001; C) A positive correlation between Thymosin beta 4 and CD4 at r=0.755, p= 0.001

The results of a chi-square test of independence: A chi-square test of independence was performed to examine the relation between the number of subjects with energy and those lacking energy (lethargy) as a symptom of vitamin D deficiency, is shown in Figure 2. Subjects with vitamin D deficiency suffer from lethargy than sufficient subjects, the difference was significant, χ^2 (1, N = 35) = 6.632, p < 0.001.

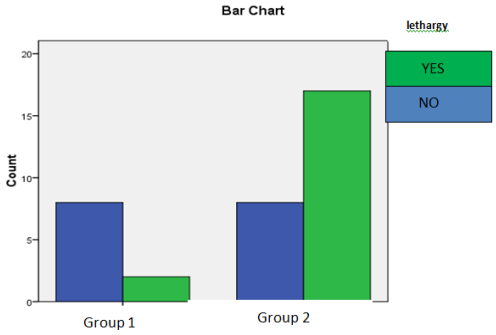

Figure 2: A bar chart showing the difference between Group 1 and 2 regarding lethargy

Discussion

In this study we identified that the majority of subjects were severely deficient in vitamin D where the ratio was 2:5 in which (28.58%) were sufficient and (71.42%) were severely deficient in vitamin D which could be attributed to blood sampling at the end of winter; an observation noted in the studies carried by both Anderson, and Pittawaw as they found vitamin D levels to depend on season [27] [28], because Vitamin D levels are in their lowest levels after winter and their higher at summer [29]. Our current study found a strongly significant correlation between vitamin D, Thymosin beta 4 and CD4. Thymosin beta 4 is the most abundant thymosin in human cells and tissues, it represents 70–80% of the total thymosin content [9] and implicated in lymphocyte maturation and differentiation [15] while vitamin D receptor VDR is found nearly in every tissue and cell type in the body [30] and resides in the cytoplasm in the absence of VDR ligands [31]. When stimulated with 1alpha,25-(OH)(2)D(2) or 1,25(OH)(2)D(3), VDR moves from the cytoplasm into the nucleus [31].

Thus, the strongly significant correlation between vitamin D, Thymosin beta 4 and CD4 found in this study may raise a speculation about a release of thymosin beta 4 secondary to vitamin D stimulated VDR in the thymus. The correlation found between vitamin D and CD4 could be explained that vitamin D has effects on adaptive immune cells because of the expression of the nuclear (vitamin D receptor) as well as vitamin D-activating enzymes in both T- and B-cells [32]. The VDR expression by these cells is very low in resting conditions but when activated, T- and B cells up-regulate VDR expression significantly, allowing regulation of up to 500 vitamin D responsive genes which influence their differentiation and proliferation [33] [34] therefore leading to a shift from a proinflammatory to a more tolerogenic immune status [35].

A recent study by Hewison who proposed that vitamin D influence on T cells function by the direct conversion of 25(OH)D to calcitriol by T-cells, and the effects of calcitriol on T-cells in which calcitriol have indirect effects on antigen presentation to T cells [36]. This study also revealed a strong positive correlation between thymosin β4 and CD4in agreement with Knutsen and colleagues in 1999 [37] which could be attributed to the fact that thymosin β4 is the predominant form of thymic hormones [38], and that its primary function is to stimulate the production of T-cells which are targets of thymosin activity [39]. In our study vitamin D was in positive correlation with CD4 - that represent helper cells - which has found to contain the significant amount of VDR [40]. Our study was in agreement with (Ritterhouse et al) [41] vitamin D regulates T-helper 1 (Th1) and dendritic cell function [42], which suggest that vitamin D support the innate and the adaptive immune system.

We didn't find any significant difference regarding zinc levels between group 1 and 2 or any correlation between zinc and vitamin D, Thymosin beta-4 and CD4 because the sources of zinc like whole grains, cereals and legumes, was available for our subjects according to questionnaire; as whole grains are high in zinc [43]**.** In addition, a study conducted by Hess, 2007 revealed that zinc levels in the serum are not an indicator marker of zinc status because it is detectable in a population with risk and severe deficiency [44]. A recent study by Chiplokara and Kawade 2012 observed that zinc deficiency is very rare but moderate is widespread [45]. Vitamin D dietary intake is highly dependent on nutritional habits. However, a study with a global perspective found that 6 to 47% of vitamin D intake comes from dietary supplements [46] [7]. Thus, without supplementation, vitamin D status strongly will depend on endogenous vitamin D production which is also influenced by latitude, skin pigmentation, season, and lifestyle such as clothing [47] [48].

In conclusion, Vitamin D is obtained from limited dietary sources and the high vitamin D deficiency found in this study emphasises the importance of increased awareness and supplementation. It is apparent that vitamin D influences Tβ4 and CD4 levels so supplementation with vitamin D is essential to support immunity. More experimental trials in laboratories are needed to measure the levels of thymosin beta 4 in the compartments of thymus by its direct stimulation with vitamin D and measuring its concentration in vitro to explore the strong correlation found between vitamin D and thymosinβ4.

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Basic Science

Vitamin D Status, Insulin Resistance, Leptin-To-Adiponectin Ratio in Adolescents: Results of a 1-Year Lifestyle Intervention

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Abstract

AIM: We aimed to study the relationships between circulating 25-hydroxyvitamin D [25(OH)D], insulin resistance and leptin-to-adiponectin (L/A) ratio in Guadeloupean children and adolescents and to analyse the changes in 25(OH)D levels after a 1-year lifestyle intervention program.

METHODS: 25(OH)D concentrations were measured via a chemiluminescence assay. Cardiometabolic risk factors, homoeostasis model assessment of insulin resistance (HOMA-IR), and adipokines were measured. The lifestyle intervention included dietary counselling, regular physical activity.

RESULTS: Among 117 girls and boys (11–15 years old, 31.6% obese), 40% had vitamin D deficiency (25(OH)D levels < 20 ng/mL). With linear regression models where 25(OH)D and HOMA-IR acted as independent variables and age, sex, BMI, L/A ratio as covariates, 25(OH)D was significantly associated with HOMA-IR alone (P = 0.036). HOMA-IR was also associated with BMI z-score ≥ 2, L/A ratio and an interaction term BMI z-score ≥ 2*L/A ratio (P < 0.001 for all). After one year, in 78 children/adolescent, mean serum 25(OH)D increased significantly from 21.4 ± 4.9 ng/mL at baseline to 23.2 ± 6.0 after 1 year; P = 0.003 whereas BMI z-score, HOMA-IR and L/A ratio decreased significantly (P = 0.003, P < 0.001 and P = 0.012; respectively).

CONCLUSION: The association between 25(OH)D and HOMA-IR, independently of obesity and the high prevalence of vitamin D deficiency should be considered in order to prevent the later incidence of T2DM. A healthy lifestyle including non-sedentary and outdoor activities could be a way for improving vitamin D status.

Introduction

Vitamin D deficiency, a public health concern, define as serum 25-hydroxyvitamin D [25(OH)D] lower than 20 ng/mL (50 nmol/L) [1], is frequent in adolescents worldwide and particularly in presence of obesity. Vitamin D is mainly synthesised from 7-dehydrocholesterol by the skin's exposure to ultraviolet B (UVB) radiation. Circulating 25(OH)D is obtained from both UVB action and dietary intake, provides an indication of vitamin D stores. Any barrier to the penetration of UVB radiation into the skin may result in a decrease in circulating 25(OH)D. Melanin pigment may absorb the ultraviolet B photons responsible for the photolysis of 7-dehydrocholesterol to vitamin D3. Vitamin D deficiency has negative consequences on indices of insulin resistance (IR) and metabolic parameters [2].

In a previous study conducted in Guadeloupean school children and adolescents (children/adolescents), we found that obesity was

associated with an adverse metabolic profile including a high frequency of insulin resistance (89%) [3].

This should also be a concern in the island of Guadeloupe (FWI), in which the prevalence of diabetes is high and estimated at 8.1% [4].

Vitamin D deficiency may be a major contributor to the obesity-associated complications with potential interactions between 25(OH)D, weight status, IR and leptin and adiponectin cytokines. These adipocytokines have opposite effects on BMI and IR. However, the leptin-to-adiponectin (L/A) ratio has been proposed as a potential clinical tool for the assessment of IR [5]. Few studies on vitamin D status and IR in adolescents measured circulating leptin and adiponectin concentrations and still less in black adolescents or in those living in sun-rich climates [6-9].

Thus, we aimed to evaluate the association of serum 25(OH)D levels with measures of obesity, insulin resistance and leptin-to-adiponectin ratio and to analyse the potential changes in 25(OH)D levels after a 1-year lifestyle intervention combining enhanced physical activity and diet counselling for the Guadeloupean children/adolescents.

Subjects and Methods

Study design and population

The present study concerned a sample of 117 children and adolescent, aged 11 to 15 years, from a previous study on overweight conducted in Guadeloupe Island (FWI) in 2013 [3]. In this study, volunteer healthy children from a middle school were enrolled to assess 1) their basal metabolic profiles according to weight status and 2) changes in these profiles after 1-year lifestyle intervention program.

The current study targeted vitamin D status in conjunction with obesity and insulin resistance.

Exclusion criteria included seizure disorders, diabetes, cardiovascular disease, pregnancy, and use of calcium or vitamin D replacement therapy.

The study was approved by the Ethic Committee (South West - Overseas III, France). Written informed consent, to participate in this study, was obtained from all children and parents. Clinical and biological examinations were performed at baseline and one year later.

The study protocol has been described in more details elsewhere [3]. Briefly, the 1-year lifestyle intervention program included 1) a nutritional information, focusing on overweight prevention and explaining healthy eating (with an increase in the consumption of fruits and vegetables and a decrease in the consumption of high-fat and high sugar meals) 2) Information on health risks for diseases related to lack of exercise and overweight 3) Encouragement for reduction of sedentary behaviors 4) advice to achieve five hours per week of school physical education and/or physical activity outside of school and 5) Participation of parents.

The school usually provided three hours per week of physical education, supervised by physical education teachers. These activities are outdoors except in bad weather. Phone calls were made to maintain adherence to the program.

Survey data. Participants were interviewed and examined at baseline and 1 year after.

Physical examination. Height in centimetres (cm) and weight in kilogrammes (kg) was measured with participants standing without shoes and lightly clothed. The body mass index (BMI) was calculated as weight/height^2 (kg/m^2). The standard deviation score of body mass index (BMI z-score) was also used [10] and a BMI z-score ≥ 2 was considered as obesity. Waist circumference (WC) in centimetres (cm) was measured and the waist- to height ratio (WHtR) was calculated. Pubertal stage was assessed according to Tanner [11]. Tanner stages 3, 4 and 5 defined pubertal/postpubertal development.

Laboratory assessments. Blood glucose level (mmol/L) was assessed using the glucose oxidase method. Serum lipid levels (mmol/L) were measured enzymatically. Serum insulin levels (µIU/mL) were measured using the COBAS electrochemiluminescence immune assay test (Roche Diagnostics, Basel, Switzerland). The homeostasis model assessment to determine insulin resistance (HOMA-IR) was calculated: [fasting insulin (µIU/mL) x fasting glucose (mmol/L) / 22.5] [12].

Plasma samples were frozen at -80 °C until measurements of leptin, adiponectin and 25(OH) vitamin D. Leptin ng/mL and total adiponectin (µg/mL) were measured with ELISA kits Biovendor® (RD191001100) and Alpco® (47-ADPHUE-01) respectively. Plasma concentrations of 25(OH)D was measured via a chemiluminescence assay (DiaSorin SA, Antony, France), which includes 25(OH)D2 and 25(OH)D3.

These measurements were made concomitantly for the samples collected at baseline and one year after (end of the study).

Definition of factors

Weight status: The children were classified according to the definition of overweight and obesity by the International Obesity Task Force (IOTF) with three categories: normal weight (BMI<IOTF-25 kg/m^2), overweight with (BMI≥IOTF-25 kg/m^2 and BMI<IOTF-30 kg/m^2) and obese (BMI≥IOTF-30 kg/m^2)

[13, 14]. *Abdominal obesity* in children was defined as WHtR greater or equal to 0.5 [15]. *Insulin resistance* was defined by a HOMA-IR > 3.16 [16]. *Vitamin D status* was classified into the following three categories for 25(OH)D: deficiency (<20 ng/mL [<50 nmol/L]), insufficiency (≥ 20 ng/mL [≥ 50 nmol/L] and < 30 ng/mL [<75 nmol/L]) and sufficiency (≥ 30 ng/mL [≥75 nmol/L]) [17].

Statistical analysis

Data are presented as numbers (percentages) for categorical variables and as means ± standard deviations (SD) or median [inter quartile range] for continuous variables. Variables with a skewed distribution were log-transformed to approach a normal distribution.

The chi-squared test and ANCOVA with adjustment for age, sex were used to test percentage and mean differences between groups according to the presence or absence of vitamin D deficiency and of insulin resistance. Non-parametric tests were used for comparison between data at baseline and at 1 year.

The Pearson correlation test was used to study the relationships between serum 25(OH)D levels, HOMA-IR and L/A ratio with other continuous variables. We also used the multivariate linear regressions in the overall study population to assess the associations of 25(OH)D concentrations and HOMA-IR index as dependent variables with age, sex, BMI z-score and L/A ratio as covariates. In addition, serum 25(OH)D concentrations or HOMA-IR were included in the models, as covariates, according to the dependent variable.

The effects of the covariates were assessed by the values of the regression coefficients corresponding to the non-standardized regression coefficients (nSRC). IBM SPSS Statistics, version 21 (SPSS, Chicago, IL) were used for data analyses. A *P value* < 0.05 was considered significant.

Results

Characteristics of the study population at baseline

A total of 117 children/adolescents of both sexes, with a mean age of 12.5 ± 0.1 years, were concerned by the present study. Among them, 59.8% were girls. Most adolescents (98%) were Afro-Caribbean (with high skin pigmentation). There were: 31.6% obese, 32.5% with overweight and 35.9% of normal weight. The frequency of children eating at school canteen was higher in normal weight (53%) than in overweight (28%) and obese (30%) children; (P = 0.027), (data not shown). School canteens follow the recommendations designed to lower fat and sugar and increase vegetables and fruit in meals. Most children/adolescents (92.3%) were at pubertal/postpubertal stage (Tanner stage ≥ 3) and none was receiving vitamin D supplement therapy. Means of serum 25(OH)D concentrations was 21.2 ± 5.1 ng/mL. Seventy-eight (66.7%) children were insulin resistant. Regarding the vitamin D status, 40% had a vitamin D deficiency, 54% a vitamin D insufficiency, and 6% had sufficient vitamin levels. The frequencies of vitamin D deficiency in obese, overweight and normal-weight were 51%, 37% and 33%, respectively; *P* = 0.233. Considering those who were obese, mean 25(OH)D levels were significantly lower than in the rest of the study sample (19.6 ng/mL *v* 21.9 ng/mL, respectively; *P* = 0.023).

Characteristics of the children and adolescents in presence/absence of vitamin D deficiency and of insulin resistance

Distribution of parameters according to presence/absence of vitamin D deficiency and of insulin resistance are summarised in Table 1**.**

Table 1: Characteristics of school-children 11 to 15 years according to Vitamin D and Insulin resistance status

		Vitamin D deficiency			Insulin resistance		
	All adolescents n =117	No n =70	Yes n =47	*P*	No n =39	Yes n =78	*P*
Age (y)	12.0 ± 1.0	11.9 ± 1.1	12.0 ± 1.0	*0.979*	11.8 ± 1.0	12.0 ± 1.0	*0.237*
Sex (Girls)	59.8%	54.3%	45.7%	*0.136*	25.7%	74.3%	***0.033***
Tanner stage ≥ 3	92.3%	94.3%	89.4%	*0.327*	92.3%	92.3%	*1*
BMI (Kg/m²)	24.2 ± 6.2	23.2 ± 5.22	25.72 ± 7.12	***0.021***	21.2 ± 4.7	25.8 ± 6.28	***<0.001***
BMI z-score	1.09 ± 1.19	0.94 ± 1.23	1.32 ± 1.10	*0.079*	0.58 ± 1.11	1.35 ± 1.15	***0.001***
Obesity (IOTF)	31.6%	25.7%	40.4%	*0.094*	10.3%	42.3%	***<0.001***
Abdominal Obesity	32.5%	15.4%	41.0%	***0.002***	27.1%	40.4%	*0.158*
Fasting blood glucose (mmol/L)	4.83 ± 0.43	4.74 ± 0.38	4.95 ± 0.46	***0.011***	4.55 ± 0.36	4.96 ± 0.39	*<0.001*
Leptin (ng/mL)	23.39 ± 20.44	20.54 ± 16.62	27.45 ± 24.52	*0.266*	13.42 ± 13.96	28.37 ± 21.39	***<0.001***
Adiponectin (µg/mL)	5.41 ± 2.61	5.86 ± 2.70	4.76 ± 2.35	***0.010***	6.56 ± 2.82	4.83 ± 2.30	***0.001***
Leptin to adiponectin ratio	5.94 ± 6.53	4.80 ± 5.05	7.55 ± 7.98	***0.026***	2.69 ± 3.50	7.56 ± 7.09	***<0.001***
HOMA-IR index	4.70 ± 3.01	4.04 ± 2.32	5.69 ± 3.63	***0.003***	--	--	--
HOMA-IR index>3.16	66.7%	28.2%	46.2%	*0.062*	--	--	--
25(OH)D (ng/mL)	21.17 ± 5.05	--	--	--	22.85 ± 4.72	20.33 ± 5.03	***0.012***
25(OH)D (ng/mL) <20	40.2%	--	--	--	28.2%	46.2%	*0.062*

Data in this table are mean ± SD and column percentage BMI, body mass index; HOMA-IR, homoeostasis model assessment-insulin resistance; 25(OH)D, 25-hydroxyvitamin D. All p-values are in italic and significant p-value are in bold italic.

BMI, WC, fasting blood glucose, triglycerides, insulin levels, HOMA IR index and L/A ratio were significantly higher whereas adiponectin levels were significantly lower in children with vitamin D deficiency or with insulin resistance than in the others. The leptin levels were significantly higher only in the presence of insulin resistance. There was no significant difference between groups for Tanner stages.

Correlation between serum 25(OH)D levels, HOMA-IR index, leptin-to-adiponectin ratio and other clinical and biological parameters

Negative correlations were noted between 25(OH)D levels and BMI FBG HOMA-IR index, leptin levels and L/A ratio. Positive correlations were noted for HOMA-IR index and L/A ratio with BMI, BMI z-score, FBG. HOMA-IR index was also positively correlated with L/A ratio, Table 2.

Table 2: Correlations between 25(OH)D, HOMA- IR index, leptin-to-adiponectin ratio and anthropometric and metabolic parameters

Variables	25(OH)D (ng/mL)		HOMA-IR index		Leptin-to-adiponectin ratio	
	r	*P*	r	*P*	r	*P*
Age (years)	- 0.03	*0.749*	0.18	*0.051*	0.31	***0.001***
BMI (kg/m²)	-0.220	***0.019***	0.50	***<0.001***	0.80	***<0.001***
BMI z-score	-0.13	*0.178*	0.39	***<0.001***	0.60	***<0.001***
FBG (mmol/L)	-0.24	***0.010***	0.46	***<0.001***	0.33	***<0.001***
HOMA-IR index	-0.29	***0.001***	--	--	0.58	***<0.001***
25(OH)D (ng/mL)	--	--	-0.29	***0.001***	-0.24	***0.011***
Leptin (ng/mL)	-0.25	***0.008***	0.52	***<0.001***	--	--
Adiponectin (µg/mL)	0.17	*0.079*	-0.38	***<0.001***	--	--
leptin-to-adiponectin ratio	-0.24	***0.011***	0.58	***<0.001***	--	--

FBG: fasting blood glucose.

Linear regression for 25(OH)D levels and of HOMA-IR index

Table 3 presents the results of the multivariate linear regression exploring the association between 25(OH)D levels and HOMA-IR with age, sex, BMI z-score ≥ 2, and L/A ratio as independent variables.

In model 1, for 25(OH)D levels, no significant association was noted with age, gender, BMI z-score ≥ 2, L/A ratio and an interaction term BMI z-score ≥ 2*L/A. However, a significant negative association with HOMA-IR was found (*P* = 0.036). The model accounted for 14% (r^2 = 0.14) of the variability in 25(OH)D levels.

In model 2, for HOMA-IR index, no significant association was noted with age and gender. HOMA-IR was negatively associated with 25(OH)D and the interaction term BMI z-score ≥ 2*L/A (*P* = 0.036; *P* < 0.001 respectively) and positively associated with BMI z-score ≥ 2 and L/A ratio (*P* < 0.001 for both*).* The model accounted for 50% (r^2 = 0.50) of the variability in HOMA-IR index.

Table 3: Multivariate linear regression for Vitamin D level and HOMA IR index in 117 adolescents

Variables	Model 1: **25(OH)D levels (ng/mL)** R^2 = 0.14		Model 2: **HOMA IR Index** R^2 = 0.50	
	nSRC (SE)	*P*	nSRC (SE)	*P*
Age (Years)	0.39 (0.47)	*0.507*	0.06 (0.23)	*0.250*
Gender (girl)	-1.85 (0.98)	*0.125*	-0.21 (0.49)	*0.379*
BMI z-score ≥2	-1.09 (1.94)	*0.956*	3.21 (0.91)	***<0.001***
25(OH)D (ng/mL)	--	--	-0.08 (0.05)	***0.036***
HOMA IR index	-0.35 (0.20)	***0.036***	--	--
L/A ratio	0.04 (0.15)	*0.761*	0.34 (0.07)	***<0.001***
BMI z-score ≥2 * L/A ratio	-0.10 (0.18)	*0.461*	-0.24 (0.08)	***<0.001***

nSRC: non-standardized regression coefficient; SE: standard error.

Changes in 25(OH)D levels, in obese children, at one year of follow-up

Overall, in 78 children/adolescents who accepted to undergo evaluation after one year, mean serum 25(OH)D increased from 21.4 ± 4.9 ng/mL at baseline to 23.2 ± 6.0 after 1 year; *P* = 0.003 whereas BMI z-score, HOMA-IR and L/A ratio decreased significantly (data not shown).

Focusing on the 27 obese children/adolescents (table 4), we observed significant decrease in the BMI z-score (*P* = 0.009) as well as in HOMA-IR (*P* = 0.011) and L/A ratio (*P* < 0.001) and, serum 25(OH)D increased significantly from 19.8 ± 4.5 ng/mL at baseline to 21.4 ± 5.0 after 1 year; *P* = 0.007.

Table 4: Clinical and biochemical parameters at baseline and after 1-year of a lifestyle intervention program in obese adolescents

	Obese adolescents N = 27		
	Baseline	One year after	*P*
BMI z-score	2.18 ± 0.27	2.08 ± 0.40	***0.009***
25(OH)D (ng/mL)	19.77 ± 4.50	21.42 ± 5.02	***0.007***
HOMA-IR index	6.32 ± 3.19	5.53 ± 3.95	***0.011***
Leptin-to-adiponectin ratio	11.24 ± 8.43	8.13 ± 7.52	***< 0.001***

Discussion

In the current study, we observed a high frequency of vitamin deficiency (40%) in our healthy children/adolescents who not taking vitamin D supplement therapy and, obese children/adolescents were found with significantly lower circulating 25(OH)D concentrations than the others. But, a linear regression model showed that serum 25(OH)D concentrations were related to insulin resistance and not significantly to obesity and L/A ratio.

After a 1-year lifestyle intervention including diet counselling and encouraging increased physical activity, 25(OH)D concentrations increased significantly after this intervention suggesting that healthy lifestyle may protect against vitamin D deficiency in children/adolescents.

Vitamin D status in children/adolescents and relation with ethnicity and weight status

The island of Guadeloupe is localised between the equator and the tropic of cancer, with a continual sunniness. Nevertheless, in this island population, mostly of African ancestry (Afro-Caribbean), our healthy children/adolescents exhibited a 40% prevalence of vitamin D deficiency. Ethnic disparities in the prevalence of vitamin D deficiency have been documented with higher frequencies, in individuals having high skin pigmentation. Turer et *al*, in US children, documented vitamin D deficiency prevalence (< 20ng/mL) of 68%, 38% and 12% in African-Americans, Latinos and Whites, respectively [18]. In an Italian study, non-white adolescents had extremely reduced median 25(OH)D levels (11.3 ng/mL) and only 5.9% of them had vitamin D sufficiency [19]. It should be noted that the prevalence of vitamin D deficiency in our adolescents is still less than that of the US African-Americans adolescents [18].

Our obese adolescents had significantly lower mean 25(OH)D levels than in rest of the study sample. Several mechanisms could explain the relationship between VitD deficiency and obesity. One hypothesis is the dilution or deposition of the ingested or cutaneously synthesised VitD in the large body fat compartments reducing its bioavailability [20, 21]. Decreased exposure to solar UV radiation and decreased outdoor activity are also potential mechanisms by limiting cutaneous VitD synthesis [22].

But, controversial results have been reported for the relationships between serum 25(OH)D levels and anthropometric measures in adolescents. Whereas some studies described significant associations with BMI, global obesity or measures of abdominal obesity [18, 23], some others did not find these associations [24, 25], suggesting that adiposity might not be the main determinant of vitamin D status in adolescents.

The multivariate linear regression for serum 25(OH)D levels, in the current study, showed no significant association with obesity and similar results were found when abdominal obesity was used in the model (data not shown), suggesting, as for other authors, that other factors, uncontrolled in the study, could have a stronger effect than obesity to decrease serum vitamin D levels [2]. Sun exposure time, the degree of skin pigmentation might also contribute significantly to vitamin D status.

Vitamin D, insulin resistance and leptin-to-adiponectin ratio

We found an inverse association between 25(OH)D levels and HOMA-IR, independently of obesity. Esteghamati et *al* also reported a linear inverse association between vitamin D and IR in non-diabetics that were independent of obesity [26]. In the linear regression analyses, while serum 25(OH)D was associated with HOMA-IR alone, HOMA-IR per se was associated with 25(OH)D levels, obesity and L/A ratio suggesting an indirect relationship between obesity and 25(OH)D concentrations.

Authors, in a paediatric study, have reported that low 25(OH)D concentrations are related to glucose intolerance and IR particularly in obese people [27]. Several mechanisms could explain the effect of vitamin D deficiency on insulin sensitivity and its role in promoting IR and glucose abnormalities: i) vitamin D receptors exist in pancreatic tissue and could be directly involved in the regulation of insulin [28], ii) Increased parathyroid hormone levels in the context of vitamin D deficiency could affect insulin sensitivity [29] iii) vitamin D deficiency would be associated with a high inflammatory response that predisposes to insulin resistance [30, 31].

The L/A ratio, a marker of IR [5], was inversely correlated with 25(OH)D concentrations but positively and more strongly correlated with BMI, BMI z-score, FBG and HOMA-IR (table 2). Leptin and adiponectin have opposite effects on inflammation and insulin resistance. High leptin levels increase the expression of pro-inflammatory and pro- angiogenic factors [32] and, in the same line, vitamin D deficiency predisposes to IR [30, 31]. In contrast, adiponectin induces the production of anti-inflammatory cytokines and improves peripheral insulin sensitivity [33]. The BMI independent positive correlation between 25(OH)D and adiponectin documented by some authors in T2DM, suggests a potential role for this adipocytokine as a link between 25(OH)D and IR [34].

Changes in 25(OH)D after 1-year lifestyle intervention

We have previously described the improvement in BMI z-score, leptin levels and HOMA-IR after the lifestyle intervention particularly in obese adolescents [3] while increasing in HOMA-IR would be expected after one year with increasing age or puberty [35]. The 25(OH)D concentrations increased significantly after the 1-year lifestyle intervention confirming that reduction of sedentary behaviours improves vitamin D status, in adolescents. High 25(OH)D levels have been positively associated with physical activity particularly when performed outdoors [36] and, adolescents who performed less than 3 hours/week of outdoor exercise were found with a higher prevalence of vitamin D insufficiency [19]. The nutrition counselling and the continual sunniness of our island may have exerted additive beneficial effects on serum 25(OH)D levels in the present study even in obese adolescents.

Limitations and strengths of the study

The potential limitations of our study include i) its small sample size, ii) the lack of information on food intake affecting serum vitamin D levels such as milk products (although milk is not generally supplemented in our country), on time of exposure to solar UV radiation and on measure of outdoor activity at baseline . Nevertheless, the present study has a number of strengths: i) the children were healthy, not receiving vitamin D supplement therapy, comparable (between vitamin D status groups) for age, gender and Tanner stage distributions and presented no associated diseases that could influence the vitamin D status, ii) the continuously warm and sunny climate and an ethnic homogeneity of the study population and iii) the longitudinal design providing data on changes in serum 25(OH)D, HOMA-IR and L/A ratio at the end of the 1-year lifestyle intervention.

In conclusion, insulin resistance that showed a strong association with vitamin D deficiency is common among obese adolescents but, obesity is not the main factor explaining vitamin D deficiency in adolescents. The improvement in 25(OH)D level could be due to increasing outdoor physical activity and also to improvement in the nutritional status.

Patricians should encourage adolescents to have a healthier lifestyle with healthy eating, non-sedentary and outdoor activities that may protect against overweight and also improve vitamin D status. Since natural foods rarely contain enough vitamin D to compensate vitamin D deficiency, vitamin D supplementation may be considered and particularly in severely vitamin D deficiency and in very obese adolescents. But, further investigations are needed to better understand the role of vitamin D in the occurrence of insulin resistance.

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O Blood Group as Risk Factor for Preeclampsia among Sudanese Women

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Abstract

AIM: To investigate blood groups and the other possible risk factors for preeclampsia among Sudanese women.

MATERIAL AND METHODS: A case – control study was conducted at Saad Abualila Hospital, Khartoum, Sudan during the period of July 2013 through December 2014. The cases were women with preeclampsia and healthy pregnant women were the controls.

RESULTS: Two hundred eighty pregnant women were enrolled (140 in each arm of the study). Around one-quarter of all women (280) were primiparae (74.0, 26.4%), the majority were housewives (201, 71.7%). Seventy-nine (28.2%) were illiterate or had no informal education. Around half of the women (130, 46.4%) had O blood group. Binary logistic regression showed association between preeclampsia and lack of antenatal care (OR = 2.75, 95% CI = 1.172–6.494, P = 0.020) as well as O blood group (OR = 1.78, 95% CI = 1.088–2.934, P=0.022).

CONCLUSION: The current study showed that women with blood group O were at higher risk of preeclampsia.

Introduction

Preeclampsia is a common complication of pregnancy, where its incidence is approximately one tenth of births [1]. It is a major cause of increased maternal and perinatal fatality worldwide [2]. The definite aetiology of preeclampsia remains ambiguous, nevertheless, previous researchers have delineated a number of risk factors including low education, primiparity, family history of hypertension , obesity, younger or advanced maternal age, and the ethnic background [3–7].

Some biochemical and haematological factors such as serum creatinine level, serum uric acid level, anaemia were also considered as predictors of preeclampsia [8, 9]. A Proper understanding of epidemiological and clinical risk factors of preeclampsia is essential for earlier diagnoses and follow-up of women at higher risk of this disease. Most published researches on preeclampsia were carried at good resource settings with only a few exceptions [3].

Previous researchers exploring blood group in women with preeclampsia revealed different findings [10]. While some studies reported the associations of different blood groups with preeclampsia, other studies failed to reach similar conclusions [11–15]. There are an extremely high maternal mortality and morbidity in Sudan, where 4.2% out of all obstetric complications and about one fifth of maternal deaths are attributed to preeclampsia/eclampsia [16–18].

The current study was carried out to assess the possible risk factors for preeclampsia including blood groups. The data obtained is of paramount importance for the health planners, health providers, and supports the previous research on preeclampsia in Sudan [19–22].

Material and Methods

A case – control study was conducted at Saad Abualila Hospital, Khartoum, Sudan. Saad Abualila Hospital is a tertiary care hospital for women who attended the prenatal care at the hospital as well as for referrals from the other clinics and hospitals. All women with high-risk pregnancy or obstetric/medical complications are referred to the hospital. Medical files were reviewed for the women during the period of July 2013 through December 2014. A case was defined as a woman who had preeclampsia, which was defined as hypertension associated with proteinuria in a pregnant woman. Hypertension was defined as blood pressure ≥ 140 mmHg systolic or diastolic blood pressure ≥ 90 mmHg diastolic emerging after 20 weeks of gestation in a woman who was normotensive before this period. Proteinuria was defined as excretion of ≥ 300 mg of protein in 24 h urine sample or ≥ 2+ on the dipstick. A consecutive file for each case was taken as control. The controls were pregnant women presented for delivery, with blood pressure values < 139/89 mmHg and no proteinuria recorded in the pregnant health card during the antenatal visits or at the time of delivery. Women with a twin pregnancy or diabetics were excluded because these are known predictors for preeclampsia [7].

The obstetric and medical history (age, parity, education, antenatal care, weight and height) were extracted from the files. Weight and height were used to calculate the body mass index, which was expressed as weight in kg/height in m^2. Blood groups subtypes were recorded from the files of both the cases and the controls and expressed as O, A, B and AB.

A total sample size of 145 participants in each arm of the study was calculated to investigate the difference rate of the O blood group in the women with preeclampsia and the controls. We assumed the rate of O blood as 56% and 40% for preeclampsia women and the controls depending on our previous work in the same setting [22]. This rate would provide 80% power to detect a 5% difference at α = 0.05, with an assumption that complete data might not be available for 12% of participants**.**

Statistics

SPSS for Windows (version 16.0) was used for data analyses. Studied variables were described with means (M) and standard deviations (SD). Proportions of the studied groups were expressed in percentages (%). The difference of mean (SD) and proportion was compared between the two studied groups using T-test and χ^2, respectively. Binary logistic regression analyses were performed, where preeclampsia was the dependent variable and medical/ obstetrics characteristics (age, parity, and residence), blood groups (O vs. non-O blood group), BMI were the independent variables. Odds ratio and 95% CI were calculated. P < 0.05 was considered statistically significant.

Ethics

The study received ethical clearance from the Research Board at the Department of Obstetrics and Gynaecology, Faculty of Medicine, University of Khartoum, Sudan

Results

Two hundred eighty pregnant women were enrolled (140 in each arm of the study). Around one-quarter of all women (280) were primiparae (74.0, 26.4%), the majority were housewives (201, 71.7%). Seventy-nine (28.2%) were illiterate or had no informal education. Around half of these women (130, 46.4%) had O blood group.

Table 1: Comparing the obstetrics and clinical characteristics of preeclamptic and controls women at Khartoum, Sudan

Variable	Preeclampsia (n = 140)	Controls (n = 140)	P
The mean (SD)			
Age, year	28.8 (6.2)	28.1 (6.0)	0.315
Parity	1.7 (2.1)	1.7 (1.4)	0.974
Body mass index, Kg/m^2	24.5 (3.0)	24.4 (2.2)	0.648
Number (%) of			
Education < secondary level	38 (27.1)	41 (29.3)	0.410
Lack of antenatal care	35 (25)	20 (14.3)	<0.001
O	86 (61.4)	64 (45.7)	
A	24 (17.1)	37 (26.4)	
B	27 (19.3)	26 (18.6)	0.007
AB	3 (2.1)	13 (9.3)	

There was no significant difference in the mean (SD) of the age, parity and BMI between women with preeclampsia and controls. Thirty-six (12.9%) and 104 (37.1) were severe and mild preeclampsia, respectively. Compared with the controls, significantly higher numbers of women with preeclampsia were illiterate and had O blood group, (Table 1, Fig. 1).

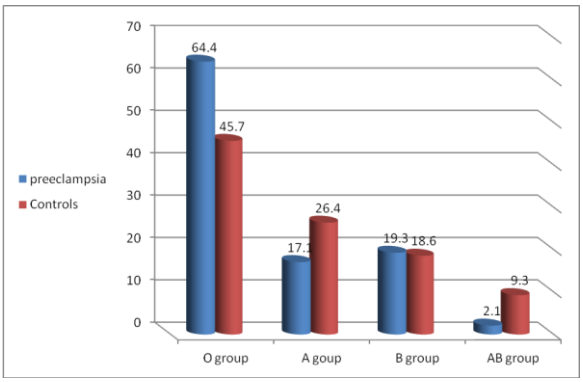

Figure 1: Percentage of blood group among preeclamptic and controls at Khartoum, Sudan

In binary logistic regression, women who had no antenatal care (OR = 2.75, 95%CI = 1.172–6.494, P = 0.020) and women with O blood group (OR = 1.78, 95%CI = 1.088–2.934, P = 0.022) were at higher risk of preeclampsia (Table 2).

Table 2: Binary logistic regression of the predictors of preeclampsia, Sudan

Variables	OR	95%CI	P
Age, year	1.05	1.001–1.113	0.048
Parity	0.88	0.739–1.050	0.158
Education < secondary level	1.19	0.647–2.203	0.571
Lack of antenatal care	2.75	1.172–6.494	0.020
Body mass index, Kg/m^2	0.98	0.897–1.090	0.821
O vs. none O blood group	1.78	1.088–2.934	0.022

Discussion

Perhaps this is the first study investigating the association between blood groups and preeclampsia in Africa. In this study, women who had no antenatal care were more likely to develop preeclampsia than those who had an antenatal follow-up. Previously, Bilano *et al.* described such a finding in their secondary analysis for the world health organisation facility based, a multi-country survey [23]. An explanation for such an association is the fact that antenatal visits help to detect and treat pregnancy-induced hypertension at an early stage before the development of complications.

An important finding of the current study is the association between blood group O and preeclampsia. This goes with the previous findings, where blood group O has been reported as a risk factor for preeclampsia among 386 cases and 342 controls [24]. Likewise, blood group A has been reported as a risk factor for preeclampsia [15].

A large Swedish cohort study showed that the blood group O is rather protective against preeclampsia [14]. Interestingly, a meta-analysis conducted by Franchini *et al.* demonstrated that the ABO system was consistently involved in preeclampsia. According to Franchini et al., the non-O blood groups are risk factors while blood group O is protective against preeclampsia [10].

There is a great controversy about whether blood groups could have an association with preeclampsia. Scott *et al.* found no association between the ABO system and preeclampsia in their case-control study on a population of 46 patients [25]. However, Scott *et al.* study had relatively small sample size compared with the other studies that established an association between the ABO system and preeclampsia. Scott *et al.,* results are further supported by Witsenburg *et al., a* report in 36 cases and 272 controls and Clark *et al.,* findings in 4250 pregnancies, 66 of which are preeclamptic women [11, 12]. A separate population-based nested case-control study exploring 248 study cases demonstrated AB blood group as a risk factor for preeclampsia [13].

In conclusion, the current study showed that women with blood group O were at higher risk of preeclampsia.

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Indexes of Insulin Resistance in Hyperinsulinemic Polycystic Ovary Syndrome in a Macedonian Cohort of Women of Reproductive Age: A Cross-Sectional Study

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Abstract

BACKGROUND: Polycystic ovary syndrome (PCOS) is complex hormonal, metabolic and reproductive disorder and is a leading cause of female infertility. Hyperinsulinemia secondary to insulin resistance plays important role in the pathogenesis of PCOS.

AIM: To assess the sensitivity of different indices of insulin resistance and their relevance in a clinical setting.

MATERIAL AND METHODS: A cross-sectional study of 43 patients with PCOS and 29 noromo ovulatory women as a control group was conducted. Standard clinical, anthropometrical and hormonal testing for hyperandrogenism was conducted, as well as oral glucose tolerance test with determination of basal and stimulated glucose and insulin values.

RESULTS: The dynamic I/G index showed the highest sensitivity and specificity, but the static indexes HOMA-IR and QUICKI, although based on only basal glycemic and insulinemic values, showed good sensitivity, 90.38% and 94.01% respectively. HOMA-IR showed significant positive correlation with the stimulated insulin values.

CONCLUSIONS: Our results support the use of static indexes in the evaluation of insulin resistance in women with PCOS in a clinical setting, offering a simple assessment of insulin resistance in PCOS, which holds great prognostic and treatment implications.

Introduction

Polycystic ovary syndrome (PCOS) is complex hormonal, metabolic and reproductive disorder affecting 10% of females of reproductive age and is a leading cause of female infertility [1]. PCOS is one of the most frequent endocrinologic dysfunctions in women of reproductive age, characterised by the association of polycystic ovaries, hyperandrogenism and chronic anovulation [2], [3]. Its aetiology remains unknown, but it is clear that hyperinsulinemia secondary to insulin resistance plays an important role in the pathogenesis of reproductive abnormalities [4]. PCOS is a diagnosis of exclusion and is defined by the Rotterdam classification from 2003 requiring at least 2 out of 3 criteria: oligo-ovulation and/or anovulation, clinical and/or biochemical hyperandrogenism and polycystic ovaries on ultrasound [5].

As early as 1980, Burgen reported that women with PCOS have basal and glucose-stimulated hyperinsulinemia and noted a significant positive linear correlation between the insulin and androgen concentrations in women with polycystic ovarian syndrome [6]. Insulin resistance and hyperinsulinemia is evident both in lean and obese patients with PCOS [7], [8]. The prevalence of insulin resistance in PCOS is approximatively 50-60%, compared to 10-20% in the general population [9]. It is still controversial whether the hyperinsulinemic state stimulates the excessive ovarian production of androgens or if the chronic hyperandrogenemic milieu promotes insulin

resistance. Despite obesity, present in more than half of the patients, decreased insulin-stimulated glucose utilisation regardless of body mass index has been demonstrated in polycystic ovary syndrome [8].

Several mechanisms have been postulated to explain the correlation between the hyperinsulinemia and hyperandrogenemia in PCOS. Studies have shown a strong negative correlation of basal insulin with the levels of sex hormone binding globulin (SHBG), suggesting that the insulin has an inhibitory effect on the hepatic production of SHBG, decreasing its levels and leading to increased bioactivity of testosterone in its free unbound form that emphasises the hyperandrogenic characteristics. Insulin resistance also predisposes to a visceral type of adiposity, reflecting the androgen-like phenotype of PCOS [10], [11]. The latest studies revealed unique post-receptor defects in the insulin signalling pathways in PCOS. Fibroblast cell lines from women with PCOS have significantly decreased insulin-stimulated glucose incorporation into glycogen, but similar insulin-stimulated thymidine incorporation, compared to cell lines from reproductively normal control women. This suggests that there is a selective defect in insulin action in PCOS that affects the metabolic, but not the mitogenic actions of insulin [4].

Insulin resistance imposes not only disturbances of the glucose metabolism but is also associated with a tendency towards atherogenic dyslipidaemia and vascular endothelial dysfunction. Approximately, 25% to 30% of women with PCOS will show impaired glucose tolerance by the age of 30 and 8% of affected women will develop type 2 diabetes annually [12]. Women with PCOS are seen to have more extensive coronary artery disease by angiography. Hypertension is also observed more frequently in these women [13]. Therefore the testing for insulin resistance becomes important and integral part of the evaluation of patients with PCOS.

The aim of this study was to assess the sensitivity of different indices of insulin resistance and their relevance in a cross-sectional study of patients with PCOS and noromo ovulatory women as a control group.

Material and Methods

A cross-sectional study was conducted at the University Clinic of Endocrinology, Diabetes and Metabolic Disorders in Skopje, Macedonia, in the period between October 2012 and May 2014. In this study, 43 patients were enrolled with hyperinsulinemic PCOS and a control group of 29 noromo ovulatory women with regular menstrual cycles, without clinical or biochemical signs of hyperandrogenism and no prior known endocrinologic diseases. PCOS was diagnosed according to the criteria from The Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group [5]. Patients presenting with thyroid disorders or neoplastic causes of hyperandrogenemia such as androgen-secreting tumours, congenital adrenal hyperplasia and Cushing's syndrome were excluded from the study.

Patients with PCOS were grouped according to body mass index (BMI) into PCOS with normal BMI ≤ 25 kg/m^2 (PCOS N) and PCOS with high BMI > 25 kg/m^2 (PCOS H).

Hormonal parameters were assessed in the follicular phase of the menstrual cycle or at any given day in women with absent menstrual cycle in the previous two or more months. Blood samples for hormonal and biochemical analyses were obtained by venipuncture between 08:00 and 10:00 h, after an overnight fast of 12 hours. Anthropometrical measurements and clinical assessment of signs of hyperandrogenism were conducted at the visit. Transvaginal ultrasound scan of the ovaries was performed at the University clinic of Gynaecology using a 6,5 MHz transducer in order to determine the total number of early antral follicles. All patients underwent an oral 75g glucose tolerance test (OGTT) after an overnight fast of at least 12 hours during which basal values of glucose and insulin were measured at baseline, as well as post-load glucose and insulin values at 60 and 120 minutes.

Serum estradiol (E_2), luteinizing hormone (LH), follicle stimulating hormone (FSH), testosterone (T), androstenedione (A), dehidroepiandrostenedione-sulphate (DHEA-s) and prolactin (PRL) levels were measured by electrochemiluminescence immunoassay on a Roche Elecsys 1010/2010 automated immunoassay analyser. Sex hormone binding globulin (SHBG) measurements were performed by enzyme-linked immunosorbent assay on an IMX Abbott semiautomatic analyser (values of these tests are not shown in results section). Insulin was measured in serum with microparticle enzymatic assay (MEA) on the semiautomatic analyser IMX Abbot. Glucose was determined in plasma with glucose-oxidase method on the glycemic analyser Beckmann. Free androgen index (FAI) was calculated using the standard formula: testosterone/SHBG × 100 (values of these tests are not shown in results section).

Established direct methods for measuring insulin sensitivity in vivo are relatively complex. Therefore, simple surrogate indexes for insulin sensitivity/resistance which is derived from blood insulin and glucose concentrations under fasting conditions (steady state) or in the postprandial state (dynamic) was calculated. Based on the results of basal and post-load glucose and insulin levels obtained during OGTT, several indexes of insulin resistance were calculated for all of the patients.

Homoeostasis model of insulin resistance (HOMA-IR) was calculated using the formula: (fasting insulin (μU/ml) x basal glucose (mmol/l))/22.5. The quantitative insulin sensitivity check index (QUICKI) was derived using the inverse of the sum of the logarithms of the fasting insulin and fasting glucose, using the formula: 1/(log(fasting insulin μU/mL) + log(fasting glucose mmol/l)). The sum of insulin measured at 0, 60 and 120 minutes divided by the sum of glucose at 0, 60 and 120 minutes was calculated as a dynamic index I/G [14].

All statistical procedures were run using the StatSoft Statistica 7 software. Statistical significance was set at $p < 0.05$. Normality of distribution was evaluated with the one-sample Kolmogorov-Smirnoff test. Comparisons of means were performed with one-way ANOVA and general linear model multi-variance. Correlations were evaluated with the calculation of the Spearman coefficient.

Results

In this study, 43 patients were diagnosed as having PCOS according to the Rotterdam diagnostic criteria and had basal or stimulated hyperinsulinemia during OGTT while the control group consisted of 29 women with normal glucose and insulin values during OGTT and normal hormonal status. The basic clinical data for the patient groups (PCOS N, PCOS H and control group) as well the results for insulinemia and glycaemia during the OGTT are shown in Table 1.

Table 1: Basal clinical, anthropometrical, hormonal and metabolic values in the three groups

	Control group	PCOS N BMI ≤ 25 kg/m^2	PCOS H BMI >25 kg/m^2
n	29	12	31
Age (years)	22.36 ± 3.82	21.42 ± 3.94	24.97 ± 6.29
BMI (kg/m^2)	23.16 ± 3.24	23.75 ± 1.22	33.13 ± 5.56*
Menstrual cycle length (days)	49 ± 2.6	77.92 ± 64.39	67.76 ± 45.29
Testosterone (ng/ml)	1.32 ± 0.77	1.5 ± 1.4	1.72 ± 0.95
Fasting insulin (μU/ml)	10.45 ± 2.63	24.15 ± 35.57	22.89 ± 7.77
Stimulated insulin at 60 min (μU/ml)	44.95 ± 20.11	148.74 ± 80.87	166.95 ± 96.68
Stimulated insulin at 120 min (μU/ml)	28.85 ± 9.17	102.65 ± 78.6	148.96 ± 102.66
Fasting glucose (mmol/L)	4.43 ± 0.58	4.75 ± 0.7	4.84 ± 0.63
Stimulated glucose at 60 min (mmol/L)	5.65 ± 1.04	6.96 ± 1.66	8.26 ± 2.32
Stimulated glucose at 120 min (mmol/L)	4.85 ± 072	5.75 ± 1.05	6.63 ± 2.06

*, $p < 0.001$.

Most of the patients were in the 21 to 30 years age group, with an average age of the patients in the PCOS H group higher (24.97 ± 6.29 years) in comparison to the PCOS N group, but not statistically significant. A statistically significant difference ($p<0.001$) was found in the BMI of patients with PCOS H in comparison to the control group. There was no statistically significant difference in the glycemic status between the groups, but still, the PCOS H group had higher average basal glycaemia values and more markedly higher glycemic excursions post load (Fig. 1).

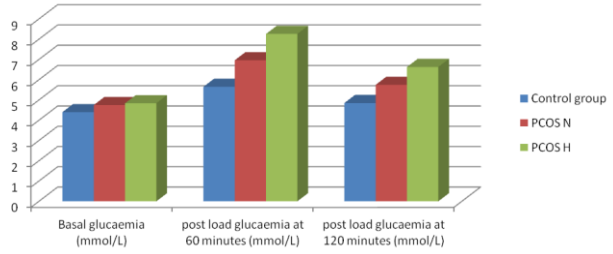

Figure 1: Basal and post load glycemic values in the groups

The PCOS group showed both basal and post-load hyperinsulinemia. The stimulated insulin values were significantly higher both in PCOS N and PCOS H groups compared to the controls, independent of obesity status, which was used as criteria for group division (Fig. 2).

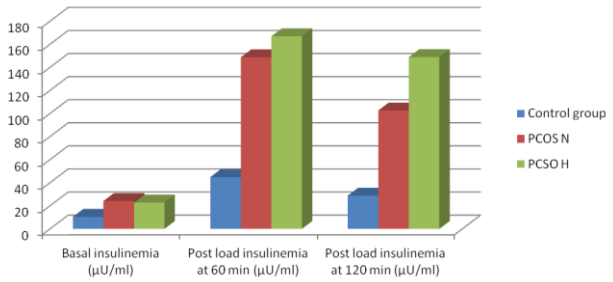

Figure 2: Basal and stimulated insulin values in the groups

There was no statistically significant difference between the values of fasting glycaemia between the groups, although higher post load glycemic excursions were noted in PCOS patients, especially at 60 minutes during OGTT. There was no significant difference in the degree of stimulated hyperinsulinemia in lean and obese patients with PCOS (Table 2).

Table 2: Statistical difference in the basal and stimulated glucemic and insulinemic values between lean and obese PCOS patients

	Mean PCOS N	Mean PCOS H	St.D. PCOS N	St.D. PCOS H	t-value	p
Age	21.42	24.97	3.94	6.29	-1.81	0.076
BMI	23.75	33.13	1.21	5.56	-5.75	<0.001*
MNZ	77.92	67.76	64.38	45.30	0.57	0.568
Testosterone	1.50	1.72	1.40	0.95	-0.56	0.579
Basal insulin	24.15	22.90	35.57	7.77	0.19	0.851
Stimulated insulin at 60'	148.74	166.95	80.87	96.69	-0.58	0.566
Stimulated insulin at 120'	102.65	148.96	78.61	102.66	-1.41	0.166
Basal glucose	4.74	4.84	0.70	0.63	-0.42	0.674
Stimulated glucose at 60'	6.94	8.26	1.67	2.32	-1.74	0.090
Stimulated glucose at 120'	5.75	6.63	1.06	2.06	-1.34	0.189

*, $p < 0.001$.

For the PCOS groups, three indexes of insulin resistance were calculated: homoeostasis model of insulin resistance (HOMA-IR) and the quantitative insulin sensitivity check index (QUICKI) as static indexes and the sum of insulinemia versus the sum of

glycaemia (I/G) as a dynamic index. All of the values were compared to the values obtained in the control group and sensitivity and specificity were calculated for each index. The results for sensitivity and specificity of insulin indexes in the entire PCOS group are given in Table 3.

Table 3: Sensitivity and specificity of indexes of insulin resistance

	I/G	HOMA-IR	QUICKI
Control group	5.65 ± 1.47	2.06 ± 0.65	0.61 ± 0.04
PCOS N	15.78 ± 11.13	5.02 ± 7.2	0.59 ± 0.14
PCOS H	17.17 ± 9.6	4.92 ± 1.75	0.49 ± 0.03
PCOS all	17.42 ± 9.91	4.2 ± 3.84	0.51 ± 0.07
Sensitivity %	96.02	90.38	94.01
Specificity %	92.64	84.09	86.21

The I/G index showed the highest sensitivity and specificity, being derived from both basal and post load values of glycaemia and insulinemia. Although the static indexes HOMA-IR (90.38%) and QUICKI (94.01%) are based on only basal glycemic and insulinemic values, they showed good sensitivity. HOMA-IR values also showed significant positive correlation with the insulin values at 60 minutes ($r = 0.42$; $p < 0.005$) and at 120 minutes post load ($r = 0.52$; $p < 0.0003$) during OGTT (Fig. 3).

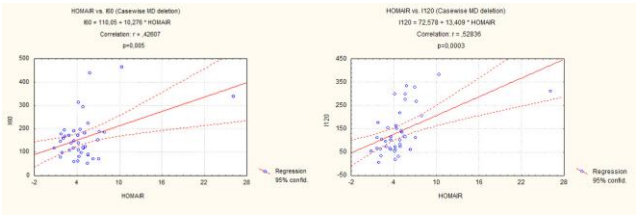

Figure 3: Correlation of HOMA-IR with post-load insulinemic values at 60 and 120 minutes

Discussion

The polycystic ovary syndrome is not only the most common underlying cause of anovulation in women in the reproductive period but is also associated with characteristic dysfunctions of the insulin action that have an important impact on the possible metabolic disturbances appearing later in life in women with PCOS. The association between the impaired carbohydrate metabolism and hyperandrogenism was first described in 1921 by Archard and Thiers as “diabetes in a bearded female” [15]. Kierland in 1947 described the characteristic skin lesion, acanthosis nigricans, which can be seen in women with hyperandrogenism and in patients with diabetes, as an epiphenomenon of the insulin resistance [16]. Dunaif et al investigated the characteristics of hyperandrogenic women with acanthosis nigricans; they found impaired glucose tolerance in 20% of the patients [17]. According to the revised criteria of the world health organisation (WHO), later studies determined the prevalence of impaired glucose tolerance of 20-40% in women with PCOS, compared to 5.3% prevalence of impaired glucose tolerance in the control population [18]. This high incidence led towards more extensive research of the role of insulin resistance in PCOS.

Insulin resistance is seen in 50-60% of women with PCOS, while almost 10% of them have a certain degree of glucose intolerance or diabetes at the time of diagnosing the syndrome [9], [19]. Pesant et al, reported that during three years follow-up period, up to 25% of women with PCOS, initially with normal glucose metabolism, will develop some degree of glucose metabolism abnormalities [20]. In our study, we have demonstrated that the fasting glycaemia is not a marker of metabolic dysregulation, since there was no statistically significant difference between the values of fasting glycaemia between the groups, although higher post load glycemic excursions were noted in PCOS patients, especially at 60 minutes during OGTT. Our data confirm that insulin resistance is not exclusively seen in obese patients with PCOS, but marked post load hyperinsulinemia was also noted in lean patients with PCOS. There was no significant difference in the degree of stimulated hyperinsulinemia in lean and obese patients with PCOS, confirming the fact that insulin resistance in PCOS is independent of obesity, which was taken as a group dividing factor.

Insulin resistance is characterised by an inability of normal amounts of insulin to achieve the normal predicted response, often in the clinical setting of central adiposity. To achieve euglycemia, the pancreas over secretes insulin [21]. Investigators define insulin resistance based on hyperinsulinemic-euglycemic clamp techniques. Hyperinsulinemic-euglycemic clamp techniques rely on an intravenous insulin infusion to maintain steady serum glucose concentrations at fasting levels to measure glucose uptake. Lower glucose uptake signifies resistance to insulin action (insulin resistance). Since the technique requires intravenous infusions, frequent blood sampling, extensive time and significant financial resources, it is experimentally useful but rarely applicable in a clinical setting [22]. Therefore, many derived indexes for assessment of insulin resistance have been proposed. Clamp techniques have been used as comparisons to validate other modes of assessment of insulin resistance.

The homeostatic model assessment of insulin resistance (HOMA-IR) has been compared to clamp techniques with good results [22]. One major limitation of HOMA rests on the fact that many adolescents with PCOS display stimulated but not fasting metabolic abnormalities. In fact, HOMA in young PCOS patients missed 50% of insulin resistance as compared to OGTT with insulin-AUC calculations [23]. I/G ratio correlated strongly with clamp-demonstrated insulin resistance in a small study of PCOS women, showing

evidence of insulin resistance in both lean and obese women with PCOS [24]. Quantitative insulin sensitivity check index (QUICKI) was developed to improve the sensitivity of fasting measurements. QUICKI has been shown to correlate well to clamp measurements in obese and non-obese patients [25]. QUICKI also demonstrates correlation with HOMA-IR. In our study, the I/G ratio showed the highest sensitivity, which was expected because the index calculations are based on both basal and simulated values of insulin and glucose, but the static indexes showed also very high sensitivity. The sensitivity and specificity of HOMA-IR were comparable to the QUICKI index, bearing in mind the fact that our studied population did not include adolescents. The correlation of HOMA-IR with the stimulated insulin values indicates that this index gives a relevant value of the level of insulin resistance in women with PCOS.

Our results are in accordance with several studies that support the use of static indexes of insulin resistance in the evaluation of insulin resistance in women with PCOS in a clinical setting. Furthermore, the method is simple, quick and easy to execute in contrast to the complicated and expensive hyperinsulinemic-euglycemic clamp. The diagnosis of insulin resistance holds great prognostic and treatment implications. All women with PCOS should be screened for the presence of insulin resistance as well as assessed for other stigmata of the insulin resistance syndrome such as hypertension, dyslipidemia, central obesity, and glucose intolerance.

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Clinical Science

Transcatheter Closure of Patent Foramen Ovale: A Single Center Experience

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Abstract

BACKGROUND: Percutaneous transcatheter closure (PTC) of patent foramen ovale (PFO) is implicated in cryptogenic stroke, transitional ischemic attack (TIA) and treatment of a migraine.

AIM: Our goal was to present our experience in the interventional treatment of PFO, as well as to evaluate the short and mid-term results in patients with closed PFO.

MATERIAL AND METHODS: Transcatheter closure of PFO was performed in 52 patients (67.3% women, mean age 40.7 ± 11.7 years). Patients were interviewed for subjective grading of the intensity of headaches before and after the PFO closure.

RESULTS: During 2 years of follow-up, there was no incidence of new stroke, TIA and/or syncope. Follow-up TCD performed in 35 patients showed complete PFO closure in 20 patients (57.1%). Out of 35 patients, 22 (62.9%) reported having a migraine before the procedure with an intensity of headaches at 8.1 ± 1.9 on a scale from 1 to 10. During 2 years of follow-up, symptoms of a migraine disappeared in 4 (18.2%) and the remaining 18 patients reported the significant decrease in intensity 4.8 ± 2.04 ($p = 0.0001$). In addition, following PFO closure the incidence of the headaches decreased significantly ($p = 0.0001$).

CONCLUSIONS: Percutaneous transcatheter closure of PFO is a safe and effective procedure showing mid-term relief of neurological symptoms in patients as well as significant reduction of migraine symptoms.

Introduction

Patent foramen ovale (PFO) is a tunnel-shaped opening between the atria that helps blood circulate avoiding the lungs during intrauterine life. Most of the PFOs spontaneously close during the first year of life, but some may remain open later in life. In an autopsy study including 965 normal hearts of the general population, the prevalence of PFO was 27.3% with progressive decline as age increases (34.3% in the first three decades, 25.4% in the 4th to 8th decade, and 20.2% in the 9th and 10th decade) [1]. Until recently PFO was thought to be an anatomical variant in different people, however, more and more evidence puts into question the benign nature of this anomaly and the relative importance of the defect is being reevaluated [2-4].

Increasing medical evidence is recognising the PFO as a conduit for a paradoxical embolism resulting in cryptogenic stroke (CS) and transitory ischemic attack (TIA) [5]. There are studies suggesting the connection of PFO with a migraine with aura, orthodeoxia-platypnea and decompression illness in divers [6-8]. The pathophysiology of the neurological events associated with a paradoxical embolism may be due to the direct transfer of venous blood through the PFO into the left heart chambers during increased right heart pressure (Valsalva manoeuvre) [9]. The venous blood contains microthrombi from the lower extremities which are usually filtered through the pulmonary capillaries,

"purifying" the blood returning to the left heart chambers before it enters the cerebral vasculature. During activities associated with increased right heart pressure (diving, squatting, coughing) the certain portion of this microthrombi filled blood enters the left heart via PFO and ends up in cerebral arteries causing microinfarcts. These can manifest as stroke, TIA or provoke migraine attacks [10, 11].

Up to 21% of women and 6% of men suffer from migraine attacks and 2/1000 people over 65 years will have an ischemic stroke [12]. Up to one-third of ischemic strokes are "cryptogenic" because the cause cannot be identified. A reason for this cryptogenic stroke could be a paradoxical embolism through a PFO. In younger patients with stroke, a 4-fold greater incidence of PFO has been detected compared to a stroke-free control group of the same age and sex [13]. Co-existence of PFO with certain interatrial septal abnormalities like atrial septal aneurysm (ASA) and Chiari's network increases the risk of stroke [9, 14-17]. Systematic reviews have suggested the significant association between a migraine and stroke [18], PFO and stroke [19] as well as PFO and migraine [20, 21].

Percutaneous closure of PFO was first described as a derivative from atrial septal defect closure in 1992 [22]. While initial experiences with different devices were disappointing, in 1997 the Amplatzer PFO Occluder was introduced and the interventional technique of transcatheter PFO closure started showing promising results [23]. There are published randomised clinical trials in transcatheter closure of PFO that failed to reach the primary endpoint [23-25]. However, the RESPECT (Randomized Evaluation of Recurrent Stroke Comparing PFO Closure to Established Current Standard of Care Treatment) trial showed a 7-fold higher stroke rate in the on-PFO closure group with extremely low complication rates of the procedure. Sub-analysis showed significant reduction of stroke rate in patients with larger sized PFO and atrial septal aneurysm [26-28]. There are 3 ongoing clinical trials with a total cohort of approximately 1700 patients in France, South Korea and Canada comparing interventional and medical management of PFO.

Close analysis of all published data shows that interventional treatment of PFO is superior to medical treatment in the prevention of stroke and with the new devices and the low complication rate of the procedure one would expect greater implication of transcatheter closure technique for PFO [30].

We set out to examine our experience with PTC treatment of PFO, hypothesising that percutaneous transcatheter closure is a safe and beneficial medical intervention for preventing stroke, TIA and reducing migraine symptoms.

Material and Methods

Between 2011 and 2013, 52 patients underwent PTC of PFO. Stroke was diagnosed in 15 (28.8%), repetitive TIA in 17 (32.7%), repetitive syncope during activities that involved Valsalva manoeuvre in 10 patients (19. 2%), migraine with aura in 7 (13.5%) and without aura in 3 (5. 8%) patients.

All of the patients underwent initial diagnostic testing with TCD. We grouped the positivity of the procedure by the number of bubbles that entered cerebral vasculature after Valsalva manoeuvre. The first group was a very positive test, that is, more than 40 bubbles, the second group was with moderate positivity, from 20 to 39 bubbles, and the third group was with mildly positive transcranial Doppler test, that is, from 1 to 19 bubbles (Fig. 1).

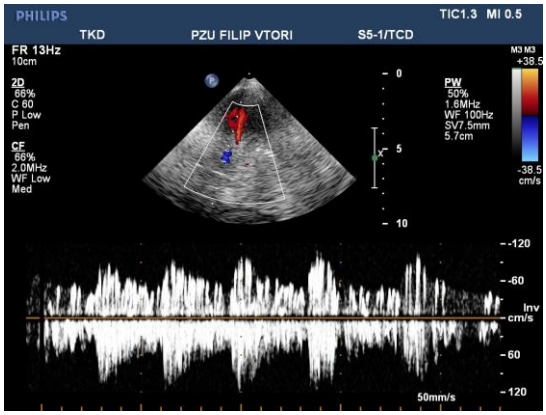

Figure 1: Transcranial doppler ultrasound (TCD) in a patient with large PFO (over 40 bubbles)

The patients with the moderate and severe positivity of TCD who had an indication for PFO closure were further referred for TEE using the agitated saline solution as a contrast, where the presence of PFO was validated and measurements for the size of the device were taken (Fig. 2). Most of our patients had cerebral imaging performed with brain magnetic resonance imaging (MRI) or multislice computerised tomography (MSCT).

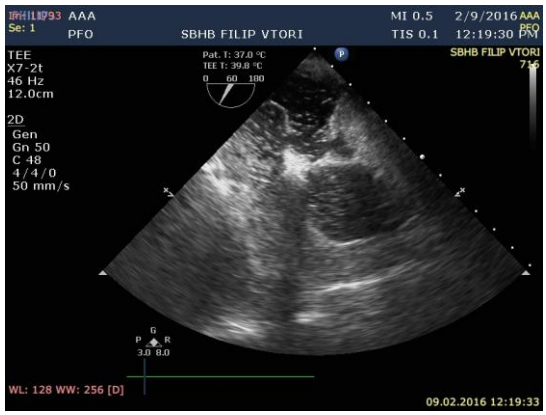

Figure 2: Transesophageal echocardiography (TEE) in a patient with PFO

The transcatheter closure of PFO was performed using local anaesthesia. The patients were informed of the procedure and a written consent was obtained. The PFO was crossed under fluoroscopic guidance through the femoral vein, with 5 F multipurpose catheter and hydrophilic wire, which was exchanged with stiff Amplatz wire. In large PFOs, we used balloon sizing before device implantation. We placed 4 (7.6%) Amplatzer PFO devices and 45 (86.5%) Cera Occluder (Fig. 3). In 3 (5.8%) patients, we implanted an ASD septal occluder, due to a large PFO. All of the patients received a prophylactic dose of antibiotics. Patients were placed on dual antiplatelet therapy with aspirin 100 mg daily and clopidogrel 75 mg daily for 3 months. After 3 months the clopidogrel was discontinued in all patients and aspirin therapy was maintained for an additional 3 months. Endocarditis prophylaxis was recommended for the first 6 months after the procedure. Discharge from the hospital took place one day after the transthoracic (TTE) or TEE examination.

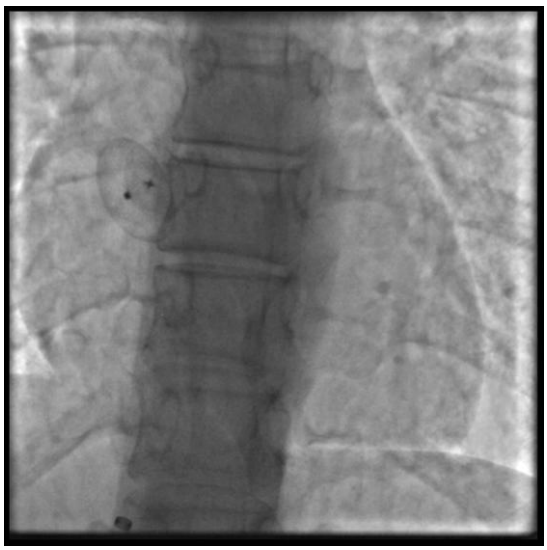

Figure 3: Percutaneous implantation of a PFO occluder

The patients had echocardiographic follow-up at 7 days, 1 and 6 months after the procedure. Follow-up TCD ultrasound was performed 6 and 24 months after the procedure. We performed electroencephalography (EEG) on all patients before the intervention. During the 2 year follow-up, patients were screened for neurological symptoms and events (CVI, TIA, syncope, migraine) during ambulatory visit according to the up-to-date guidelines of referent professional societies. After the 24 month follow-up period, we did a follow-up TCD, EEG and questionnaire interview for headaches in 35 patients. The remaining 17 patients were either unable or refused to come for follow up appointments and were only screened for neurological receives (TIA, stroke or syncope) according to their official clinical diagnosis by telephone interview. The thirty-five patients were interviewed for migraine severity graduation before and after the procedure. The evaluation was based on a questionnaire with a subjective grading of the intensity of headaches on a scale of 1 to 10, as well as frequency (1-10) of more than 10 attacks per month, and less than one time per month.

The study was approved by our ethics committee and participants signed informed consent documents.

Statistics

Continuous variables are expressed as mean value + SD and categorical variables as counts and percentages. Wilcoxon's signed rank test was used to compare migraine characteristics before and after percutaneous PFO closure. 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

In 52 patients an occluder was successfully placed using the PTC technique. There were two minor complications (femoral hematoma treated conservatively and hypotension in one patient). We had one patient that complained of chest pain that started 24 hours after the intervention and lasted for a month. We assigned the symptom as a possible side effect of the device implantation.

The follow-up TCD performed in 35 of the patients showed complete PFO closure in 20 (57.1%), whereas 12 patients (34.3 %) had mild (1-19 bubbles) and 3 patients (8.6 %) had moderate (20-39 bubbles) residual shunt. Over the 2 year follow-up, none of the patients had the recurrence of the neurological symptoms represented as CVI, TIA and/or syncope.

From 35 patients that were interviewed, 22 (62.9%) reported having a migraine before the procedure. Subjective grading with the questionnaire showed the average intensity of headaches to be 8.1 ± 1.9, on a scale from 1 to 10 (Table 1). Following the procedure, over a 2 year of follow-up, symptoms of a migraine ceased completely in 4 patients (18.2%) and the remaining 18 (81.8%) patients reported the significant decrease in intensity to 4.8 ± 2.0 ($p = 0.0001$) (Table 1). As for the frequency of headaches, before the procedure, it was graded as less than one attack per month in 3 patients (13.6%), between 1 and 10 attacks per month in 5 patients (22.7%), and more than 10 attacks per month in 14 patients (63.6%). After the procedure, frequency of headaches decreased significantly with 15 patients (88.2%) having attacks less than once per month, 1 patient (5.9%) having attacks between 1 to 10 times per

month, and 1 patient (5.9%) having attacks more than 10 times per month (p = 0.0001) (Table 1).

Table 1: Comparison of intensity and frequency of the headaches before and after PFO closure

	Before closure	After closure	*p* Values*
Migraine intensity (0-10)	8.14 ± 1.9	4.8 ± 2.0	0.0001
Frequency of headaches attacks (n/%)			
<1 per month	3 (13.6%)	15 (88.2%)	
1-10 per month	5 (22.7%)	1 (5.9%)	0.0001
>10 per month	14 (63.6%)	1 (5.9%)	

*Before *vs*. after closure.

In all patients who had EEG before the PFO closure, where findings were normal in 2 (3.8%) of the patients, 20 (38.5%) patients presented with irritative changes, 6 (11.5%) patients had changed with focal activity and paroxysmal discharge was seen in 24 (46.1%) patients. We performed follow-up EEG in 35 of the patients after 24 months of transcatheter closure of the PFO. EEG findings improved in 23 (65.7%) of the patients and in 12 (34.3%) there were no changes in the EEG after the intervention.

Discussion

Whilst CLOSURE I (Evaluation of the STARFlex Septal Closure System in Patients with a Stroke and/or Transient Ischemic Attack due to Presumed Paradoxical Embolism through a Patent Foramen Ovale) [24] as the first randomized trial published on PFO closure showed that closure with a device did not offer a greater benefit than medical therapy alone for the prevention of recurrent stroke or TIA, results of later randomized trials showed that concept of PFO closure to reduce recurrent strokes has been proven [31, 32]. The difference was subjected to mainly shorter fixed 2-year follow-up period and especially lower quality of STARFLex device used in CLOSURE I in comparison to Amplatzer devices used in later studies. The meta-analysis of Stortecky et al. included four randomised trials that included 2963 patients with 9309 patient-years. The Amplatzer device showed superiority compared to the other two devices (STF and HLX) and patients who were allocated to PFO closure with Am player were less likely to experience a stroke than patients on medical therapy [rate ratio (RR) 0.39; 95% CI: 0.17-0.84]. However, PFO closure with any of the three devices showed better probability in preventing stroke compared to medical therapy [29]. The study of Wahl et al. [25] showed that PFO closure performs better than medical treatment. In our study, there was no recurrence of CVI or TIA during a 24-month follow-up period.

The PREMIUM trial (Prospective Randomized Investigation to Evaluate Incidence of Headache Reduction in Subjects With Migraine and PFO Using the AMPLATZER PFO Occluder to Medical Management) was a randomized trial conducted in 29 centers in the United States and enrolled patients between 18 and 64 years of age who had diagnosis of migraine, ranging from 6 to 14 attacks per month, with or without aura. The patients had PFO documented with TCD and their symptoms were unresponsive to medical therapy. A total of 226 patients had the femoral puncture and while some of them received the occluder, the remaining underwent the sham procedure. Patients and neurologist were blinded to the randomization. The results of the trial did not meet the pre-specified primary endpoint of 50% reduction in migraine attack frequency in 117 patients compared to 103 who received a sham procedure and medical management. However, some secondary endpoints did achieve significance and 8.5%-11% of patients had complete migraine remission. Most of the patients who had complete remission had a migraine with aura. The conclusion of the study was that a migraine with aura might need different therapeutic approach than a migraine without aura and that maybe the key to different responses to PFO closure lies in the different anatomies of PFO and adjacent structures [33]. In our study migraine ceased completely in four patients and three of them were with a migraine with aura.

The PRIMA trial (Percutaneous Closure of PFO in Migraine with Aura) was conducted in 20 cities around the world and was prospective, randomised trial that included 107 participants with a migraine with aura who failed medical therapy. Fifty-three of them had PFO closure with Amplatzer device and 54 received medical therapy. PFO closure was associated with the mean reduction of migraine days of -2.9 compared to -1.7 for medical therapy (P = 0.17). There was numerical but not statistical significance. The responder rates were 37.5% for interventional treatment group and 14. 6% for conservative treatment group (P = 0.02). There was a complete resolution of a migraine in 10% of the interventional group compared to 0% in medical therapy group (P < 0.05). However, looking only at patients free of migraine attacks with aura the difference was pronounced with 40% in the PFO closure group and 10% in the control group (P = 0.004). In PRIMA, the reduction of migraine attacks with aura and migraine days with aura was greater than the reduction of all types of migraine attacks and days. Therefore, this unequal and greater effect on migraine with aura is likely a true effect of PFO closure. There was only one vascular major complication and one transient atrial fibrillation reported from the interventional procedure [34].

A Recent meta-analysis conducted by Kent at al. showed that among patients with PFO and cryptogenic stroke, closure reduced recurrent stroke

and had statistically significant effect on the composite of stroke, TIA and death in the adjusted analysis [35].

In our study 22 out of 35 patients had migraine attacks. Ten of them went through the procedure only because of a migraine, but 12 had additional CVI, TIA or repetitive syncope. The closure of the PFO significantly reduced the intensity and the frequency of the headaches (Table 1). Four patients (18.2%) reported the complete absence of headaches, while two patients (9%) reported no change in intensity; however, they did report lower frequency of occurrence. The rest of them reported the significant reduction of intensity and frequency.

The interventional procedure of PFO closure is relatively efficient, depending on the type of the occluder used, with a very low complication rate [36-38]. In our experience, we had only two minor complications (3.8%) and no major complications. There was one case of larger hematoma due to a femoral puncture and it was treated conservatively. The second minor complication was a patient with hypotension and confusion after the procedure, which was resolved with intravenous fluids. The follow-up MSCT of the brain showed no new vascular lesions.

The limitations of our study included the use of a non-randomized sample, a small sample size and the possibility of recall bias for the patient-reported symptoms.

In conclusion, in spite of all the published data until today, we still do not have clear evidence-based recommendations for PFO closure [39, 40]. However, with the usage of new devices and recently published studies, PFO closure procedure is more beneficial than harmful. Calculations say that in young people with a life expectancy of 50 years, 2.4 PFOs need to be closed in order to prevent one stroke. The availability of new devices and the very low complication rates of the procedure suggest that perhaps we should be more proactive towards PFO screening and closure. Percutaneous transcatheter closure of PFO is a safe and effective procedure showing mid-term relief of neurological symptoms (CVI, TIA), and significant reduction of migraine symptoms. However, there is a necessity of more studies showing results of long-term follow-up.

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Clinical Science

The Prevalence of Allergic Rhinitis, Eczema and Asthma in Students of Guidance Schools in Mazandaran Province, Iran

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Abstract

BACKGROUND: Eczema, allergic rhinitis and asthma are common chronic allergic disorders in childhood.

AIM: The aim of this study was to determine the prevalence of common allergic disorders among Iranian guidance schools students in Mazandaran Province, northern Iran.

METHODS: This analytical cross-sectional study was performed on 3000 children aged 11-14 years old during 2012-13 according to ISAAC study. Of 3000 recruited children 1576 (52.54%) were female and 1424 (47.46%) were male. Data gathered by ISAAC first phase questionnaire analysed by SPSS software 20.

RESULTS: The prevalence of wheezing, allergic rhinitis symptoms (sneezing and pruritus) and atopic dermatitis symptoms (pruritus skin lesion) were 30.5%, 30% and 15% respectively. History of pets contact and smoking was positive 6.6% and 36 % respectively. About 52% was born with caesarian section. There was wheezing in 32.5% during sport. The diagnosis of asthma, allergic rhinitis and eczema were 12.2%, 28.5% and 15% respectively. Eczema, asthma and allergic rhinitis were significantly more common in boys students ($p < 0.05$).

CONCLUSIONS: The results of this study showed that asthma, allergic rhinitis and eczema have a high prevalence and they are more common in boys.

Introduction

Asthma, allergic rhinitis (AR) and eczema or atopic dermatitis (AD) are the most common chronic diseases in childhood [1]. Allergic disorders are encountered with variable prevalence in different parts of the world. The prevalence of childhood asthma and other atopic disorders has been increased in recent decades [2]. In Previous epidemiological studies, the prevalence of asthma among elementary and guidance schools students in this region was reported 12-17% [3, 4] but in a meta-analysis, the prevalence of asthma in Iranian children was 7.6% and 10.7% in elementary and guidance school students respectively [1]. Another study estimated that asthma affects 2.7 to 35.4% of children (in average 13.4%) in Iran [5]. More than 300 million people in the world currently have asthma [5].

Clinical manifestations of AR include rhinorrhea, pruritus; sneezing and congestion and the disease is more common among children. The pooled prevalence of AR in children 6-7 years of age was 11.9% and in children aged 13-14 was 21.2 % [6].

AD is the most common chronic dermatologic disease in children associated with pruritus and marked by remissions and exacerbations. In a meta-analysis study, the prevalence of eczema in children aged 6-7 years and 13-14 years was 5.98% and 6.52%, respectively [7].

Allergic disorders pose the heavy social-economic burden on family and society. Asthma is the most common disease that observed in emergency rooms, hospital admission and abstinence of school. It

has a high economic burden on patients, their family, society and healthcare resources in different countries [5].

Although the exact etiologies of allergic diseases are unknown, both genetic and environmental factors have been included [8]. In children with asthma, environmental factors especially viral infection (24-34%), indoor and outdoor includes; air pollutants, cold, tobacco smoking, moulds and pets are main trigger factors [9].

The aim of this study was to determine the prevalence of common allergic disorders including asthma, allergic rhinitis and eczema and also to evaluate a few environmental factors such as BMI, smoking and delivery among students of guidance school in the region of northern Iran.

Material and Methods

We conducted an analytic cross-sectional prospective study which has been carried out on urban guidance students (11-14 years old) in the north of Iran from September 2012 to march 2013. The children who could not complete the questionnaire were excluded. Each school was considered as a cluster, and 20 guidance schools were selected randomly. Standard ISAAC core questions for wheezing, rhinitis, and eczema was used [10]. From 3250 students, 3050 questionnaires were obtained. The questionnaire return rate was 93.84%. Finally, 3000 questionnaires (1576 for girls and 1424 for boys) were collected and analysed. Students from guidance school answered the questionnaire associated with their parents. Written informed consent was obtained from participants and schools managers. Our study approved by local ethics committee of Mazandaran University of Medical sciences.

The boys and girls were matched for body mass index (BMI) [11]. Also, data were collected about kitchen design (open and closed), any passive smoking at the home or non-passive smoking; delivery based on normal delivery (NVD) and cesarian section(c/s) and animal contact based on indoor or outdoor contact and without animal contact from our participants. The family numbers, room numbers including bedroom and living room and housing characteristics were considered.

Data were analysed with SPSS 20 and statistical analysis tests such as Chi-Square test and odds ratio was calculated. We used logistic regression to find the effect of explanatory variables on asthma. P- Value less than 0.05 was statistically significant.

Results

The weight of the participants was between 25-92 kg (Mean 48.42 kg) and the height of the children was 120-182 (mean155.99) cm. The BMI, family and room numbers were 9.26-43.79 (mean 19.88), 2-9 (mean 4) and 1-4 (mean 2) person respectively. Table 1 showed analysis of kitchen, smoking exposure, kind of delivery and animal contact.

Table 1: Prevalence of allergic rhinitis, eczema and asthma in students of guidance schools

Disease	Total sample	Number of patients	Prevalence (%)	95% Confidence Interval	
				Lower	Upper
Allergic Rhinitis	2979	847	28.32	26.82	30.09
Eczema	2962	359	12.12	10.96	13.35
Asthma	2969	362	12.2	11.04	13.42

Children who have passive smoking are 1.8 times more likely to develop asthma (PV <0.001) (OR=2.88 with CI= 2.13-3.88). Room number leads to an increase of asthma in about 44 % (PV < 0.001) (OR = 0.694, CI = 0.570-0.844).

Out of total asthma patients (362; 12.2%), 222 (15.9%) were boys and 140 (8.9%) were girls. The prevalence of asthma was more common in boys than girls (PV=0.0001) in our study. Table 2 has shown asthma symptoms based on ISAAC questionnaire.

Table 2: Prevalence of allergic rhinitis, eczema and asthma in students of guidance schools by gender

Disease	Gender	Total sample	Number of patients	Prevalence (%)	p-value
Allergic Rhinitis	Boys	1003	406	40.47	<0.001
	Girls	1976	441	22.31	
Eczema	Boys	982	168	17.10	<0.001
	Girls	1980	191	9.64	
Asthma	Boys	984	144	14.63	0.006
	Girls	1985	218	10.98	

We use logistic regression to find the effect of explanatory variables on asthma. Overweight and obesity were more common in non-asthmatic patients' 47% and 15% respectively verse 39% and 9% in asthmatic patients respectively (PV = 0.002).

Association between a family number and asthma was not significant using logistic regression. (P-Value=0.45). The relationship between asthma and BMI in all students has shown in Table 3.

Table 3: Evaluation of environment factors in asthma prevalence using multivariate logistic regression

environment factors	B(regression coefficients)	S.E.*	P-Value.	Odds Ratio	**95% Confidence Interval**	
					Lower	Upper
Open kitchen (Yes/NO)	-0.111	0.151	NS**	0.895	0.666	1.202
Family. Number (NO)	0.142	0.087	NS	1.153	0.973	1.366
Room. Number (NO)	-0.494	0.111	0.000	0.610	0.491	0.759
Animal Contact(Yes/NO)	0.051	0.387	NS	1.052	0.493	2.245
Secondary Smoker (Yes/NO)	0.609	0.130	0.000	1.839	1.425	2.373
Born (Cesarean/Normal)	-0.12	0.143	NS	0.88	0.67	1.17

* Standard Error of Mean; **. Not Significant.

Allergic rhinitis

Sneezing and rhinorrhea were seen in 29.8% of our allergic rhinitis patients in 12 last months but ever sneezing and rhinorrhea was seen in 22.4%. Nasal pruritus and eye involvement such as redness, Lacrimation was observed in 35.6% (Table 4).

Table 4: Asthma symptoms according ISSAC questionnaire in guidance school students

Asthma symptoms	Boys (%)	Girls (%)	Total (%)	P-value
Ever Wheeze				
Yes	596 (20)	153 (5)	749 (25)	< 0.001
No	803 (27)	1420 (48)	1503 (75)	
Total	1399 (47)	1573 (53)	2972 (100)	
Current Wheeze				
Yes	812 (27)	102 (4)	914 (31)	< 0.001
No	586 (20)	146 (49)	2053 (69)	
Total	1398 (47)	1570 (53)	2968 (100)	
Sleep disturbance related Wheeze				
Yes	847 (29)	70 (3)	917 (32)	< 0.001
No	548 (18)	1490 (50)	2038 (68)	
Total	1395 (47)	1560 (53)	2955 (100)	
Speech Disorder				
Yes	286 (10)	151 (5)	437 (15)	< 0.001
No	1113 (37)	1415 (48)	2528 (85)	
Total	1399 (47)	1566 (53)	2965 (100)	
Ever asthma				
Yes	222 (8)	140 (5)	362 (13)	< 0.001
No	1176 (39)	1431 (48)	2607 (87)	
Total	1398 (47)	1571 (53)	2969 (100)	
Exercise related Wheeze				
Yes	837 (29)	139 (5)	976 (34)	< 0.001
No	558 (18)	1425 (48)	1983 (66)	
Total	1395 (47)	1564 (53)	2959 (100)	
Dry cough at night				
Yes	542 (18)	308 (11)	850 (29)	< 0.001
No	855 (29)	1254 (42)	2109 (71)	
Total	1397 (47)	1562 (53)	2959 (100)	

The prevalence of allergic rhinitis was more common in boys (n = 736, 51.7%: vs.; n = 174, 11%), PV = 0.0001. Association between using an open kitchen and allergic rhinitis was significant and persons who had open Kitchen had odds ratio 1.868 times more than others to have allergic rhinitis (P < 0.001).

Association between smoking and allergic rhinitis was significant and secondary smoker had odds ratio 3.11 times more than others to have allergic rhinitis (P <0.001). There was no significant relationship between others explanatory variables and allergic rhinitis.

Eczema

There was no relationship between BMI and prevalence of eczema (PV = 0.052). Association between smoking and eczema was significant and secondary smoker had 2.96 times more odds than others to have eczema using logistic regression (P < 0.001). There were no significant association between others explanatory variables and Eczema.

Discussion

Asthma

Asthma is the most common chronic disease in the pediatric population [12). The prevalence of asthma has doubled over the past several decades [13]. In the present study, the prevalence of asthma was more common among boys than girls (2:1 ratio) similar other studies [1, 3, 4]. Also, in the present study, the prevalence of asthma was higher than the other studies such as Thailand [13]. The prevalence of asthma was more variations between countries than within countries (15). The higher prevalence of asthma was reported in United Kingdom, New Zealand, and Australia but the lowest asthma prevalence reported from several eastern European countries, China, and some other countries in Southeast Asia. Generally, asthma was more common in affluent countries than in more developing countries [16].

Although the prevalence of asthma in some studies was different between urban and rural areas [17, 18, 19] but the results of our study in the same region in the north of Iran and the results reported by others were similar between the two areas [4, 20]. The prevalence of asthma in guidance school was 29.9% in rural area in this region [20]. Therefore there is no significant difference between rural and urban in asthma prevalence in our region that was similar the study by Gunner et al [21]. Urban and rural areas in our region are not substantially different climate. Environmental factors such as allergens, viral infections, pollutants, lifestyle, socioeconomic status, geographical area, diet, have been similar between two areas in this region.

Our study showed that the prevalence of wheezing in a 12 months period was 30.5%, so about one-fourth of our children had probability asthma. Our study showed that ever and current wheezing prevalence decreased with increasing age. Asthma diagnosis was 12.2 percent in our study. It has moderate prevalent in Iran and Middle East. In another study by Ghaffari et al., 12% of elementary school students had asthma with predominantly in boys [22]. Based on the previous studies in this region, the prevalence of asthma is more common in guidance school children than in elementary school students.

The prevalence of asthma is an average of 9 and 13 percent in Iran [1]). The average prevalence rate of asthma in the Middle East is 10.7% (11% in Oman and Palestine and around 17% in Kuwait) [1]. The prevalence of asthma in the north of Iran is more common than other regions of Iran and also it is more common or the same as some other countries such as United Kingdom (14.9%) and Malaysia (13 %) and less than other countries such as Austria (32%), United States of America (24.4%), and Singapore (27.4%) [1]. There is evidence that overweight and obesity are associated with children asthma [23]. It has been suggested that obesity results more severe, increased exacerbation, and poorer control asthma. Obesity rates have increased significantly in children in the world. A study showed that the risk of obese

children having asthma was two times higher than for children with normal body weight. But our study showed that overweight and obesity were lower in asthmatic or in patients with wheezing (p = 0.002) [23]. Also, Alvarez Zallo et al study showed no relationship between obesity and asthma in the guidance school children group [24]. There is more conflict in a relation between overweight and obesity with asthma in children. More studies are needed in children and adolescents to confirm the relation between overweight and obesity with asthma and better understand how body fat distribution impacts the obesity-asthma relationship.

Sleep disturbance related wheeze, speech disorder, exercise related wheeze and dry cough at night were more common in boys than girls (p < 0.001). The asthma prevalence was more common in boys than girls in our study (significantly, PV = 0.0001). Susceptibility to developing asthma among passive smoking guidance school children were 2.8 times more common than the non-passive smokers children (CI, 95% = 2.13-3.88) (PV = 0.001). Therefore, exposure to passive smoking is a significant risk factor for developing asthma. Of course, reversely, also for a room asthma leads to an increase of about 44% (P < 0.001). (OR = 0.694, CI = 0.570-0.844). In our study, kind of kitchen, type of delivery and contact with pets could not find any association with children asthma.

Allergic rhinitis

The prevalence of allergic rhinitis was 30% in our study. Based on a meta-analysis study in Iran, the prevalence of allergic rhinitis in children of aged 13-14 was 21.2%. Therefore AR is more common in the north of Iran with high humidity and warm weather. In another study in this region, the prevalence of allergic rhinitis in guidance school was 18.1% and 21.7% in rural and urban areas in this region respectively [6, 20]. Prevalence of AR is not more common in urban than the rural area.

In this study, the prevalence of rhinitis symptoms in the both age groups was higher than children in Croatia, city of Zagreb and is lowers than in children in Thailand [20]. Wide variations in the prevalence of rhinitis were observed across centres with a higher prevalence in Austria, Madrid, Cartagena and Bilbao, lower prevalences were reported from Barcelona, Castellon and Pamplona [20]. Our results showed that 12-month and lifetime prevalence rates of allergic nasal symptoms and hay fever, particularly in the older age group was even higher. One study in the global ISAAC Phase reported more than fourfold worldwide variation in the prevalence of allergic nasal symptoms in both age groups [17]. However, we believe that 12- month prevalence of nasal symptoms (not the combination of nasal symptoms) in a great majority of children living in our region is sensitised to house dust mite. It may be expected that many of them have perennial allergic rhinitis without associated significant conjunctival symptoms [18]. The incidence of allergic rhinitis has been increasing for the last few decades in the world [20]. AR is a major risk factor for developing asthma. Our study showed the similar results. AR and asthma have had significant effects on quality of life and other numerous complications.

We found no significant relationship between BMI and guidance school children with or without AR in our study (Table 4), P = 0.281). In another study, there was evidence that overweight and obesity was not associated with children AR [23]. Our research showed that prevalence of AR was more common (significantly, P = 0.0001) in boys than girls. We found that kind of kitchen, type of delivery and contact with pets could not find any association with children AR.

Eczema

Atopic dermatitis (AD) is an increasingly chronic dermatologic disease. Fifteen percent of our cases have had eczema. The pooled prevalence of eczema in children in guidance school age was 6.52% (range 4.1 to 24.3%) in Iran [7].

The prevalence of eczema in our study is much higher than the average in our country. A previous study in this region showed that the prevalence of eczema was 5.5% among rural guidance school children [20]. But in another study in urban guidance school children the prevalence of eczema was 8.2% in this region [6]. Our results related to the eczema symptoms in both age groups are higher than previously reported study in former socialist Europe and lower than that in Scandinavia and the United Kingdom [20].

Similar to our study, Hogewoning et al.'s in Ghana, Gabon and Rwanda and also Guner et al in Turkey reported that the prevalence of eczema had no significant difference in urban and rural areas [21, 25]. Our study showed no significant relation between BMI and guidance school students with or without eczema. There is evidence that overweight and obesity are associated with children eczema [23]. In addition, our study revealed that the eczema prevalence was more common in guidance school`s boys than girls (P = 0.0001). We found that kind of kitchen, type of delivery and contact with pets had any association with children eczema.

In conclusion, it can be concluded that the prevalence of asthma, allergic rhinitis, and eczema symptoms (except a dry cough at night) in boys were higher than in girls. This study also shows that asthma, allergic rhinitis, and eczema in guidance schools children from the capital of Mazandaran province northern Iran are more common than an average of our country. A major possible limitation of

the study is parental reporting on disorder symptoms (asthma, AR and eczema) could be influenced in data analysis.

Authors` contributions

SB participated in the conception of the study and acquisition of the data. ST contributed to acquisition of the data and distribution of questionnaires. DZ contributed to conception, design, acquisition of the data, and distribution of questionnaires. JY contributed to analysis, interpretation of data and drafting, and JGH contributed in conception, design, drafting and final approval.

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Clinical Science

Academic Achievement and Psychosocial Profile of Egyptian Primary School Children in South Sinai

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Abstract

BACKGROUND: Population of South Sinai has suffered from negligence for many years. Solving educational problems of this population is the main concern nowadays.

AIM: To assess academic achievement in primary school children in South Sinai in relation to intelligence and psychosocial profile.

SUBJECTS AND METHODS: A descriptive cross-sectional survey was conducted on 407 Bedouin and urban students randomly selected from twelve public primary schools in six cities in South Sinai. Intelligence was assessed using Goodenough-Harris test. The midyear Arabic language and Arithmetic scores were used to assess academic achievement. The teachers completed a Pediatric-Symptom Checklist for evaluation of children behaviour.

RESULTS: A statistically significant difference in academic achievement ($P < 0.001$), total psychosocial scores, ($P < 0.05$), and externalization ($P < 0.05$) was found between urban and Bedouin students with significant gender differences ($P < 0.05$). Highly significant positive correlations were observed between IQ percentile and mid-year Arabic language scores and Arithmetic scores ($P < 0.001$), and significant negative correlations with the total score of PSCL and its subscale scores (externalising, inattention, and internalising behaviour) ($P < 0.001$) among the students.

CONCLUSION: Comorbid academic and psychosocial dysfunction in primary school children were observed in South Sinai. A national strategy to minimise the educational gap between Bedouin and urban areas should be implemented.

Introduction

South Sinai is the least populated governorate of Egypt located in the east of the country, encompassing the southern half of the Sinai Peninsula [1]. Population in Sinai has unique circumstances, which is far different from that of other Egyptian urban or rural communities. They live at the periphery of Egypt, with the population of 21 per m^2 of the total area away from the centre and away from the basic services [2, 3]. They are growing up in contexts characterised by dry weather, deficient water supply, and higher rates of illiteracy, weak economies, limited educational opportunities, pervasive poverty, and other socio-cultural transformations [4]. This environment can have negative impacts on students' development as reported in similar communities Urban and Bedouin children attend public government-financed schools located in their communities. Most of the teachers (60%) have Bedouin origin [5, 6].

Many factors affect the academic performance of school children amongst are the cognitive function of the children and psychosocial behavioural factors. Environmental factors also affect academic achievement [7, 8].

There have been few such studies in South Sinai. Therefore, the aim of this study was to assess factors that affect academic achievement in primary school children in South Sinai in relation to intelligence and psychosocial profile. The ultimate goal was putting an intervention plan to reverse, or at least retards the progress of educational and

psychosocial problems and its sequels in this target population.

Subjects and Methods

Study design

This descriptive cross-sectional, school-based study was conducted as a part of the project "Improvement of health and nutritional status of children and adolescents living at South Sinai", which is funded by the European Union (EU) as a collaborative research with the National Research Center in Egypt. The study was performed in the period of 2009-2010. The sample size was calculated to detect the mean differences in the scores of the factors probably affecting scholastic achievement.

Setting of the study

This study is a school-based that was conducted in twelve public primary schools in six cities in South Sinai (Ra's-Sedr, Abo is, Abo Zenimah, El Tor, Saint Catherine, and Nweiba), Egypt. The study population represents the social variations in South Sinai, (210 Bedouin students, families are original inhabitants of Sinai, and 197 urban students, some of their families moved from the Nile valley to work in Sinai).

Subjects

The study included 407 Bedouin and urban students (203 males and 204 females). Students recorded to have any mental disorder, chronic disease, visual or auditory impairment were excluded, as well those who were absent or refused to participate.

Ethical considerations

The study was approved by the Ethical Committee of the National Research Centre. Permission to conduct the research in the selected schools was obtained from the Egyptian Ministry of Education. In addition, a verbal consent was obtained from each parents' participant through the school authority; they were informed about the questionnaires being used in the study and accepted their sharing in the study.

Procedures

The subjects of the study completed a self-report assessment questionnaire which included the following items:

I-Demographic and personal data such as age, sex, residence and origin whether Bedouin or Urban.

II-Assessments:

a) A medical examination was done to explore any health problem.

b) Cognitive function assessment: Using draw a man test. Draw a man test was developed by Goodenough [9]. The test is preferred as a screening tool for cognitive assessment as it's easily applied, not consuming time can be applied to large numbers of children at the same time. The scoring for the Draw a man test is straightforward and objective. A drawing receives 1 point for the presence and correct quantity of each of various body parts, such as head, eyes, mouth, ears, arms and feet. The performance of this drawing task relies on various cognitive, motoric, perceptual, attentional, and motivational capacities. Previous research proved that performance on the Goodenough Draw a Man Test correlated moderately with scores on time-consuming comprehensive IQ tests, and the test was both reliable and valid [10, 11].

c) Educational achievement assessment: The educational achievement was assessed using the mean scores of midyear tests of Arabic language and arithmetic which represent the essential educational components that the students must pass in order to enter higher levels of education.

d) Psychosocial function assessment: Children's psychosocial function was evaluated using a teacher completed Pediatric Symptom Checklist-17 (PSCL-17) [12]. It is a psychosocial screen designed to facilitate the recognition of hyperactivity, attention, emotional, conduct, and social problems, relational difficulties with peers, anxiety/depression, and aggressive behaviour [13].

A total score of 15 or higher suggests the presence of significant behavioural or emotional problems. It consists of 3 subscales: internalising subscale, externalising subscale, and attention subscale [14]. The PSC-17 subscales have obtained reasonable agreement with validated and accepted parent-report instruments for internalising, externalising, and attention problems [15]. Teachers had filled checklists for 390 students out of the 407.

Results

Our present study comprised 407 students (210 Bedouin and 197 urban students), aged 6 to 14 years. Although the mid-year Arabic language and arithmetic scores were statistically highly significantly lower in Bedouin group compared to urban group ($P < 0.001$), no significant differences between the two groups were found in IQ percentile ($P > 0.05$). Total

PSCL and externalisation scores were significantly higher in Bedouin group compared to urban group (P < 0.05), while no significant differences were found in the internalisation and attention scores (P > 0.05). Comparison of IQ percentile, academic achievement and psychosocial variables of the students among Bedouin and urban students was shown in Table 1.

Table 1: Comparison of age, cognitive and academic achievement variables in Bedouin and urban students

Variables		Bedouin group (N = 210)	Urban group (N = 197)	Bedouin Vs. urban groups
		Mean ± SD	Mean ± SD	P value
Age (yrs.)		9.77 ± 1.4	8.78 ± 1.34	0.06
Cognitive and academic achievement	IQ percentile	57.78 ± 32.21	63. 46 ± 30.66	0.068
	Mid-year Arabic score	60.33 ± 20.11	76.67 ± 20.3	0.00**
	Mid-year arithmetic score	53.25 ± 17.85	73.07 ± 20.76	0.00**
Psychosocial variables	Internalization score	2.77 ± 2.42	2.56 ± 2.26	0.373
	Attention score	3.8 ± 2.7	3.46 ± 2.25	0.183
	Externalization score	4.3 ± 3.67	3.46 ± 3.03	0.015*
	Total psychological score	10.84 ± 7.85	9.35 ± 6.72	0.043*

*Significant difference at p < 0.05, **highly significant difference at p < 0.001.

Our present study comprised 203 males and 204 females. The mean IQ percentile was statistically significantly lower in males compared to females (P < 0.05). As regard total PSCL scores, externalisation and attention scores, they were statistically highly significantly higher in males compared to females (P < 0.001) while no significant differences were found in the internalisation score (P > 0.05). Comparison of cognition, academic achievement, and psychosocial variables of the students of the Bedouin and urban students according to sex was shown in Table 2.

Table 2: Comparison of cognitive, academic achievement, and psychosocial variables of the Bedouin and urban students according to sex

Variables		Males group (N = 203)	Females group (N = 204)	Males vs Females groups
		Mean ± SD	Mean ± SD	P value
Cognition	IQ percentile	52.98 ± 31.45	67.94 ± 29.35	0.000*
Academic achievement	Mid-year Arabic score	65.32 ± 21.37	71.18 ± 21.81	0.007*
	Mid-year arithmetic score	60.26 ± 22.31	65.37 ± 20.78	0.018*
Psychosocial	Internalization score	2.67 ± 2.40	2.67 ± 2.29	0.989
	Attention score	4.15 ± 2.39	3.0 ± 2.09	0.000**
	Externalization score	4.42 ± 3.56	3.36 ± 3.1	0.002**
	Total psychological score	11.17 ± 7.63	9.05 ± 6.94	0.004**

*Significant difference at p < 0.05, **highly significant difference at p < 0.001

Categorization of children according to IQ percentile scores showed that 98 students (24.3%) were superior, 63 (16.6%) were above average, 174 (42.8%) were average, 35 (8.7 %) were below average, 30 (7.4%) were borderline and 7 (1.7%) students were intellectually deficit. Thus, about 83.7% of the whole sample have average and above average IQ compared to 9.1% having poor intelligence.

Analysis of results of PSCL revealed that, out of 390 students, 110 students (28.2%) exceeded the cutoff level of the total PSCL score that means that 28.2% of the sample suffers from psychosocial problems. About 88 students (22.7%) had symptoms suggestive of externalising behaviour, 56 (14.4%) having symptoms suggestive of inattention and hyperactivity behaviour, and 87 (22.5%) having symptoms suggestive of internalising behaviour. The symptoms suggestive of psychosocial disorders and externalising behaviour were found to be significantly higher in Bedouin group compared to urban group (P < 0.05). Table 3 shows a distribution of psychosocial problems among students in relation to their residence.

Table 3: Distribution of psychosocial problems among students in relation to their residence

Variables	Total Students (N = 390) N (%)	Bedouin group (N = 205) N (%)	Urban group (N = 185) N (%)	P value
Internalization behavior	87 (22.5%)	49 (24.0 %)	38 (20.8%)	0.44
Inattention	56 (14.4%)	36 (17.6%)	20 (10.8%)	0.058
Externalizing behavior	88 (22.7%)	57 (27.9%)	31 (16.8%)	0.00**
Total psychosocial dysfunction	110 (28.2%)	67 (32.7%)	43 (23.2%)	0.039*

*Significant difference at p < 0.05, **highly significant difference at p < 0.001.

Categorization of children according to academic achievement showed that the scores of the Bedouin students were statistically significantly lower compared to Urban group (P < 0.001). About 19.6% students failed in the Arabic language (27% in Bedouin versus 11.6% urban), and 28.2% students failed in arithmetic (41.1% Bedouin, versus 14.4% urban). Comparison of educational achievement among Bedouin and urban students was shown in Table 4.

Table 4: Comparison of educational achievement among the students in Bedouin and urban groups

	Variables	Total Group (N = 407) N (%)	Bedouin Group (N = 210) N (%)	Urban Group (N = 197) N (%)	P value
Arabic	Failed	19.6%	27%	11.6%	0.00**
	Passed	80.4%	73%	88.4%	
Mathematic	Failed	28.2%	41.1%	14.4%	0.00**
	Passed	71.8%	58.9%	85.6%	

*Significant at p < 0.05, **highly significant at p < 0.001.

Highly significant positive correlations were observed between IQ percentile and mid-year Arabic language scores (P < 0.001) and also Arithmetic scores (P < 0.001), and significant negative correlations with the total score of PSCL and its subscale scores (externalising, inattention, and internalising behaviour) (P < 0.001) among the students.

Table 5: Correlations of academic achievement test scores, psychosocial variables and IQ percentile score among the students

Variables		Academic achievement tests	
		Mid-year Arabic scores	Mid-year Arithmetic scores
IQ	IQ percentile	0.299**	0.186**
Psychosocial	Internalization scores	-0.49**	-0.326**
	Attention scores	-0.449**	-0.257**
	Externalization scores	-0.414**	-0.242**
	Total psychosocial scores	-0.501**	-0.305**

*Significant difference at p<0.05, **highly significant difference at p<0.001

Correlations of academic achievement test scores, psychosocial variables and IQ percentile score among the students were shown in Table 5.

Discussion

Studies addressing the issue of co-morbid school underachievement and psychosocial problems in schoolchildren are often lacking in Sinai. Institutions offering special education facilities for children with school underachievement are also limited and they often do not have school-based mental health program established. Therefore, the aim of this current study was to explore the prevalence and assess the association between educational achievement and psychosocial problems among children in public schools in South Sinai.

Our study showed that 83.7% of the studied groups had normal IQ and only 9.1% had the poor cognitive function; with no statistically significant difference between Bedouin and urban inhabitants ($P > 0.05$). There was a gender difference with lower cognitive function in boys ($P < 0.05$). Our findings are consistent with several studies that have been reported gender differences in intelligence. They support gender differences in specific cognitive abilities; some support females and some support males [16]. Adrian and Buchanan [17] have examined gender differences in intelligence in 20 countries, studies from China through to Germany and Scotland, and their results showed that males give significantly higher estimates than females for over intelligence. They stated also this difference is consistent across countries and population although there are wide differences in level.

In the present study, the means of academic achievement were statistically significantly lower in males compared to females ($P < 0.05$), that is consistent with Watkins et al. [18], who revealed sex differences on the academic achievement subtests. Males outperformed than females on subtests (arithmetic, information, and matrix reasoning), while females' performance was better than males only on the digit of symbol substitution. Habibollah, et al., [19], studied the academic achievement distribution in 80,000 students; there was no significant difference between males and females regarding academic achievement.

In this study, Bedouin students had statistically highly significant lower educational achievement scores compared to Urban group ($P < 0.001$). Almost 20 % of the whole sample were failed in Arabic language test (27% of Bedouin versus 11.6% of Urban), and 28.2% students were failed in arithmetic test (41.1% of Bedouin versus 14.4% of Urban group). Similar results were reported in studies from Nigeria comparing rural and urban areas [20, 21]. Boniface et al., [21] reported that more than two-thirds of schoolchildren in rural areas of Malaysia had lower educational achievement compared to those in urban areas.

Intelligence plays an important role in school achievement. Highly significant positive correlations were observed between IQ percentile and mid-year Arabic language scores ($P < 0.001$) and Arithmetic scores ($P < 0.001$). Our findings are consistent with Ritu & Sheikh [22] that have been reported that IQ scores often predict school achievement, children with higher IQ scores will have better achievement tests, school grades, and complete more years of education. They have been shown that performance on intelligence tests is correlated with school achievement.

However, having an average IQ is not a guarantee to be academically successful. In this study, although 83.7% of the studied subjects had average and above average IQ, almost 20% of students were failed in language test and 28.2% of students were failed in the Arithmetic test. These results are in agreement with Ormrod et al., [23], who stated that high scores on an intelligence test do not always mean someone is going to do well academically, and vice-versa. This is because intelligence tests gauge someone's ability to critically think and reason abstractly, while educational achievement is a combination of these abilities and the ability to be motivated, plan time well and many other factors as family resources, parental support are also involved.

Psychosocial dysfunction contributes to disability among children [24]. Anselmi et al. [25], reported that 21.1% of Brazilian children exceeded the cutoff level for clinical significance on the total PSCL score, 14.7% exceeded criteria on the internalising subscale, 16.2% on the externalising subscale, and 21% on the inattention subscale. In our study, analysis of PSCL-17 results revealed that 28.2% of the studied children had psychosocial dysfunction according to the cutoff level of the PSCL-17. Almost 23% students exceeded on the externalising subscale, 22.5% students exceeded criteria on the internalising subscale and 14.4% students exceeded on the inattention and hyperactivity subscale.

In the present study, symptoms suggestive of psychosocial disorders and externalising behaviour were found significantly higher in Bedouin group compared to urban group ($P < 0.05$). These data are proportionate with Polaha et al., [26] who showed such problems are predominant in rural Children. Meanwhile, our findings were not in the agreement with the results of Elbedour et al., [27] who reported that Bedouin children had higher levels of fear, inattention, and hyperactivity behaviour than urban children. Over than 50% reporting fears were related to the family situation (e.g. being punished by their

father) and too unrealistic threats (e.g. ghosts). In our opinion, this pattern would seem better explained by differences in the socialisation goals of these societies.

In this study, there was a gender difference with higher scores suggesting higher psychosocial problems in boys ($P < 0.05$). Our findings are consistent with several studies as Akpan et al., in Nigeria, Eapen et al., in Emirate, and Gupta et al., in India that have been reported gender differences and more males than females having behavioural disorders [28-30]. The prevalence of behavioural problems was also evaluated among 620 in the United Arab Emirates aged 6 - 18 years; 11.8% scored above the cut-off point, indicating behavioural problems. Conduct problems were more prevalent among boys and emotional problems among girls [29]. In our study, total PSCL, externalisation and attention scores were statistically highly significantly higher in males compared to females ($P < 0.001$) while no significant differences were found in the internalisation score ($P > 0.05$).

The prevalence of psychosocial dysfunction in children with academic underachievement documented by past studies [31-33] carried out in developed parts of the world ranges from thirty-one to forty-one percent. Academic performance is often associated with co-morbid behavioural problems which often develop during childhood period, and had been known to persist into the adulthood in most cases. Children who do poorly at school may be under a lot of stress and cope with it either by externalising their feeling as behaviour problem while others might internalise it [28]. Our results showed that psychosocial problems had the negative association with academic performance both in Arabic language and arithmetic ($P < 0.001$) in all students.

Other risk factors may help in contributing these findings such as lower socioeconomic status, lack of well-equipped schools, expert teachers, psycho-educational services, and insufficient infrastructure (including electricity). Moreover, most students in primary schools in Sinai are daily confronted with obstacles of coping with academic achievement and serious emotional strains as long walk to school. The results were in accordance with previous studies in Egypt, and other countries as in India, Nigeria and Malaysia [34-37], that were discussing potential determinants of educational achievement, and psychosocial problems in rural and urban school children.

In conclusion, comorbid academic and psychosocial dysfunction in primary school children was observed in South Sinai. Effective interventions programs must involve activation of the role of psychosocial counsellors and teachers in schools, promoting complementary relation between parents and children to build resilience against conditions that produce psychosocial and learning disabilities. It is recommended that government should increase the allocation of funds, and the school should be equipped with different facilities including a computer laboratory with internet access, a library, and visual and science laboratories to facilitate learning and improve academic performance in the schools in South Sinai. Encouraging sports practice and establishing playgrounds is mandatory.

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Clinical Science

Assessment of Increase in Aortic and Carotid Intimal Medial Thickness in Type 1 Diabetic Patients

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Abstract

AIM: To assess aortic and carotid intima-media thickness (aIMT and cIMT) in diabetic patients.

PATIENTS AND METHODS: The study included 75 type 1 diabetic patients and 30 age and sex matched healthy volunteer. A blood sample was taken for analysis of HbA1 and lipid profile and the urine sample was taken for analysis of albumin/ creatinine ratio. aIMT and cIMT via ultrasound were also done.

RESULTS: cIMT & aIMT were significantly higher in diabetics. aIMT was found to be significantly higher than cIMT in diabetic patients (0.72 ± 0.11 vs. 0.52 ± 0.06, P = 0.0001). Ten of our patients (14%) with normal cIMT revealed significantly increased aIMT. aIMT had a significant positive correlation with age of patients, waist/hip ratio & cIMT.

CONCLUSION: Diabetic patients had increased aIMT and cIMT with a relatively greater increase in the aIMT than in the cIMT. Because atherosclerosis begins first in the intima of the aorta, these data suggest that the aIMT might provide the best currently available noninvasive marker of preclinical atherosclerosis in children. We recommend frequent follow up of diabetic patients for early detection of diabetic complication.

Introduction

Juvenile diabetes has been stated as one of the major risk factors for atherosclerosis and its complications and diabetic patients have about 3-fold increased risk of developing atherosclerotic disease [1].

Atherosclerosis starts in childhood in the form of intimal fatty streaks where lipids accumulate in the intima of arteries and these fatty streaks occur in the aorta in almost every child over the age of 3 years [2]. Autopsy study of individuals aged 15 to 34 years showed that atherosclerotic lesions tends to affect certain arterial segments, especially the abdominal aorta where lesions mostly affected the dorsolateral wall of the aorta just proximal to its bifurcation [3] but atherosclerotic lesions occur later in life in the coronary and carotid arteries than in the aorta, and there is a strong association between atherosclerosis in the abdominal aorta and coronary arteries [4].

Recent improvements in ultrasound machines with increased resolution and accuracy have allowed identification of early vascular changes that can be assessed noninvasively using ultrasound. Aortic & carotid intima-media thickness (cIMT) testing via B-mode ultrasound is a safe, simple, and inexpensive method for evaluating cardiovascular (CV) risk by measuring the combined thickness of the intimal and medial layers of the arterial wall. Use of IMT testing can also detect marked thickening of the arterial wall, possibly indicating plaques or atheromas that are associated with accelerated atherosclerotic disease and increased risk for coronary artery disease, myocardial infarction, and stroke [5]. Many studies have been detected increased IMT in young diabetic children [6-8]. In young and middle-aged adults, increased IMT was also associated with

cardiovascular risk factors [8-11].

Since atherosclerotic changes occur earlier in the abdominal aorta than in the carotid arteries, measurement of aortic intima-media thickness (aIMT) may be a more sensitive indicator in younger individuals allowing early detection of atherosclerotic lesions, thereby allows risk factor modification at younger age. McGill et al. [3] suggested that risk factor control in youth would be the most effective strategy for preventing heart disease in the 21st century.

In the study reported herein, we measured aIMT and cIMT in adolescents and young adults to identify the relationship of concurrently measured cardiovascular risk factors with both of these ultrasound-derived IMT measures.

Patients and Methods

Patients

The study included 75 children with type 1 diabetes mellitus (DM) attending the endocrine clinic, National Research Centre, Cairo, Egypt. The control group consisted of 30 ages and sex matched healthy normal volunteers. The control group was the healthy friends and relatives of our patients.

Age of patients was 12-23 years (17.99 ± 2.59), duration of diabetes was 5-20.5 years (10.91 ± 3.54), onset of diabetes was 1.5-17 years (7 ± 3.28), insulin dose/kg was 0.28-3.05 U/kg (1.26 ± 0.44 U/kg), and BMI (Kg/m^2) was 16.89-34.31 (24.44 ± 3.89) and nobody used insulin pump. All diabetic patients were on intensive insulin therapy regimen.

None of our patients had acute diabetic complications, for example, diabetic ketoacidosis (DKA) or hypoglycemia, none suffering from cardiac diseases, for example, congenital, rheumatic heart and left ventricular dysfunction. Also patients receiving drugs for cardiovascular disease and patients on metformin or multivitamins were excluded.

Study design and protocol

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

All the studied patients were subjected to history taking including age of patients, sex, age of onset of diabetes, duration of diabetes, type and dose of insulin therapy, and family history of diabetes.

We asked about the presence of any symptoms of cardiac, renal, neurological affection, or presence of any type of autonomic dysfunction. We also asked about history of taking drugs other than insulin.

Clinical examination

Patients and controls were subjected to general, cardiac, chest, and neurological examination. Blood pressure was measured three times for patients and controls after 5 min rest in the sitting position on both upper limbs with the use of automatic manometer (Omron M4 Plus; Omron Health Care Europe, Hoof drop, The Netherlands). The mean value of the second and the third measurement was calculated.

The measurements taken on the dominant limb were analyzed.

Anthropometric measurements in the form of weight, height, waist circumference (WC), and hip circumference (HC) were taken for each participant. The weight and height of the participants were measured up to 0.01 kg and 0.1 cm using a Seca Scale Standing Balance and a Holtain Portable Anthropometer (Holtain, Ltd, Crymmych, UK). Body mass index (BMI) was calculated as weight (in kg) divided by height (in m) squared. Waist circumference was measured at the level of the umbilicus with the participant standing and breathing normally; hip circumference was measured at the level of the iliac crest, using non-stretchable plastic tape to the nearest 0.1 cm. The waist/hip ratio and waist/height ratio (cm/cm) were calculated. Each measurement was taken as the mean of three consecutive measurements, using standardized equipment [14, 15]. The landmarks, instruments used, and techniques followed were those recommended by the international biological program [14, 15].

Laboratory investigation

All patients and controls underwent the following tests. For cholesterol measurements, venous blood was sampled after a 12 h fast. Serum total cholesterol was determined by a commercial kit (Boehringer-Mannheim, Mannheim, Germany) [16]. High-density lipoprotein (HDL) cholesterol was separated from the serum by precipitation of the other lipoproteins with a heparin/manganese procedure [17]. Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald equation. The concentration of triglycerides (Tg) was measured in a TechnoCon AutoAnalyzer II (TechnoCon Instruments, Tarrytown, NY, USA). Glycosylated hemoglobin (HbA1) was done every 3 months and the mean value was calculated per year. It was measured using high

pressure liquid chromatography (Nichols Institute, Van Nuys, CA, USA) [18]. Screening for microalbuminuria was assessed in fresh morning urine samples by measuring albumin/creatinine ratio by enzyme linked immunosorbent assay (ELISA) kit provided by Orgentec Diagnostika, Gmbh (Mainz, Germany) [19].

cIMT assessment

A single experienced vascular sonographer, who was blind to the clinical and laboratory data of the study subjects, performed all imaging studies. The images were obtained using a General Electric medical ultrasonographic machine (model: Vivid 7 Pro, GE Healthcare Vingmed ultrasound AS-NI90, Horton, Norway) equipped with 7.5–10 MHz linear-array transducer. Imaging of the carotid arteries was performed in the cardiovascular ultrasound laboratory with the subject resting in the supine position with his/her neck extended, and the head turned 45 degrees toward the contralateral side. Care was taken to have the vessel as perpendicular as possible to the plane of ultrasound beam to ensure optimal imaging of the vessel wall in its longitudinal axis with the least possible pressure in order not to compress the overlying jugular vein and to allow expansion of the carotid artery in all directions. A longitudinal section of the common carotid artery 1 cm proximal to the carotid bulb was imaged to achieve consistent site of measurement, and a resolution box function was used to magnify this part of the artery. Three maximal IMT measurements of the far wall of the artery at 3-mm intervals were obtained starting at 1 cm proximal to the bulb and moving proximally.

The reported IMT for each side is the average of these three measurements and the reported IMT for each subject was the average of the six measurements (three measurements from the right and three from the left common carotid artery). Generally, images are recorded in the plane where the maximal cIMT can be visualized. Magnification of the vessel wall allows easy identification of the intimal-medial complex, defined by the border between the echolucent vessel lumen and the echogenic intima and the border between the echolucent media and echogenic adventitia. Image frames were selected on the basis of areas where the intima-media complex was best visualized and appeared the thickest, irrespective of the cardiac cycle, with manual assessment by the sonographer using electronic calipers online [6].

Measurement of aIMT

The abdominal aorta was first identified in the upper abdomen using a 7.5 MHz pediatric phased array transducer, and it was then followed distally until the aortic bifurcation was reached. The depth (anterior-posterior direction) and location (cranio-caudal direction) of the distal 15 mm of the abdominal aorta was measured from these images and used as an aid to locate the aortic intima-media complex using a 10 MHz linear array transducer. For the assessment of aIMT, the image was focused on the far wall (dorsal arterial wall of the most distal 15 mm of the abdominal aorta), and gain settings were used to optimize image quality. Images 15 mm in width were magnified using a resolution box function. The dorsal arterial wall of the most distal abdominal aorta was chosen for the area of interest because post mortem series have shown it is the most lesion-prone site [12]. Generally, images are recorded in the plane where the maximal aIMT can be visualized. Magnification of the vessel wall allows easy identification of the intimal-medial complex, defined by the border between the echolucent vessel lumen and the echogenic intima and the border between the echolucent media and echogenic adventitia. Image frames were selected on the basis of areas where the intima-media complex was best visualized and appeared the thickest, irrespective of the cardiac cycle, with manual assessment by the sonographer using electronic calipers online. At least 3 measurements were taken, followed by averaging the three measurements of each patient to calculate aIMT [**7**].

Table 1: Descriptive statistics of demographic, blood pressure and anthropometric data of diabetic patients included in the study

Variables	Minimum	Maximum	Mean	Std. Deviation
Demographic data:				
Age of patients (yrs)	12.00	23.00	17.99	2.59
Duration of disease (yrs)	5.00	20.50	10.91	3.54
Onset of disease (yrs)	1.50	17.00	7.00	3.28
Insulin dose (U/Kg)	0.28	3.05	1.26	0.44
Blood pressure:				
Systolic blood pressure (mmHg)	100.00	150.00	118.45	13.33
Diastolic blood pressure (mmHg)	50.00	100.00	76.55	10.057
Anthropometric data:				
Weight (kg)	32.00	96.00	64.40	12.01
Height (cm)	135.00	181.80	162	10.31
Waist circumference (cm)	50.00	105.00	82.83	11.21
Hip circumference (cm)	74.00	121.00	94.60	10.32
Midarm circumference (cm)	21.00	3205.00	75.14	379.53
Body mass index (kg/m^2)	16.89	34.31	24.44	3.89
Waist/ hip ratio	0.57	0.99	0.88	0.08
Waist / height ratio	0.28	0.65	0.51	0.07

Statistical analysis

Statistical analysis was conducted using Statistical Package for Social Science (SPSS) program version 15.0 (Chicago, IL, USA); t-test or Mann-Whitney U-test (for non-normally distributed data) for independent variables was done. Pearson's (for normally distributed data) or Spearman's (for non-normally distributed data) correlation was also used.

Results

Descriptive statistics of demographic data, blood pressure, anthropometric and clinical data were in Tables 1 & 2.

Table 2: Descriptive statistics of clinical data of patients included in the study

Variables	N	%
Dyspnea	5	7
Orthopnea	3	4.2
Paroxosmal nocturnal	3	4.2
Palpitation	22	18.3
Chest pain	13	18.3
Dyspnea	18	25.4
Sensory lose	4	5.6
Syncope	1	1.4
Cough on exersion	5	7
Gastointestinal symptoms	5	7
Lower limp edema	1	0.8
Intermittent claudication	9	12.7
Weak muscle power	1	1.4
Postural hypotension	5	7
Urinary symptoms	11	15.5
Numbness	27	38
Parathesia	12	16.9
Tremors	2	2.8
Bluring of vision	9	12.7
Easy fatigability	1	1.4

Diabetic patients had significantly increased LDL (p = 0.01), oxLDL (p = 0.0001), albumin/creatinine ratio (p = 0.001), cIMT (p = 0.0001) & aIMT (p = 0.0001).

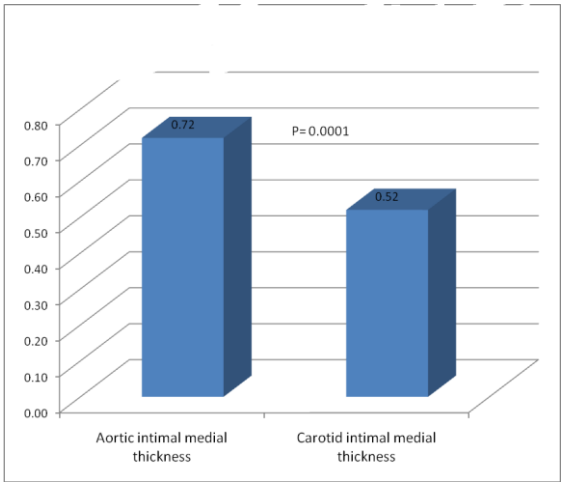

Figure 1: Comparison between aortic intimal medial thickness and carotid intimal medial thickness of diabetic patients

cIMT & aIMT were significantly higher in diabetics (0.52 ± 0.06 vs. 0.4 ± 0.03, P = 0.0001 and 0.72 ± 0.11 vs. 0.46 ± 0.04, P = 0.0001 respectively). aIMT was found to be significantly higher than cIMT in diabetic patients (0.72 ± 0.11 vs. 0.52 ± 0.06, P = 0.0001) (Fig. 1, 2, 3 & 4).

Ten of our patients (14%) with normal cIMT revealed significantly increased aIMT (Fig. 5). aIMT had significant correlation with age of the patients (P = 0.0001), waist/hip ratio & with cIMT (P = 0.01) and positively correlated with the duration of diabetes but didn't reach statistical significance (P = 0.08) (Fig. 6 Table 4).

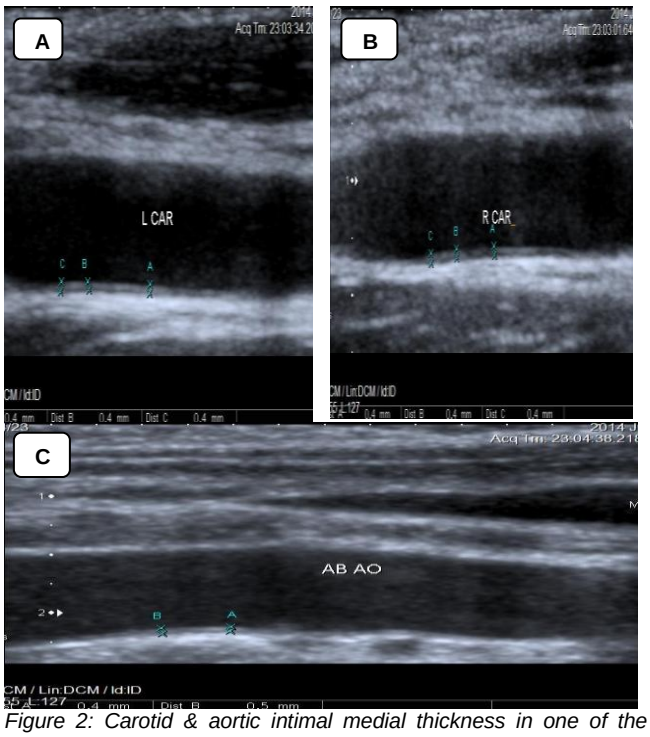

Figure 2: Carotid & aortic intimal medial thickness in one of the controls.A & B: showing normal Rt. & Lt. cIMT of one of the controls. cIMT= 0.4mm. C: showing normal aIMT of one of the controls. aIMT= 0.4-0.5mm

Discussion

In the current study, diabetic patients had significantly increased LDL, oxLDL, albumin/creatinine ratio, cIMT & aIMT.

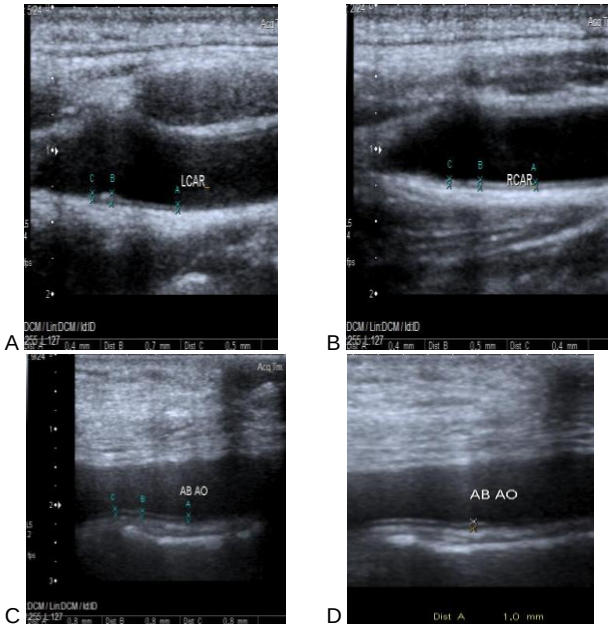

Figure 3: Carotid & aortic intimal medial thickness in one of the patients. A: showing normal Rt. cIMT of one of the patients. cIMT = 0.4-0.5 mm. B: showing increased Lt. cIMT of the same patients. cIMT = 0.4-0.7 mm. C &D: showing increased aIMT of the same patient. aIMT = 0.8-1 mm

cIMT and aIMT were significantly increased in diabetics compared with healthy control children, but the difference was greater for aIMT as aIMT was found to be significantly higher than cIMT in diabetic patients.

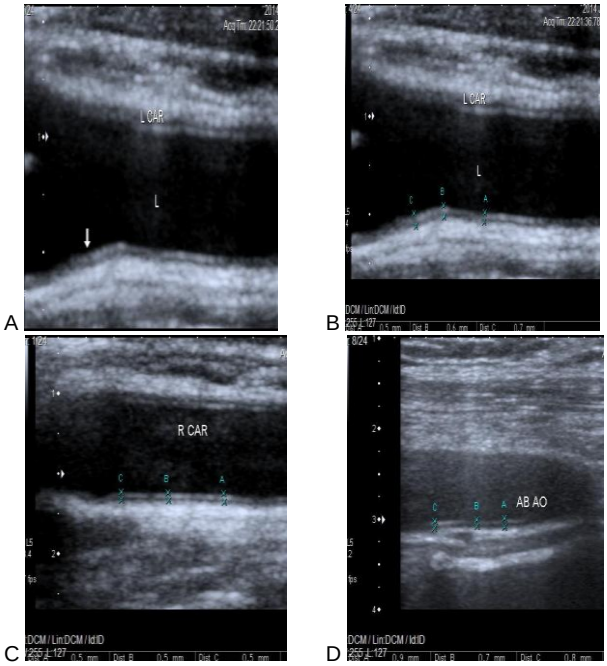

Figure 4: Carotid & aortic intimal medial thickness in one of the patients. A & B: showing increased Lt. cIMT of one of the patients. cIMT = 0.5-0.7 mm (arrow). C: showing increased aIMT of the same patient. aIMT = 0.7-0.9 mm. D: showing normal Rt. cIMT of the same patients. cIMT = 0.5 mm

Ten of our patients (14%) with normal cIMT revealed significantly increased aIMT. aIMT had significant correlation with age of the patients, waist/hip ratio & with cIMT and positively correlated with the duration of diabetes but didn't reach statistical significance (P = 0.08).

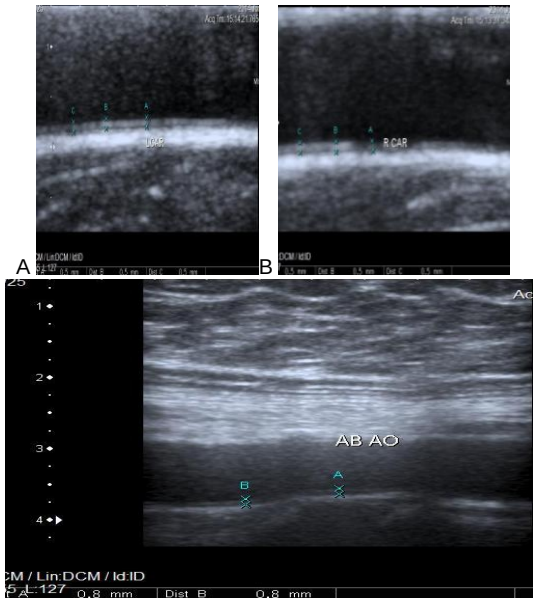

Figure 5: Carotid & aortic intimal medial thickness in one of the patients. A & B: showing normal Rt. & Lt. cIMT of one of the patients. cIMT = 0.5 mm. C: showing increased aIMT of the same patient. aIMT = 0.8 mm

The present study shows that diabetic children have significantly increased aortic IMT & carotid artery IMT compared with normal control.

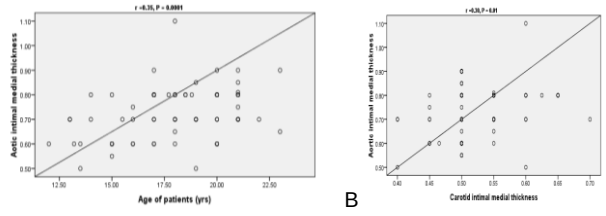

Figure 6: Correlation between age of diabetic patients and aortic intimal medial thickness (A). Correlation between aortic and carotid intimal medial thickness of diabetic patients

Our findings are consistent with observations reported in a postmortem study that have indicated a relation between early atherosclerotic lesions and diabetic state [3] and with findings of Järvisalo et al. who reported that type I diabetes predisposes to increased subclinical atherosclerosis at a very early age and that type 1 diabetes is an independent risk factor for increased IMT in children [13].

Table 3: Comparison between data of diabetic patients and controls (N = 75)

	Patients		Controls		
Variables	Mean	SD	Mean	SD	P-value
Body mass index (kg/m^2)	24.44	3.89	21.86	6.47	0.3
Waist/ hip ratio	0.88	0.08	0.68	0.07	0.03
Waist /height ratio	0.51	0.07	0.48	0.10	0.3
Cholesterol (mg/dl)	194.86	63.65	176.41	37.27	0.1
Triglyceride (mg/dl)	106.59	53.12	89.24	49.95	0.1
HDL-c (mg/dl)	49.31	16.35	48.07	36.12	0.8
LDL - c (mg/dl)	116.49	39.10	94.16	34.66	0.01
Albumin / creatinine ratio (µg/g)	69.69	81.29	8.45	10.68	0.001
OxLDL	43.34	14.21	26.58	13.74	0.0001
Carotid intimal medial thickness	0.52	0.06	0.41	0.03	0.0001
Aortic intimal medial thickness	0.72	0.11	0.46	0.04	0.0001

Also our findings are in agreement with the finding of McGill et al [12] that atherosclerosis develops first in the intima of abdominal aorta and they showed that aIMT may be used as a noninvasive ultrasound marker of preclinical atherosclerosis in children.

Table 4: Correlation between demographic, anthropometric, laboratory data and carotid intimal medial thickneass and aortic intimal medial thickness in diabetic patients

Variables	r	P-value
Age of patients (yrs)	0.35	0.0001
Duration of disease (yrs)	0.21	0.08
Onset of disease (yrs)	0.14	0.24
Systolic blood pressure (mmHg)	0.03	0.82
Diastolic blood pressure (mmHg)	0.05	0.71
BMI (kg/m^2)	0.11	0.36
Waist/hip ratio	0.27	0.03
Waist/ height ratio	0.16	0.18
Cholesterol (mg/dl)	0.19	0.13
Triglyceride (mg/dl)	0.15	0.25
HDL-c (mg/dl)	0.07	0.62
LDL-c (mg/dl)	0.06	0.67
HbA1 (%)	-0.03	0.79
Albumin/ creatinine ratio (µg/gm)	0.16	0.27
Insulin dose (U/kg)	0.01	0.93
Carotid intimal medial thickness	0.30	0.01
OxLDL	0.05	0.67

In the present study, aIMT was found to be significantly higher than cIMT in diabetic patients (P = 0.0001) and Ten of our patients (14%) with normal

cIMT revealed significantly increased aIMT. Our findings are in agreement with *Järvisalo et. Al [7] and with* an autopsy study [3] that showed that atherosclerotic lesions tend to affect certain arterial segments, especially the dorsolateral wall of abdominal aorta just proximal to its bifurcation and reported that atherosclerotic lesions occur later in life in the coronary and carotid arteries than in the aorta.

McGill et al [12] reported that fatty streaks develop in different areas of the abdominal aorta but those on the dorsal surface of the distal abdominal aorta develop and progress most rapidly to become raised lesions, so we focused on this on this site in the present study, being the most lesion-prone site seen in autopsy study. Since fatty streaks develop in the aortas of adolescents but raised lesions occurs after the age of 20 years [12], so the increased aIMT seen in diabetic adolecents in the present study likely reflect increased fatty streak formation.

In our study, the increased aIMT in diabetics was positively correlated to waist/hip ratio. Our findings are consistent with observations of Dawson et al [14] who reported that aIMT was associated with body mass index (BMI), and waist/hip ratio. Also Rathsman et al [15] reported increased cIMT in diabetic adolescents with positive correlation between cIMT and waist circumference [15]. Similarly McCloskey et al [17] concluded that increased infant weight and adiposity at birth, as well as increased early weight gain, were positively associated with aortic intima-media thickness. In the current study, increased aIMT had significant correlation with age of the patients (P = 0.0001) but not correlated to other cardiovascular risk factors. Similarly, Jarvisalo et al [7] reported association between age & aIMT in diabetic children. In line with our findings, a Japanese study of 60 Japanese children age 5 to 14 years [16], cIMT was not associated with any cardiovascular risk factors but did increase with age.

Diabetic children show increased IMTs compared with healthy controls with more pronounced increase in the aIMT than in the cIMT. Since atherosclerotic changes starts first in the intima of the aorta, our data suggest that the aIMT might provide the best currently available noninvasive marker of preclinical atherosclerosis in diabetic children so we recommend early and close observation of children with diabetes for detection preclinical atherosclerosis.

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Clinical Science

Cigarette Smoking and Oxidative Stress in Patients with Coronary Artery Disease

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Abstract

AIM: To determine whether cigarette smoking, as a risk factor for CAD, affects (anti)oxidant status.

MATERIAL AND METHODS: The study included patients with CAD, divided according to their smoking status and the number of cigarettes smoked during a day. Biological markers of oxidative stress (concentration of oxidants and activity of antioxidant enzymes) were measured in all subjects.

RESULTS: The study included 300 patients with CAD, (average age of 63 ± 11 years), predominantly males. Of the total, 34.0% were active smokers, 23.0% were former smokers, and 43.0% were non-smokers. Most of the active smokers smoked 1-20 cigarettes/day. In terms of concentration of oxidants (MDA and HP) there was not a significant difference between smokers versus non-smokers. As for the activity of antioxidant enzymes (SOD, CAT and GPX), a statistically significant difference was found in the activity of GPX among the active smokers with CAD and the non-smokers with CAD (p = 0.039).

CONCLUSION: Smoking as a risk factor for CAD is closely associated with increased oxidative stress, and the number of cigarettes smoked plays an important role in increasing the level of oxidative damage and reducing antioxidant defence.

Introduction

Smoking and cigarette smoke are among the dominant risk factors for premature or accelerated development of peripheral, coronary and cerebral atherosclerotic vascular disease. Cigarette smoke contains more than 4000 identified ingredients [1] including nicotine, ammonia, acrolein, phenols, acetaldehyde, polycyclic aromatic hydrocarbons, polyphenols, then carbon monoxide, nitrogen oxides, hydrogen cyanide, trace metals [2, 3].

Two main phases have been identified in cigarette smoke, tar phase and gas phase. The two phases are rich free radicals, and non-radical oxidants. Superoxide radical (.O2-), and particularly hydroxyl radical (.OH), and peroxyl (.ROO) are able to initiate oxidative damage in the form of lipid peroxidation [4].

Because oxidative modification dominates the current concept of the pathogenesis of atherosclerosis, many studies, including that of Marangon et al. [5], have been focused on oxidative stress as potentially clinically relevant factor where cigarette smoke is associated with cancer and atherogenesis. They considered that smokers have a triple threat, first as they actively smoke cigarettes, second because of unhealthy nutrition with reduced

intake of antioxidants and finally because of consumption of large amounts of alcohol during smoking [6]. As a result, they increased oxidative stress and reduced antioxidant protection.

It is believed that smoking causes increased oxidative stress because of several mechanisms, including direct damage by radical species and the inflammatory response caused by cigarette smoking [7].

Peroxyl radicals and reactive nitrogen species cause direct damage stimulating lipid peroxidation and oxidation of proteins and DNA bases; aldehydes can deplete GSH (reduced glutathione) and modify protein -SH and -NH2 groups; cigarette smoke tar phase hydroquinone/quinine complexes diffuse across cell membranes, give rise to semiquinones and lead to the formation of superoxide radicals and hydrogen peroxide (H2O2) [8].

The damage caused by ROS (reactive oxygen species) in cigarette smoking occurs as an imbalance between production and detoxification of these species. Defence against oxidative stress is provided by a system of enzymes and antioxidants capable of preventing excess production of ROS and neutralizing free radicals [9].

Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) are an important line of defence against oxidative cell damage preventing lipid peroxidation, and protein and DNA oxidation.

The aim of this study was to determine whether cigarette smoking as a risk factor for coronary artery disease (CAD), affects (anti)oxidant status, and induces oxidative stress. For this purpose, we compared the biological markers of (anti)oxidant status (concentration of oxidants and antioxidant enzymes activity) in patients with CAD divided according to their smoking status, and the number of cigarettes smoked in active smokers.

Materials and Methods

The study included 300 patients with CAD, admitted to the University Clinic of Cardiology in Skopje.

Patients were examined for risk factors for CAD, with particular reference to smoking as a risk factor. Patients were divided into appropriate subgroups. According to their smoking status, the patients were separated into non-smokers, active smokers and former smokers. Non-smokers were those who during their life had never started smoking. Active smokers were those who smoked daily, over several years. According to the number of cigarettes smoked in 24 hours, active smokers were divided into: patients who smoked 1-20 cigarettes/per day, patients who smoked 21-40 cigarettes/per day and > 40 cigarettes/per day. Former smokers were those who during their life had actively smoked, but due to various influences stopped smoking.

The following parameters were determined in all patients: erythrocyte concentration of thiobarbituric acid reactive substances (TBARS), expressed as malondialdehyde (MDA), concentration of total hydroperoxides (HP) in plasma, both products of lipid peroxidation, and also the activity of erythrocyte antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX).

Fasting blood samples were collected by venepuncture of all participants, in tubes that contained EDTA as an anticoagulant. The blood was centrifuged at 4000 g for 10 minutes at 4ºC, immediately after venepuncture.

Plasma and leukocytes layer were separated, and the erythrocytes were washed three times with two volumes of saline. Then, a known volume of washed red cells were lysed with ice-cold distilled water (1:4), were left for 15 minutes in a refrigerator at a temperature of 4ºC, and cell debris was removed by centrifugation (10 minutes at 2000 g at a temperature of 4ºC). Samples of plasma and red cell lysates were kept at a temperature of -70ºC until the analysis.

Results

Basal characteristics of the study population

In our study we have included 300 patients with coronary artery disease. Within the total number of subjects, 187 (62.3%) patients were diagnosed with acute coronary artery disease and 113 (37.7%) patients with chronic (ischemic) coronary heart disease.

Patients had an average age of 63 ± 11 years, and were predominantly male (194 or 64.67% men and 106 women or 35.33%).

Risk Factors for CAD

Risk factors for CAD that were present in patients are shown in Fig. 1. All patients were divided according to their smoking status: 102 (34%) active smokers, 69 (23%) former smokers and 129 (43%) non-smokers. The group of 102 (34%) active smokers was divided into subgroups according to the number of cigarettes they smoked per day and night, which is shown in Fig. 1.

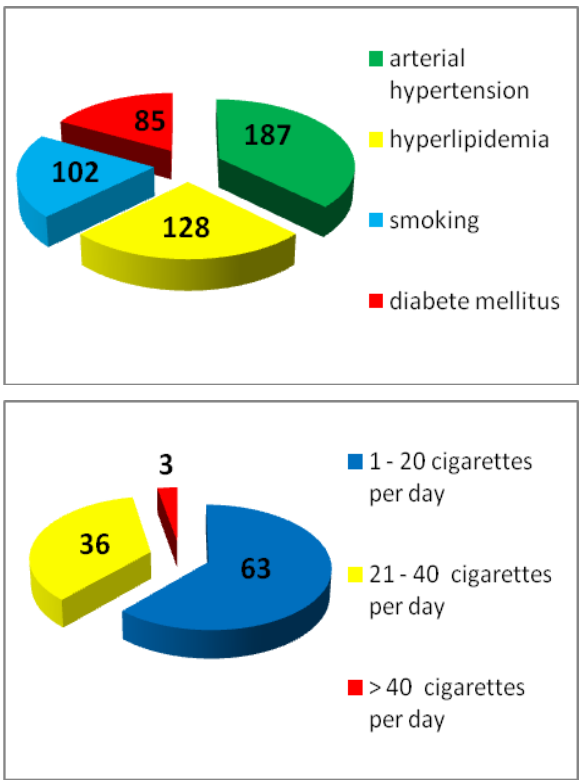

Figure 1: Risk factors for CAD (top) and active smokers by number of cigarettes smoked per day (bottom)

Biological markers of oxidative stress and smoking

We measured concentration of malondialdehyde (MDA) and the concentration of total hydroperoxides (HP) in patients with CAD, divided according to their smoking status. The results are presented on Table 1.

Table 1: Concentration of MDA and total HP (respectively) in patients with CAD, depending on smoking status

	Pts	Min	Max	Mean	Standard deviation
MDA (nmol/ml) | former smokers	69	20.153	65.915	33.791	7.605
MDA (nmol/ml) | non-smokers	129	17.260	81.857	33.474	8.178
MDA (nmol/ml) | active smokers	102	20.908	108.508	35.187	10.943
(ANOVA, p = 0.344) (Kruskal-Wallis ANOVA, p = 0.313).					
Total HP (CARR U) | former smokers	69	126.000	530.542	283.770	70.042
Total HP (CARR U) | non-smokers	129	152.753	510.103	268.875	69.398
Total HP (CARR U) | active smokers	102	111.658	484.103	289.204	78.551
(ANOVA, p = 0.180) (Kruskal-Wallis ANOVA, p = 0.077).					

Statistical analysis of these results showed that there was no statistically significant difference in mean concentration of MDA and total HP in patients with CAD according to their smoking status.

Concentration of MDA and the concentration of total HP were highest in patients with CAD who were active smokers and lowest in patients with CAD who had never smoked cigarettes.

Then we measured the activity of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) in patients with CAD, according to the smoking status. The results are presented on Table 2.

Table 2: Activity of SOD, CAT and GPX in patients with CAD, according to the smoking status

	Pts	Min	Max	Mean	Standard deviation
SOD (U/ml) | former smokers	69	10.477	571.631	128.620	114.651
SOD (U/ml) | non-smokers	127	12.118	560.085	139.121	122.408
SOD (U/ml) | active smokers	101	10.477	525.138	124.602	99.219
(ANOVA, p = 0.349) (Kruskal-Wallis ANOVA, p = 0.213)					
CATALASE (U/L) | former smokers	68	0.313	136.242	62.517	37.084
CATALASE (U/L) | non-smokers	123	0.313	147.374	67.066	40.728
CATALASE (U/L) | active smokers	94	0.430	133.137	63.889	36.780
(ANOVA, p = 0.728) (Kruskal-Wallis ANOVA, p = 0.765)					
GPx (U/ml)| former smokers	21	0.717	27.484	7.108	7.376
GPx (U/ml)| non-smokers	58	0.158	32.241	7.434	6.741
GPx (U/ml)| active smokers	38	0.158	10.510	4.362	3.030
(ANOVA, p = 0.042) (Kruskal-Wallis ANOVA, p = 0.038)					

Statistical analysis of these results showed that there was a statistically significant difference in the mean activity only of GPX in patients with CAD according to their smoking status.

In addition, the analysis showed that the activity of SOD was lowest among patients with CAD who were active smokers, and highest in patients with CAD who had never smoked cigarettes.

Active smokers according of number of smoked cigarettes per day

According to the number of smoked cigarettes per day, we examined if there is a difference in the concentration of MDA and total HP. The results are shown in Table 3.

Table 3: Concentration of MDA and total HP in patients with CAD who are active smokers according to the number of smoked cigarettes per day

	Pts	Min	Max	Mean	Standard deviation
MDA (nmol/ml) | > 40 cigarettes per day	3	21.301	55.982	35.606	8.208
MDA (nmol/ml) | 1 - 20 cigarettes per day	63	20.908	108.508	34.995	12.431
MDA (nmol/ml) | 21 - 40 cigarettes per day	36	28.108	43.964	34.212	8.535
(ANOVA, p = 0.954) (Kruskal-Wallis ANOVA, p = 0.673)					
Total HP (CARR U) | > 40 cigarettes per day	3	158.356	530.542	290.077	70.683
Total HP (CARR U) | 1 - 20 cigarettes per day	63	126.000	499.013	272.709	69.808
Total HP (CARR U) |21 - 40 cigarettes per day	36	219.138	347.661	284.062	64.272
(ANOVA, p = 0.499) (Kruskal-Wallis ANOVA, p = 0.371)					

Statistical analysis shows that there was no statistically significant difference of mean concentration of MDA and total HP in patients with CAD according to the number of smoked cigarettes.

However, the concentrations of both parameters are highest in patients with CAD who smoke more than 40 cigarettes/day.

Regarding the difference in the activity of antioxidant enzymes, SOD, CAT and GPX in patients with CAD who were active smokers, according to the number of smoked cigarettes per day, we received the following results which are shown in Table 4.

Table 4: Activity of SOD, CAT and GPX (respectively) in patients with CAD who are active smokers according to the number of smoked cigarettes per day

	Pts	Min	Max	Mean	Standard deviation
SOD (U/ml) | > 40 cigarettes per day	3	36.952	96.256	68.102	29.765
SOD (U/ml) | 1 - 20 cigarettes per day	62	12.875	525.138	125.458	101.932
SOD (U/ml) | 21 - 40 cigarettes per day	36	10.477	397.393	124.984	98.553
(ANOVA, p = 0.954)					
CATALASE (U/L) | > 40 cigarettes per day	3	18.417	50.876	29.901	18.192
CATALASE (U/L) | 1 - 20 cigarettes per day	58	0.313	147.374	70.462	42.368
CATALASE (U/L) | 2 -40 cigarettes per day	33	11.562	137.062	64.478	38.019
(ANOVA, p = 0.221) (Kruskal-Wallis ANOVA, p = 0.214)					
GPx (U/ml)| > 40 cigarettes per day	1	0.158	9.950	3.647	2.678
GPx (U/ml)| 1 - 20 cigarettes per day	22	1.370	10.510	5.412	3.393
GPx (U/ml)| 21 - 40 cigarettes per day	15	4.355	4.355	4.355	3.001
(ANOVA, p = 0.225) (Kruskal-Wallis ANOVA, p = 0.170)					

Statistical analysis shows that there was no statistically significant difference in activity of SOD, CAT and GPX in patients with CAD according to the number of smoked cigarettes.

The activity of SOD, CAT and GPX is lowest in patients with CAD who smoke more than 40 cigarettes/day and highest in patients with CAD who smoke 1-20 cigarettes/day.

Discussion

The results of our study clearly demonstrate that patients who smoke cigarettes have higher oxidative damage and reduced antioxidant status than those who never smoked. Our results showed that the concentrations of MDA and HP were higher in smokers versus non-smokers.

In their study, Metta et al. [10] proved that there is an imbalance between oxidants and antioxidant enzymes in patients who had been admitted in the intensive coronary unit with a diagnosis of ischemic coronary heart disease. According to their smoking status, patients were divided to non-smokers and smokers. There was a statistically significant difference in the activity of GPX, SOD and CAT among smokers versus non-smokers. In addition, MDA levels in plasma were significantly increased in smokers compared to non-smokers.

Also in studies of Lykkesfeldt et al. and Block et al. [11, 12] concentration of MDA in plasma and antioxidant profile were tested the in two groups of patients: smokers and non-smokers. It was proved that there is no significant difference in the antioxidant profile between the two groups, but there was a significant effect of smoking on the concentration of MDA in plasma.

Jansen et al. [13] studied the influence of smoking on the levels of several biomarkers of oxidative stress, antioxidant status and redox status, including plasma hydroperoxides. Using different assays, they confirmed that smokers have elevated concentrations of oxidative stress biomarkers and compromised antioxidant status.

Exposure to environmental tobacco smoke (ETS) is associated with increased risk of developing cardiovascular disease. A meta-analysis conducted in 1999, published a total relative risk of 1.25 (95% CI = 1.17, 1.32) and showed a dose-response relationship [14]. Even very short exposure to ETS can produce changes in platelet activation and endothelium-dependent vasodilation [15]. Cigarette smoke contains high concentrations of toxic gases and tiny particles that are easily inhaled [16]. Therefore, exposure to ETS leads to a risk of cardiovascular events associated with active smoking. The study of Megson et al. [17] proved that patients with acute myocardial infarction (AMI) who were exposed to ETS and admitted to the intensive care unit had increased oxidative stress, as demonstrated by two independent plasma biomarkers (malondialdehyde and protein carbonyl). By this way they proved the role of ETS in the development of atherosclerosis and the occurrence of AMI. In conclusion, they put forward that exposure to cigarette smoke is associated with increased oxidative stress, by significantly higher concentrations of MDA in plasma. Exposure to cigarette smoke was associated with an increased risk of death from all causes, cardiovascular deaths, and fatal/nonfatal acute myocardial infarction within 30 days of admission [18]. From here, it is plausible that oxidative stress may be involved in the mechanism by which exposure to cigarette smoke increases the risk of CAD.

In our study, active smokers who smoke > 40 cigarettes/day have higher oxidative stress than those who smoke 1-20 cigarettes/day or do not smoke, which means the number of cigarettes smoked is a significant risk factor for increased oxidative stress.

Morbidity and mortality from CAD are very high in Turkey [19]. EUROASPIRE III study showed that 20% of subjects who were hospitalized with CAD in Turkey are less than 50 years of age. Turkey has the highest prevalence of early CAD of all populations. The main reasons are the low levels of cholesterol in high density lipoprotein (HDL-cholesterol) and high prevalence of smoking, which are more common in Turkey than in other countries [20]. A study of Aksoy et al. [21], where they examined indices of oxidative stress and severity of CAD in young patients who were hospitalized with AMI, proved that oxidative stress is an important factor for the severity of CAD in young smokers. Elevated levels of biomarkers of oxidative stress reflected the seriousness of the disease and vascular damage associated with cigarette smoking in the early onset CAD.

The study of Bikkad et al. [22] confirmed the

view of increased lipid peroxidation and oxidative stress in smokers, expressed by increased concentration of MDA with a parallel reduction of antioxidant enzymes SOD and GPX. In this study, the level of SOD was significantly lower in smokers compared with non-smokers. The mechanism by which smoking causes low levels of blood SOD is unknown. Increased lipid peroxidation and antioxidant depletion in smokers may contribute for biomolecular vascular endothelial damage. The same study also reported a significant reduction in the level of GPX in smokers in comparison to the non-smokers. GPX seems to have a major role in the prevention of oxidative stress; it can also be an important anti-atherogenic enzyme. This enzyme is responsible for the removal of hydrogen peroxide and organic hydroperoxides formed during cellular oxidative metabolism.

In conclusion, smoking as a risk factor for CAD is closely linked to the increased oxidative stress. The number of cigarettes smoked per day plays an important role in increasing the level of oxidative damage and reducing the antioxidant defence, which results in increased oxidative stress.

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Complications of Laparoscopic Cholecystectomy: Our Experience from a Retrospective Analysis

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Abstract

AIM: The aim of this study was to evaluate the intraoperative and postoperative complications of laparoscopic cholecystectomy, as well as the frequency of conversions.

MATERIAL AND METHODS: Medical records of 740 patients who had laparoscopic cholecystectomy were analysed retrospectively. We evaluated patients for the presence of potential risk factors that could predict the development of complications such as age, gender, body mass index, white blood cell count and C-reactive protein (CRP), gallbladder ultrasonographic findings, and pathohistological analysis of removed gallbladders. The correlation between these risk factors was also analysed.

RESULTS: There were 97 (13.1%) intraoperative complications (IOC). Iatrogenic perforations of a gallbladder were the most common complication - 39 patients (5.27%). Among the postoperative complications (POC), the most common ones were bleeding from abdominal cavity 27 (3.64%), biliary duct leaks 14 (1.89%), and infection of the surgical wound 7 patients (0.94%). There were 29 conversions (3.91%). The presence of more than one complication was more common in males (OR = 2.95, CI 95%, 1.42-4.23, $p < 0.001$). An especially high incidence of complications was noted in patients with elevated white blood cell count (OR = 3.98, CI 95% 1.68-16.92, $p < 0.01$), and CRP (OR = 2.42, CI 95% 1.23-12.54, $p < 0.01$). The increased incidence of complications was noted in patients with ultrasonographic finding of gallbladder empyema and increased thickness of the gallbladder wall > 3 mm (OR = 4.63, CI 95% 1.56-17.33, $p < 0.001$), as well as in patients with acute cholecystitis that was confirmed by pathohistological analysis (OR = 1.75, CI 95% 2.39-16.46, $p < 0.001$).

CONCLUSION: Adopting laparoscopic cholecystectomy as a new technique for treatment of cholelithiasis, introduced a new spectrum of complications. Major biliary and vascular complications are life threatening, while minor complications cause patient discomfort and prolongation of the hospital stay. It is important recognising IOC complications during the surgery so they are taken care of in a timely manner during the surgical intervention. Conversion should not be considered a complication.

Introduction

Adopting laparoscopic cholecystectomy in a treatment of symptomatic cholelithiasis introduced a new spectrum of associated intraoperative and postoperative complications. Minor complications (biliary and non-biliary) are usually treated conservatively. Major complications (biliary and vascular) are life threatening and increase mortality rate, therefore creating the need for conversion to open surgical approach in order to treat them. The frequency of complications associated with laparoscopic cholecystectomy varies from 0.5 to 6% [1-4]. The most serious complications are associated with high mortality rate: injury of common bile duct with an incidence of 0.1-0.6% [5, 6], injuries of large blood vessels 0.04-1.22% depending on the study [7]. The most common complication is iatrogenic perforation of the gallbladder with spilt gallstones with an incidence of 10-30% [8]. Injuries during the laparoscopic cholecystectomy can be prevented by

precise operative technique, clear visualisation of anatomical landmarks, and careful dissection of tissues. Intraoperative cholangiography should be used in case of a dilemma [4, 9, 10].

Male gender, age, presence of systemic inflammatory response syndrome (defined by elevated inflammatory parameters- elevated white blood cell count and C- reactive protein), acute inflammation of the gallbladder and preoperative ultrasonographic finding of increased thickness of the gallbladder wall, and/or presence of gallbladder empyema, are all factors that increase risk for possible development of intraoperative laparoscopic complications, and the possibility of needing a conversion [11-14].

The aim of this study was to evaluate the intraoperative and postoperative complications of laparoscopic cholecystectomy, as well as the frequency of conversions.

Patients and Methods

We retrospectively analysed medical records of 740 patients who were diagnosed with cholelithiasis and had laparoscopic cholecystectomy in the General Hospital in Berane, Montenegro, in the time period between 2005 and 2014. The analysis included operative protocols, anesthesiology records, the medical history which included the history of the disease, documented laboratory findings and imaging results.

We analysed the type and frequency of intraoperative and postoperative complications, as well as factors that increase the risk for development of complications. We noted causes and incidence of conversions and the way they resolved. We noted gender, age, body mass index (BMI), white blood cell count, and level of C-reactive protein (CRP), preoperative ultrasonographic findings, pathohistological findings of the surgically removed gallbladder, as well as their correlation with the occurrence of complications.

The patients were divided into groups according to their age (older than 65, and younger than 65), gender (male, and female), BMI (greater than 25 kg/m^2, and less than 25 kg/m^2), white blood cell count (greater than 10 x 10^9/l, and less than 10 x 10^9/l), and CRP level (greater than 5 ml/l, and less than 5 ml/l). Subsequently, the correlation between these factors and type/frequency of intraoperative and postoperative complications were analysed. All surgically extracted gallbladders were examined by pathophysiologists in order to confirm the diagnosis of acute cholecystitis, chronic cholecystitis or presence of malignancy. Subsequently, correlations between these pathohistological findings and type/frequency of intraoperative and postoperative complications were analysed.

An ultrasonographic exam was performed 24 hours before each surgery. In order to simplify the analysis of the correlation between ultrasonographic findings and possible complications, all ultrasonographic findings were grouped into three groups: group I- chronic cholecystitis, group II- acute cholecystitis, gallbladder empyema, increased gallbladder wall thickness > 3 mm, and group III- gallbladder with fibrous changes and a calculus with > 2 cm in diameter. We used a standard four-port technique in all surgical interventions.

In order to test the differences between the groups and correlation between the presence of the risk factors and outcomes of the surgical interventions regarding the complications, we used Chi-square test, Fisher test, Mann-Whitney test. We used a multivariate regression analysis to determine the most important predictors of complications during and after the surgery. The results were considered statistically significant if the $p < 0.05$. The statistical analysis was performed by using statistical package SPSS v. 19.

Ethics

Each subject signed the acceptance of the study protocol, in which the Ethical Principles for Medical Research Involving Human Subjects (the Helsinki Declaration) were clearly stated.

Results

Out of the 740 patients in the study, 502 were female (67.8%), and 238 were male (32.2%). The median age was 51 years, including participants that were 16 to 98 year old. There were 97 patients (13.1%) with intraoperative complications (IOC) (Table 1). The most common complications noted were: iatrogenic perforations of the gallbladder- 39 (5.27%), bleeding from the tissues adjacent to the gallbladder 21 (2.83%), gallstones spilt into the peritoneal cavity 15 (2.02%).

Intraoperative bleeding from the cystic artery occurred in 5 (0.67%), bleeding from the port in 9 (1.21%) and bleeding from the ligaments of the liver in 4 patients (0.54%). The transection of the common bile duct, a major complication, occurred in in only one patient (0.13%) This complication caused conversion to open procedure and was resolved by hepatopathy with a T-drain. IOC was more frequent in males (34 males, or 25.21%) compared to females (63 females, or 7.37%).

Table 1: Intraoperative complications (IOC)

	IOC	Σ
N	97	13.1%
IOC – types		
Bleeding from tissues adjacent to gallbladder	21	2.83%
Bleeding from cystic artery	5	0.67%
Iatrogenic perforations of the gallbladder	39	5.27%
Injuries to the common bile duct	1	0.13%
Bleeding from the abdominal wall (port)	9	1.21%
Spilled gallstones	15	2.02%
Bleeding from the ligaments of the liver	4	0.54%
Lesions of the omentum	3	3.09%

There were 70 patients (9.45%) with postoperative complications (POC) (Table 2). The most common postoperative complications were: bleeding from the abdominal cavity more than 100 ml/24h (in 27 patients or 3.64%), bile leaks through the drain > 50-100 ml/24h (14 patients, or 1.89%). Less frequent complications were surgical wound infection (7 patients, or 0.94%), incisional hernia at the place of port (3 patients, or 0.40%), and intra-abdominal abscess caused by residual calculus in the abdominal cavity (2 patients, or 0.27%). In the postoperative period, one case of subhepatic collection and 2 cases of abscess formed around retained calculi were treated by laparotomy and they subsequently resolved. Hematoma of the abdominal wall around the working port was noted in 5 patients (0.67%). Choledocholithiasis was noted in 3 patients (0.40%), and this was resolved by endoscopic papillotomy. Carcinoma of the gallbladder was confirmed by pathohistological analysis in 4 patients (0.54%). POC was more frequent in males (26 patients, or 18.48%) compared to females (44 patients, or 5.17%).

Table 2: Postoperative complications (POC)

	POC	Σ
N	70	9.45%
POC - types		
→ Bleeding from abdominal cavity >100 ml/24h	27	3.64%
→ Bile leaks >50-100 ml/24h	14	1.89%
→ Subhepatic collection	3	0.40%
→ Surgical wound infection	7	0.94%
→ Incisional hernia	3	0.40%
→ Hematoma of the abdominal wall	5	0.67%
→ Gallbladder carcinoma	4	0.54%
→ Retained calculus in choledochal duct	3	0.40%
→ Lost gallstones (abscess)	2	0.27%
→ Choleperitoneum	2	0.27%

Both IOC and POC were more common in males compared to females, and this difference was statistically significant (χ^2 = 0.548, p<0.01). There were 29 conversions (3.91%), and they were more common in males (19 males, 7.98%) compared to females (10 females, or 1.99%). This difference was also statistically significant (χ^2 = 6.743, p<0.05). The causes for conversions are shown in Table 3.

Table 4 shows analysed variables and their correlations with an occurrence of POC and IOC. In addition, it shows the correlation between the noted risk factors and the need for a conversion. The multivariate regression analysis showed the most important predictive factors for the occurrence of IOC, POC, and conversions with the confidence interval of 95%.

Table 3: Causes of conversions

	Male	Female	Σ
Conversions- causes	19 (7.98%)	10 (1.99%)	29 (3.91%)
Difficult access to Calot's triangle. Identification of anatomical structures	7	5	12 (41.37%)
Bleeding from the tissues adjacent to gallbladder	2	0	2 (6.89%)
Spilled gallstones	2	1	3 (10.34%)
Gallbladder empyema	3	2	5 (17.24%)
Mirizzi II	2	1	3 (10.34%)
Bleeding from the vascular supply	2	0	2 (6.89%)
Transection of the common bile duct	1	0	1 (3.45%)
Impacted calculus	0	1	1 (3.45%)

The occurrence of more than one complication was more common in males compared to females (OR 2.95, CI 95% 1.42-4.23, p < 0.001), in the group with increased white blood cell count (OR 3.98, CI 95% 1.68-16.92, p < 0.01), and the group with increased levels of CRP (OR 2.42, CI 95% 1.23-13.54, p < 0.01).

Table 4: **Correlation between examined variables and incidence of complications**

Variable	N/%	IOC	POC	CONV	P value
Age					
<65	552 (74.6%)	63 (11.41%)	44 (7.97%)	10 (1.82%)	<0.05
>65	188 (25.4%)	34 (18.08%)	26 (13.82%)	19 (10.10%)	
Gender					
Male	238 (32.2%)	34 (25.21%)	26 (18.48%)	19 (7.98%)	<0.001
Female	502 (67.8%)	63 (7.37%)	14 (5.17%)	10 (1.99%)	
BMI					
<25	295 (39.8%)	12 (4.06%)	9 (3.05%)	2 (0.67%)	<0.001
>25	445 (60.1%)	85 (13.7%)	61 (13.7%)	27 (6.06%)	
White blood cell count					
<10X10^9/l	452 (61.08%)	29 (6.41%)	20 (4.42%)	2 (0.44%)	<0.01
>10X10^9/l	288 (38.91%)	68 (18.%)	50 (17.36%)	27 (9.37%)	
CRP					
<5	392 (52.95%)	34 (8.67%)	21 (5.35%)	3 (0.76%)	0.0001
>5	348 (47.02%)	63 (18.%)	49 (14.08%)	26 (7.47%)	
Pathohist report:					
Acute cholecystitis	201 (27.1%)	67 (33.3%)	50 (24.8%)	26 (12.93%)	<0.001
Chronic cholecystitis	539 (72.8%)	30 (5.5%)	20 (3.7%)	3 (0.55%)	
Ultrasound findings:					
Group I (chronic cholecystitis)	419 (56.6%)	19 (4.53%)	10 (2.38%)	1 (0.23%)	
Group II (empyema, gangrene, wall thickness >3mm)	235 (31.3%)	65 (27.6%)	46 (19.5%)	26 (11.06%)	<0.001
Group III (gallbladder wall fibrosis, calculus >2cm)	86 (11.6%)	13 (15.1%)	14 (16.2%)	2 (2.32%)	

In addition, the ultrasonographic findings of empyema, gangrene of the gallbladder wall, and increased gallbladder wall thickness > 3 mm (group II) is a significant predictor of complications and

conversion (OR = 4.63, 95% CI 1.56-17.33, p < 0.001). Pathohistologic analysis of the surgically extracted gallbladder with the diagnosis of acute cholecystitis was also the significant predictor for complications and conversion (OR 1.75, 95% CI 2.39-16.46, P < 0.001).

Discussion

Laparoscopic cholecystectomy became the preferred method for the treatment of symptomatic cholelithiasis. Laparoscopic cholecystectomy has many advantages over the standard open cholecystectomy: minimal trauma, decreased pain, shorter hospital stay, satisfactory cosmetic outcome, quick recovery, and return to work. However, numerous studies have shown this that laparoscopic cholecystectomy is associated with a higher frequency of complications compared to the standard open cholecystectomy including lesions to the common bile duct, injury to the vascular and visceral structures during the application of a Veress needle, and a trocar with fatal outcomes [1-4, 15].

Review of recent literature shows that the incidence of injuries to the common bile duct is 0.1-0.6% [5, 6, 9]. Nuzzo et al [9] analysed complications of laparoscopic cholecystectomies done in 184 hospitals in Italy in the time period from 1998 to 2000 and reported 235 (or 0.41%) injuries of the common bile duct. In the presented study, we report one case of the common bile duct transection (0.13%) that was corrected by choledochoeneteroanastomosis with the Roux-en-Y loop. Although recent publications lead to the conclusion that injuries of the common bile duct are more commonly encountered with the laparoscopic procedure, the controversy related to this issue is still present [10, 16, 17]. Tanitia e al. [16] analysed data from 13,305 laparoscopic cholecystectomies that were done over a period of 13 years and found that 52 (0.32%) cases had a transection of the common bile duct.

As laparoscopic cholecystectomies gained wider acceptance, the spectrum of complications associated with this procedure also became wider. Vascular injuries are the most common ones, and after the complications of anaesthesia, they are the second leading cause of mortality and morbidity in laparoscopic surgery [18-20]. Our study shows that there were 21 patients with bleeding from the tissues adjacent to the gallbladder, 5 from the cystic artery, 4 with bleeding from the ligaments of liver, and 9 from the abdominal wall during the placement of ports. Although we did not have major vascular complications, we had 4 conversions because of the bleeding.

Both biliary and nonbiliary complications take an important place in the published studies. The most common biliary complications described are lesions of the common bile duct, lesions of the right hepatic duct, and perforation of the gallbladder with spilt calculi. Vascular injuries, injuries to the intestine, diaphragm, and iatrogenic pneumothorax represent the most important non-biliary complications.

In our study, there were 14 patients with the bile leak > 50-100 ml/24 h in the postoperative period. Other studies have shown that the injuries that are most commonly seen are minor injuries to the gallbladder, and ducts of Luschka with bile leaks, smaller bleeds with hematomas of the abdominal wall at the place of port, or in the tissues adjacent to the gallbladder. Although major injuries to the great blood vessels like the aorta, inferior vena cava, or iliac artery are rare, they are associated with high mortality rate [7, 18-20]. A study by Kaushik R [7] reports that complications with bleeding occur at a rate at up to 10%. In this study, he analysed 10,320 publications in English, and showed results from seven medical centers by seven authors with more than 1,000 laparoscopic cholecystectomies each. Khan reported 2 complications with bleeding (0.04%) out of 4,975 laparoscopic surgeries. Marakis G et al [20] reported 15 (1.22%) out of 1,225, and Kaushik R, 6 (0.49%) out of 1,233 laparoscopic cholecystectomies [7]. Intraoperative bleeding can be caused by insertion of the trocar, dissection of the gallbladder and the structures of the Calot's triangle. Postoperative bleeding can be caused by the removal of clips or ligatures and due to necrosis of the wall caused by effects of term cauterization.

The experience of the surgical team with the operative technique and equipment are important factors in preventing the complications. Surgeons who performed less than 100 laparoscopic cholecystectomies had more complications compared to surgeons with the greater number of surgeries [21, 22]. Contrary to that, there are other studies that show that surgeons with the greater number of laparoscopic surgeries have more complications [23].

Perforation of a gallbladder with gallstones spilt into the peritoneal cavity is a frequent complication, especially when associated with acute cholecystitis and larger gallstones [14, 17, 24]. Z'graggen K et al. [17] published a prospective study on 10,174 patients and showed that 1.4% complications were due to spilt gallstones. The estimated rate of gallbladder perforation is 10-30%. Duca et al [8] reported that the incidence of iatrogenic perforation of the gallbladder was 1,517 (15.9%) out of 9,542 patients who underwent laparoscopic cholecystectomy. In our study, we report 39 (5.27%) iatrogenic perforations of the gallbladder. Out of that, 15 cases (2.02%) were associated with spilt gallstones, which is in accordance with studies published by others.

Studies show that the most common complications after spilt and retained calculi in the abdominal cavity are: intra-abdominal abscesses, fistulas, and tumefactions of the abdominal wall [25-27]. Dasari BVM et al. [26] reported spilt calculi in 19.8% laparoscopic cholecystectomies in their study. In our study, we report abscess collections during the postoperative period in 2 cases (0.27%). They required laparotomy and evacuation. In addition, we report that spilt gallstones during surgery were a cause for conversion to open procedure in 3 cases (10.34% out of all conversions).

In recent publications, the incidence of injuries to the intestine varies between 0.07 to 0.7%. Intestinal injuries are usually caused by insertion of the trocar, dissection of adhesions from previous surgeries, or from the present inflammation. Frequently, they are not recognised intraoperatively [15]. Some authors report intestinal ischemia, as well as an evisceration of the section of intestine through a port [28, 29]. None of the cases from our study had intestinal injuries.

Surgical wound infection is a complication that occurs with higher frequency in open cholecystectomy compared to laparoscopic cholecystectomy [30, 31]. In our prospective study, we report 7 (0.94%) patients with the operative wound infection. Three patients (0.40%) had the incisional hernia, which agrees with studies published by other researchers. Boni et al. [30] reported that incisional complications were less commonly encountered in laparoscopic cholecystectomies compared to open cholecystectomies (mean 1.1% vs. 4.0%).

Hernias at the port insertion site have been reported in many papers with the incidence between 0.14% and 22%. Bunting DM [32] analysed 7 studies published in English, that were completed in the time period between 1995 and 2010, and that included 5984 patients who had laparoscopic cholecystectomies. This analysis reports 99 (on average 1.7%) cases of a hernia at the port insertion site as a postoperative complication. In the 7 studies that were included in this analysis, the incidence of this postoperative complication varies from 0.3% to 5.4%. The most common causes for the development of an incisional hernia were increased BMI, a diameter of the trocar duration of the surgery, a presence of a preexisting hernia, severity of inflammation, widening of the port for extraction of a gallbladder, and the age of the patient [32, 33].

In modern laparoscopic surgery, conversion is not considered to be a complication, but instead a way for the surgeon to safely finish the surgery. Therefore, the surgeon should have a low threshold for conversion [14, 26, 27]. In our study, we report 29 conversions (3.91%). Conversions were more frequent in males (7.98%) compared to females (1.99%), which agrees with studies published by others. Marakis G. et al. [20] published results of a 12-year study that included 1,225 patient who had laparoscopic cholecystectomies. This study reports 19 (1.5%) major complications, and 7.4% conversions. A meta-analysis on 14,545 laparoscopic cholecystectomies by Yang TF et al. reports 940 (6.41%) conversions [12]. This analysis shows that older age, male gender, acute cholecystitis, a gallbladder wall thickness > 3 mm and history of previous surgeries is all predictive factors for conversion.

The rate of conversion reported in today's literature are 2-15% [34]. In cases with acute inflammatory process reported rates of conversion increase up to 35% [13].

In conclusion, intraoperative complications and postoperative complications associated with laparoscopic cholecystectomy have their own specific characteristics. They are more common in patients with older age, male gender, with increased levels of markers of inflammation (white blood cell count and CRP), and in cases of acute cholecystitis confirmed by pathohistology. In addition, a preoperative ultrasonographic finding of gallbladder empyema, or gallbladder wall thickness > 3 mm, suggests that there might be an increased probability for the development of complications. Major vascular complications like the injury of the common bile duct, bleeding from the aorta, inferior vena cava or iliac blood vessels, are life threatening, and the surgeon is required to do a conversion. Conversions in these cases should not be perceived as a failure, but instead as a necessary procedure that will increase patient safety and likelihood for a favourable outcome.

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Clinical Science

Midshaft Clavicular Fractures - Osteosynthesis with Minimally Invasive Technique

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Keywords: minimally invasive plate osteosynthesis (MIPO); Midshaft Clavicle Fractures; Superior anterior Clavicle Plate.

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Abstract

BACKGROUND: Fractures of the clavicle are one of the most common fractures in modern orthopaedics and traumatology practice. Knowing the mechanism of trauma, and it's pathophysiological elements, it's clear distinction and it's individual features are essential to the development of more new and effective methods for their treatment, and the minimising of postoperative complications.

AIM: The aim of this paper was to present the results of our patients treated with minimally invasive plate osteosynthesis (MIPO).

MATERIAL AND METHODS: Between January 2011 and March 2013, 12 patients were treated with MIPO technique. The mean age was 47.5 years (range, 16-79 years). Outcomes and complications of clinical treatment were reviewed.

RESULTS: All fractures healed within a mean period of 4.9 months (range, 2-10 months). Regarding complications, there was no occurrence of implant failure or deep infection. There were no nonunions, but one 79-year-old man had a delayed union. Almost of all the cases didn't need bending of the plate. Seven plates were removed by their hopes. And there weren't any cases that required new incisions.

CONCLUSIONS: A pre-contoured plate anatomically configured to fit the clavicle was easier to apply. MIPO technique for midshaft clavicle fractures may be a good option.

Introduction

The clavicle is a membranous bone [1]. The main arterial supply to the clavicle is primarily periosteal [2]. Therefore, extensive periosteal stripping of the fracture site may cause complications, such as nonunion or infection [3]. Thus we have been performing minimally invasive plate osteosynthesis (MIPO) for displaced midshaft clavicle fractures [4].

The aim of this paper was to present the results of our patients treated with minimally invasive plate osteosynthesis (MIPO).

Materials and Methods

Under general anaesthesia, the patients were placed in a beach chair position. The C-arm was placed to take anteroposterior, oblique, and craniocaudal views of the clavicle. A superior anterior plate (DePuy Synthes, Oberdorf, Switzerland) was inserted. The function of the plate was "bridging plate". C-arm imaging in three positions was used to check fracture reduction. The craniocaudal view was the most important. That view confirmed bridging of the fracture zone in correct alignment with the plate well position to the S-shaped bone [5].

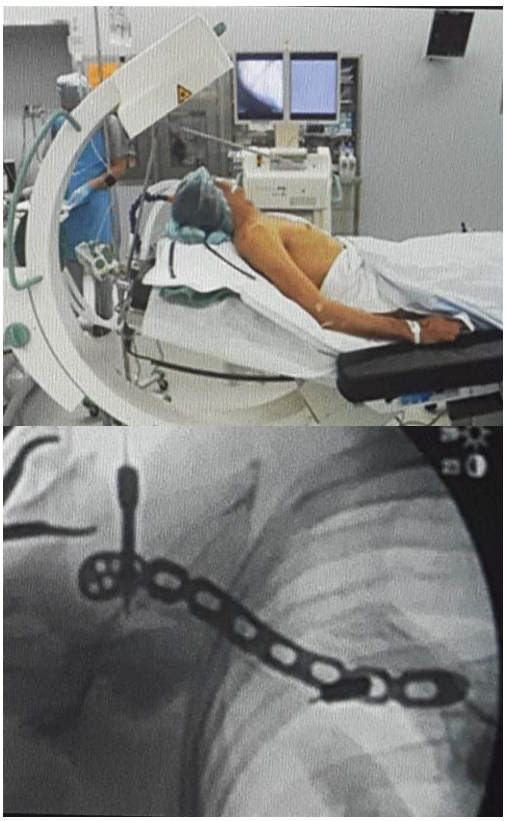

Figure 1: C-arm position of craniocaudal view

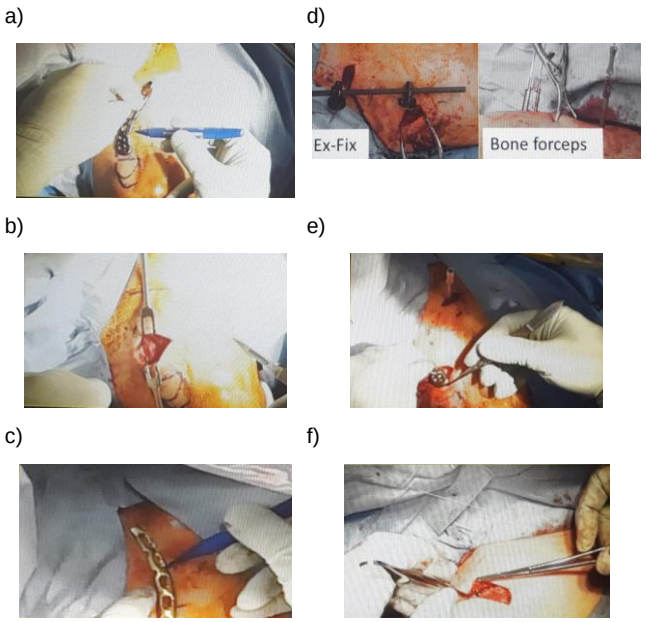

Figure 2: Surgical steps. a) A small longitudinal incision was made at the distal end or proximal end of the clavicle; b) The Platysma were incised; c) Superior Anterior plate was inserted then the incision was made the other side; d) Indirect fracture reduction and temporally fixation with Kirschner wires; e) Then a fracture fixation was performed using the appropriate number of cortical screws and locking head screws; f) Layered closure was performed to repair the platysma

Surgical Steps

Surgical steps are shown in Fig. 2 [6]. In general, a) A small longitudinal incision was made at the distal end or proximal end of the clavicle; b) The Platysma were incised; c) Superior Anterior plate was inserted then the incision was made the other side; d) Indirect fracture reduction and temporally fixation with Kirschner wires; e) Then a fracture fixation was performed using the appropriate number of cortical screws and locking head screws; f) Layered closure was performed to repair the platysma (Fig. 2).

Between January 2011 and March 2013, 12 patients were treated with MIPO technique. The mean age was 47.5 years (range, 16-79 years). Outcomes and complications of clinical treatment were reviewed.

Results

All fractures healed within a mean period of 4.9 months (range, 2-10 months). Regarding complications, there was no occurrence of implant failure or deep infection. There were no nonunions, but one 79-year-old man had a delayed union. Almost of all the cases didn't need bending of the plate.

Seven plates were removed by their hopes. And there weren't any cases that required new incisions.

Discussion

Traditionally, clavicle fractures have been treated nonoperatively [7]. However, recent studies have shown a high prevalence of symptomatic malunion and nonunion after nonoperative treatment of midshaft clavicular fractures [8]. Thus, operatively treated cases have increased. However, some complications have been described.

These complications may partly be caused by extensive periosteal stripping of the fracture site [9]. This study aims to assess the outcomes of midshaft clavicular fractures treated by our minimally invasive plate osteosynthesis technique (MIPO) [4].

MIPOs aims to preserve the biology at the fracture site, to maximise the healing potential of the bone, and to facilitate early and pain-free recovery [10]. To accomplish this, the fractures are reduced indirectly.

The clavicle is S-shaped. Thus, conventional plate bending may be difficult. Superior anterior plates have an anatomical design. There are two types of the

plate [11]. So they were very useful (Fig. 3).

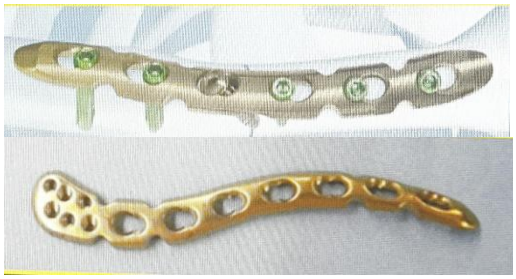

Figure 3: LCP Superior Anterior clavicle plate and LCP with lateral extension

In conclusion, a pre-contoured plate anatomically configured to fit the clavicle was easier to apply. MIPO technique for midshaft clavicle fractures may be a good option.

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Endoscopic Anatomy and Features of Anterior Cervical Foraminotomy by Destandau Technique

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Keywords: Anterior cervical foraminotomy; cervical disk herniation; minimally invasive spine surgery; endoscopic surgery.

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Abstract

BACKGROUND: Minimally invasive spine surgery limits surgical trauma and avoids traditional open surgery so in the majority of cases, recovery is much quicker and patients have less pain after surgery.

AIM: The authors describe an endoscopic approach to anterior cervical foraminotomy (ACF) by Destandau's method.

MATERIAL AND METHODS: Anterior cervical foraminotomy by Destandau's method is carried out under general anaesthesia. A 3 cm transverse skin incision is used just slightly past the anterior border of the sternocleidomastoid's muscle laterally. After exposing and dissecting superficial cervical fascia, platysma muscle, and deep cervical fascia, Endospine material designed by Destandau will be inserted. As from this moment, the procedure will continue using endoscopy.

RESULTS: the Endoscopic approach to anterior cervical foraminotomy by Destandau's method offers a convenient access to the cervical foraminal stenosis with fewer complications and negligible morbidity and gives maximum exposure to discal space with the goal of minimising cutaneous incision.

CONCLUSION: Contrary to the other minimally invasive approaches, the visual field in foraminotomy by Destandau technique is broad and depending on the workability of Endospine an adequate access to cervical disc is possible.

Introduction

The development of the endoscopic approach to lumbar disc herniation by Destandau in the early 1990s first and then to degenerative cervical spine disorders transformed both the treatment and patients' recovery. Anterior cervical foraminotomy by Destandau technique (ACFD) follows two major principles of minimally invasive surgery which are minimal iatrogenic trauma and extreme efficiency. Due to these two principles, ACFD seems to consider firstly, an adequate size of incision (scar does not exceed 3cm so it is less traumatic and more cosmetic and takes a fast and safe pathway) and secondly, and ensuring adequate target accessibility. Thanks to this minimization, ACFD shortens the recovery of the patient and there is virtually no postoperative restriction. Also, return to professional and sports life is possible in almost all cases. Furthermore, since then, more than 400 patients underwent surgery using this technique in the Endoscopy Center of Spine Surgery in Bordeaux from 2002 to 2014.

In this paper, we explain in details, endoscopic anatomy and features of anterior cervical foraminotomy by Destandau technique

Material and Methods

The surgery takes place under general anaesthesia. Supine position with the neck in gentle extension and the head held by a horseshoe-shaped pillow is required for this procedure. A preliminary x-ray image is obtained to determine the point of the

skin incision and verify the correct level of disk space once a special localisation device with two arms designed specifically for this purpose is applied. The target disc is centred on the monitor screen of the image intensifier. The localisation device is placed in position and its position modified until the two arms are projected onto the disc (Fig. 1).

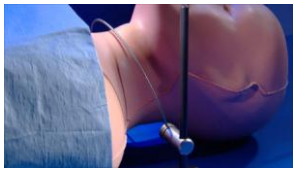
a)  b) 

Figure 1: a) Patient position; b) identification of correct level of disk space

Shadowless lighting will be set according to the spotted path of the disc space. This trajectory will guide the surgeon to find the correct level of the disk (Fig. 2).

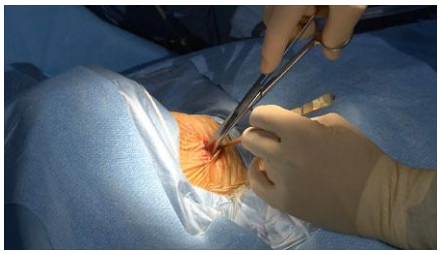


Figure 2: Shadowless lighting will be set according to the spotted path of the disc space

The skin incision is horizontal and approximately 3cm. It runs just slightly past the anterior border of the sterncleidomastoid muscle laterally extending to the midline. Subsequently, the superficial fascia is identified and sectioned. The platysma muscle is divided horizontally and the first layer of the deep cervical fascia is dissected until the omohyoid muscle and the pre-tracheal fascia is exposed. Afterward, the surgeon palpates and identifies the carotid artery by his (her) index and follows dissection superiorly and inferiorly by finger palpation to separate the neurovascular bundle on the outside from trachea and oesogaphus inside. This is where anterior cervical spine can be palpated and identified. A Faraboeuf or a Richardson appendiceal retractor is placed medially to explore prevertebral layer of deep cervical fascia longus colli muscles and the anterior longitudinal ligament. An 18-gauge spinal needle is positioned in the looked-for level. A lateral intraoperative radiograph is made to confirm the level. To have access laterally to the ventral surface of the cervical spine and uncovertebral joint, we prefer to coagulate and cut the medial border of longus colli muscle instead of exerting tension on the fascia. We consider that the latter action has a higher risk for the sympathetic trunk located in this fascia, slightly more lateral at the anterior surface of the longus colli muscle, Cloward self-retaining retractors are placed in the lateral and medial position to protect oesophagus and carotid. From this time on, Endospine will be inserted and will be placed between retractors. Endospine is the material designed by Destandau. It is composed of a speculum and an inner portion. This portion serves to support the endoscope (4 mm) and includes three other channels: one for suction, the other for surgical instruments and the last for root nerve retractor (Fig. 3). The first speculum will be placed.

Figure 3: Endospine with three channels, red arrow for surgical instruments; black arrow for suction; double arrow: for nerve root retractor

The inner portion is attached to the speculum and the rest of the procedure is carried out under endoscopic control. The first instrument used is an endoscopic drill (5 mm diameter 125 length drill). Drilling starts at the level of the disk or slightly above it, following the direction of the disk in an anteroposterior direction. This drilling is extended in order to perform a hole of approximately 8 mm. The soft tissues are resected using a 45° angled kerisson rongeur or a disk forceps. When the posterior border of a vertebral body is reached and the posterior longitudinal ligament is uncovered bone resection will be expanded laterally using a 45°, 2 mm Kerrison rongeur. At that time, disk herniation will be identified. To get an assurance that there is no adherence between the disk herniation and dural sac, the surgeon can use a hook that can be dragged between the herniation disk and dural sac. After that the disk herniation removed by hook and disk forceps (Fig. 4).

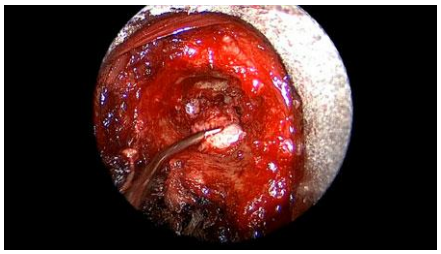


Figure 4: Using a hook, the herniation is dissected and removed by a disk forceps

If any another spinal cord compression by disk fragments is noticed, they will be removed by Kerrison rongeur. The foraminotomy is completed using 4.5 mm diameter 125 length drill and 2 mm Kerrison rongeur. Care must be taken to continue the

foraminotomy from lateral to medial to avoid inadvertent injury to the vertebral artery. The surgeon should be assured that Uncus is sufficiently open.

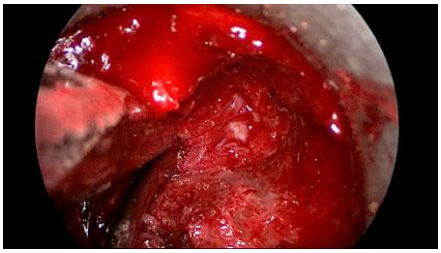


Figure 5: Nerve root is completely decompressed and exposed from the spinal canal to the vertebral artery

The procedure can be considered as being closed after complete exposure and decompression of nerve root from the spinal canal to the vertebral artery. It is not necessary to remove soft tissues adherent to the sheath of nerve because they did not exert any pressure on the nerve and their removal may lead to a dural tear.

Coagulation will be made and Endospine will be removed and the procedure comes to a closure.

Discussion

The anterior approach to the cervical spine for discectomy was described in 1955 by Robinson and smith [1]. Cloward modified this technique with direct decompression of the spinal cord and nerve root by removal of osteophytes in addition to discectomy. Since then, anterior decompression of cervical spine becomes common place.

Alternative mini-invasive approaches have been developed for cervical discectomy and foraminotomy over the last years of the previous century [2-14]. Endoscopic approach to cervical disc herniation and foraminotomy by Destandau's method gives maximum exposure to discal space with the goal of minimising cutaneous incision. Contrary to the other minimally invasive approaches, the visual field in ACFD is broad and depending on the workability of Endospine an adequate access even to two cervical levels is possible (Fig. 6).

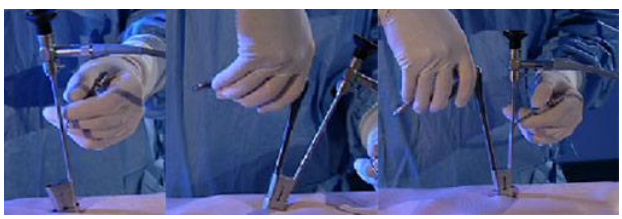


Figure 6: Endospine follows the movements of the instrument, the end of which is permanently in the field of the endoscope and gives a broad visual field

The nerve root is decompressed under direct vision and unlike conventional anterior decompression under the microscope, the surgeon does not need to stop and change the position of the surgical operating microscope The use of x-ray will be lessened to only two times in the beginning of the intervention and one more time preoperatively to check the level.

Thanks to the particular shape of the speculum, through which the surgery is performed, the risk of anatomical structures injury is minimised. There is no necessary to maintain the retractor when the speculum is placed. In this way the risk of oesophagus and carotid artery injury can be minimised because of the proximity of Vertebral artery during foraminotomy must be taken to not injury that. The other advantages of ACFD consist in disc sparing, avoiding fusion, and preserving motion segments. ACFD is logically indicated for foraminal and lateral disc compression, but it can be used for fulfilling adequately spinal cord decompression. ACDF is not indicated in the cases of circumferential disc herniation unless there was foraminal stenosis. Radiculopathy will be often disappeared very soon following surgery. Patients will be lifted the day of the surgery. There are no contraindications to twist the neck. The cervical collar will not be used. Patients will be discharged the day after surgery. The postoperative visit will not be necessary.

More than 400 patients underwent surgery using this technique in the Endoscopy Center of Spine Surgery in Bordeaux from 2002 to 2014. The results, Baseline characteristics will be published shortly in another paper.

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Treatment of the Aged Patients at a Large Cardiac Rehabilitation Center in the Southern Brazil and Some Aspects of Their Dropout from the Therapeutic Programs

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Keywords: Age; Elderly; Dropout; Cardiac Rehabilitation Program; Coronary Artery Disease.

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Abstract

AIM: This paper aims to assess the dropout rate in different age groups through the example of the large cardiac rehabilitation centre affiliated with the Institute of Sports Medicine, University of Caxias do Sul.

MATERIAL AND METHODS: A historic cohort study comprising the following groups: Non-Old < 65 (n = 141); Young-Old 65-74 (n = 128); and Middle-Old 75-84 years old (n = 57). The exercise program lasted 48 sessions and dropout was defined as attendance of 50% of sessions or less. Logistic binominal regression was performed to assess the risk of dropout. For all analyses, a two-tailed P value of < 0.05 was used.

RESULTS: The total dropout rate was 38.6%. The Young-Old and Middle-Old groups showed lower dropouts compared to Non-Old patients (p = 0.01). Young-Old has 96% less risk for dropout compared to Non-Old group (adjusted odds ratios = 1.96 [1.16–3.29]). Furthermore, patients underwent the Coronary Artery Bypass Graft showed a lower rate of dropout (p = 0.001). The absence of CABG involved three times more risk of dropout (p = 0.001).

CONCLUSION: The Non-Old and the Middle-Old patients showed higher dropout rates compared to Young-Old. To ensure the best possible rehabilitation and to improve patients´ participation in CR, these programs should be adjusted to the needs of patients in terms of their age.

Introduction

The increase in the life expectancy of the population has been in part driven by reduced mortality of aged people [1]. The number of people aged 65 or more is expected to grow from ca. 524 million in 2010 to almost 1.5 billion in 2050, with most of the increase in the developing countries. In Brazil, for example, the same demographic ageing which took more than a century in developed France occurred in two decades [2]. In a descriptive study covering the period from 1970 to 2010 the increase in the ageing index (AI) of 268% was determined for Brazil in general, with still higher AI established for the state of Rio Grande do Sul (RS) [3]. According to the data from the National Public Health Care System, in the RS, during 1991-2012 absolute number of seniors increased by 652,655 corresponding to the rise of the proportion of elderly people from 8.9% to 13.6% [4].

Despite the increase in longevity, the higher proportion of comorbidities distinguishes the older from the younger population [1]. It's known that the elderly people have 2-3 times higher incidence of acute myocardial infarction (AMI) than the younger ones. They also tend to have more complications associated with prolonged hospital stays, low physical activity and hence, suffer substantially higher fatality

rates due to coronary artery disease (CAD) [5]. Furthermore, cardiovascular disease (CVD) is by far the most important cause of hospitalisation among the elderly, and CAD is the leading cause of death in Brazil. A recent study showed that CVD accounted for 43.1% of all deaths that occurred between 2006 and 2010 in Brazil [6]. Because of high rates of morbidity and mortality, primary and secondary prevention programs are important strategies not only for alleviating cardiovascular risk factors but also for decrease the mortality and improvement of life quality for elderly patients [5].

Over the past four decades, rehabilitation programs have been recognised as an important tool in the medical care of patients with CVD. Preventive strategies for clinical practice should be developed based on cardiovascular rehabilitation programs because:

1) CVD is the leading cause of death in most countries; it is a major cause of disability and contributes significantly to increase healthcare costs.

2) Atherosclerosis can develop slowly over decades and its clinical manifestations are only seen in advanced stages of the disease.

3) Most CVD is closely associated with lifestyle.

4) Risk factors such as obesity, smoking, diabetes mellitus and hypertension have increased in the last decades.

5) The cardiac rehabilitation programs (CRP) are able to minimise risk factors and reduce morbidity and mortality [7].

Despite the low representation of older people in most experimental and observational studies on cardiac rehabilitation (CR), there seems to be clear that CR has the most beneficial effect of on younger patients compared to elder ones [8, 9]. At the same time, the risk of adverse reactions and complications for the elderly during the exercise sessions is similar to that of younger patients. Even though the age is not a determining factor for physical, functional and psychosocial response to a CRP, the existing stereotypes often prevent health professionals from prescription these exercises to the aged patients [8]. It seems clear that for many reasons the most important predictive factor for not entering in a CRP is the age [9]. In that respect, the strongest possible recommendations have an extremely high importance for entering the CRP.

CR is indicated for all patients with different presentations of CAD as it is known to have a positive effect on the treatment [7]. Nevertheless, elderly patients are less likely to be referred to a CRP [10]. It was shown that attendance to a CRP in a long-term reduces all-cause mortality [11]. However, the majority of older than 70 years referred to a CR program still avoids any training session [11, 12]. The term "old" often defines patients >65, despite the fact that most specialists consider patients 65-75 years old relatively young, while those >75 or 80 are "very elderly" and for them the CR data are still less available [13].

The dropout rate of aged patients from CR programs in Brazil was not comprehensively analysed and no differentiation by age group was carried out.

Thereby, this study aims to analyse the dropout rate of old people in a Brazilian CRP on the example of the large CR centre affiliated with the Institute of Sports Medicine (University of Caxias do Sul, RS). Comparative analysis of different age groups was also the aim of the present work.

Materials and Methods

Ethical aspects

This historic cohort study was conducted in the Cardiac Rehabilitation Service of the Sports Medicine Institute (SMI) at the University of Caxias do Sul (UCS), Brazil. The research was approved by the Research and Ethics Committee of the Cenecista Faculty of Bento Gonçalves. All patients had given informed consent.

Study Population

All patients were referred and attended the CRP between March 2011 and June 2016. They were divided into groups according to age: Non-Old (< 65 years); Young- Old (65-74 years); Middle-Old (75-84); and Oldest-Old (≥ 85 years), which is consistent with previous studies [14]. The exclusion criterion was the absence of data or discrepancy in the medical records.

Measures

The clinical characteristics assessed were: AMI with hospital report; CAD proved by cine coronary angiography; heart failure (HF) with an echocardiogram and coronary artery bypass grafting (CABG) and percutaneous transluminal coronary angioplasty (PTCA) with stent placement, both with hospital report. Also, risk factors analysed were: hypertension, diabetes mellitus, dyslipidemia, all three considered from a report of attending physician; tobacco was self-reported at medical interview.

The body mass index (BMI) was classified as corresponding to normal (≤ 24.99), to overweight (25-29.99) and to obesity (≥ 30). The exercise capacity (EC) was assessed according to the percentiles of maximal oxygen uptake (VO_2max) of the study

sample. The functional capacity classification varies according to age and gender. So, the calculation was done separately according to the type of assessment, exercise testing (ergometry) or cardiopulmonary exercise testing (ergo spirometry). Both tests were carried out using Micromed Biotecnologia Ergo PC Elite version 3.3.6.2. Protocol ramp was used in all exercises. Dropout was defined as attending 50% of the rehabilitation program or less, which is consistent with previous studies [15].

Cardiac Rehabilitation

A multidisciplinary rehabilitation program of 48 sessions of training was offered to each patient after being referred by an assistant physician. The program included: exercise training, dietary counselling by nutritionists, smoking cessation, and psychological support. Patients enrolled through National Health System (NHS) trained twice a week and those enrolled in Private Health Plan (PHP), trained 3 times a week. Before the training, the patients were seen by the nurse to assess vital signs. The session training lasted 60 min and it was conducted by physical educators. The exercise training program consisted of a combination of aerobic and strengthening exercises. In the end of the session, the physiotherapist coordinated stretching the major muscle groups.

Data collection and Statistical Methods

Data collection was performed using a spreadsheet online of Google Drive® from March 2013 to June 2015, in the SMI of UCS, a university service of CR. Statistical analysis was made with SPSS® software 22.0 version with descriptive statistics for sample characterization. The results were presented as a mean and standard deviation, absolute numbers for frequency and percentages values.

Clinical characteristics between age groups at baseline were compared using ANOVA (quantitative analyses) and by Chi-square for categorical variables. The logistic regression binominal was performed to assess the risk of dropout according to age groups, and to verify the confounding factors.

First, it was verified the correlation of the dropout with each variable by the obtaining the odds ratio. In the following steps, those variables were selected whose value level was less than 0.20 according to the descriptive test of significance. Then, the adjusted odds ratio was performed using logistic regression model. For all analyses was used a two-tailed P-value <0.05.

Results

Clinical Characteristics According to Age Groups

Out of 362 patients, 31 were excluded because of lack of information or disagreement of data in the medical records. Moreover, only 5 patients were Oldest-Old (≥ 85 years) and because of no sufficiency of this sample, these patients were excluded as well. Thus, in total the sample consisted of 326 patients. 141 of them were Non- Old Patients (<65); 128 were Young-Old people (65-74 years), and 57 were Middle- Old people (75-84 years old). The patients enrolled by NHS constituted 52.3% and by PHP, 47.7%. The overall mean age was 63.82 years (± 11.66) and 180 patients (55.2%) were men. Clinical characteristics according to age groups are summarised in Table 1.

Table 1: Clinical characteristic according to age: non-old group (< 65 y), young-old-group (65-74 y) and of the middle-old group (75-84 y)

Mean (sd[2])		Non-Old (n = 141)	Young-Old (n = 128)	Middle-Old (n = 57)	P value
Age		52.99 (8.57)	69.21 (2.85)	78.49 (2.61)	NA[1]
Body Mass Index		28.34 (5.29)	29.12 (5.97)	28.29 (4.89)	0.79
Ergometry VO_2 Max: ml/kg/min		26,09 (9,55)	21.30 (9.91)	14.42 (5.55)	<0.01
Ergospirometry VO_2 Max: ml/kg/min		20,47 (6,33)	17.18 (4.76)	14.02 (6.06)	<0.01
Prevalence at baseline (%)					
Gender Male		77 (54.6%)	54 (57.8%)	29 (50.9%)	0.66
Heart Failure		30 (21.3%)	33 (25.8%)	13 (22.8%)	0.64
Coronary Artery Disease		111 (78,7%)	98 (76.6%)	41 (71.9%)	0.32
Acute Myocardial Infarction		68 (48,2%)	44 (34.4%)	20 (35.1%)	0.03
CABG[3]		36 (25.5%)	40 (31.3%)	12 (21.1%)	0.82
PTCA[4] with stent		46 (32.9%)	34 (26.6%)	22 (38.6%)	0.73
Diabetes		34 (24.1%)	47 (36.7%)	22 (38.6%)	0.01
Hypertension		98 (69.5%)	106 (82.8%)	49 (86%)	<0.01
Dyslipidemia		80 (56.7%)	84 (65.6%)	33 (57.9%)	0.56
Smoker	Current	22 (15.7%)	19 (14.8%)	3 (2.5%)	0.05
	Ex-smoker	57 (40.7%)	40 (31.3%)	21 (36.8%)	
	Non-smoker	61 (43.6%)	69 (53.9%)	34 (59.6%)	

y: years; 1: not applicable; 2: standard deviation; 3: coronary artery bypass grafting; 4: percutaneous transluminal coronary angioplasty.

The clinical characteristics were comparable and a similar clinical status can be seen in both groups. Most significantly older people differ from more often cases of hypertension, diabetes, and by a higher number of non-smokers ($p < 0.05$). According to enrollment, the Non-Old group more often was covered by NHS, while in the Middle- Old group PHP prevailed. These results tend to be significant ($p = 0.07$). Older people were less prone to AMI and had lower EC in both tests ($p < 0.05$).

Dropout in Cardiac Rehabilitation Program

The overall mean of attendance at exercise sessions of the rehabilitation program was 28.94 (± 13.94), a frequency mean of 60.29%. Regarding dropouts, 120 patients (36.8%) abandoned the exercise program (≤ 24 sessions). Table 2 shows the clinical characteristics according to the dropout rates which differ significantly for different age groups.

Table 2: Clinical characteristics according to drop out in cardiac rehabilitation

		Dropout (n = 120)	Non-Dropout (n = 206)	P value
Enrollment	National Health System	70 (58.3%)	101 (49%)	0.10
	Private Health Plan	50 (41.7%)	105 (51%)	
Gender	Men	64 (53.3%)	116 (56.3%)	0.60
	Women	56 (46.7%)	90 (43.7%)	
Age	Non-Old	62 (51.7%)	79 (38.3%)	0.01
	Young-Old	35 (29.2%)	93 (45.1%)	
	Middle-Old	23 (19.2%)	34 (16.5%)	
Heart Failure		28 (23.3%)	48 (23.3%)	0.99
Coronary Artery Disease		93 (77,5%)	157 (76.2%)	0.79
Acute Myocardial Infarction		50 (41,7%)	82 (39.8%)	0.74
CABG[1]		19 (15.8%)	69 (33.5%)	<0.01
PTCA[2] + stent		42 (35%)	60 (29.1%)	0.27
Tobacco	Current	20 (16.7%)	23 (11.2%)	0.20
	Ex-smoker	46 (38.3%)	72 (35%)	
	Non-smoker	54 (45%)	111 (53.9%)	
Diabetes		35 (29.2%)	68 (33%)	0.47
Hypertension		92 (76.7%)	161 (78.2%)	0.75
Dyslipidemia		70 (58.3%)	127 (61.7%)	0.55
Exercise capacity	0-25th	31 (25.8%)	50 (24.3%)	0.47
(Percentile VO_2 max)	26-50th	32 (26.7%)	51 (25.5%)	
	51-75th	24 (20%)	81 (24.8%)	
	76-100th	33 (27.5%)	81 (24.8%)	
Body Mass Index	Normal (≤ 24.99)	27 (22.5%)	45 (21.8%)	0.82
	Overweight (25-29.99)	53 (44.2%)	98 (47.6%)	
	Obesity (≥ 30)	40 (33.3%)	63 (30.6%)	

1: coronary artery bypass grafting; 2: percutaneous transluminal coronary angioplasty.

Thus, the group Young-Old showed lower dropout rate compared to non-old patients (p = 0.01). Furthermore, patients underwent CABG, showed a lower rate of dropout at the end of the program (p = 0.001). The variables Enrolled, Tobacco, Age, and CABG and, thus, were selected to the binary logistic regression.

Logistic Regression Model for Dropout in Cardiac Rehabilitation

Logistic backwards regression was performed to identify the confounding factors. The remaining significant variables are shown in Table 3.

Table 3: Logistic regression model for dropout in cardiac rehabilitation

		Adjusted Odds Ratio	95% CI	P value
Enrollment	Private Health Plan (reference)	1.00		
	National Health System	1.45	0.89–2.35	0.12
Tobacco	Current (reference)	1.00		
	Ex-smoker	1.24	0.59–2.63	0.56
	Non-smoker	1.69	0.81–2.52	0.15
Age	Non-Old, <65 y (reference)	1.00		
	Young-Old, 65-74 y	1.96	1.16–3.29	0.01
	Middle-Old, ≥ 75	1.06	0.55–2.04	0.84
CABG[1]	Yes (reference)	1.00		
	No	2.76	1.53–4.95	<0.01

1: coronary artery bypass grafting.

The type of enrollment and tobacco status did not show any significant correlation with dropout. The age, on the other hand, showed that Young-Old people have 96% less risk of drop out compared to Non-Old (AOR: 1.96 [1.16–3.29]). The absence of CABG, in turn, increases the risk of dropout by almost three times (AOR: 2.76 [1.53–4.95]).

Discussion

The study consisted of 326 patients who attended the CRP of the SMI at the UCS. The patients were referred by physicians of the NHS and of PHPs. As expected, EC decreased with age of patients while comorbidities enhanced. The overall dropout was 36.8% and the age was seen as an important predictor for dropout. At the end of exercise program, the non-old patients showed higher dropout rate compared to young-old patients. The women did not show any difference regarding dropout. At the same time, CABG reduced the risk of dropout.

The prevalence of males in the literature varies due to differences in selection of participants in the studies, but it is clear that men, among older patients, are also most in this type of service [8, 17-19]. Our findings show same results. Our results have shown high frequencies of the risks of CVDs, especially of hypertension which corroborates with data of other studies [5, 17, 20]. The different clinical characteristics depending on age were expected and observed in our study. The ergo spirometry proved that VO_2 max in very old patients is very rare. The functional capacity of a person decreases significantly with advanced age [21]. Some studies showed lower functional capacities of older people at baseline in CRP. Nevertheless, this age group seems to respond better to an exercise program, and tend to have superior relative improvements of aerobic capacity [22-24]. The effects of aerobic capacity were not a goal of the present study which only described EC at baseline in the program. A significant decrease of VO_2 max was observed in the linear test, either by exercise testing or cardiopulmonary exercise test. Other comorbidities, as diabetes and hypertension noted in Table 1, were more common in older patients because they have more pathologies and this is in concordance with previous studies [9, 25].

The dropout rate of the elderly in CR programs worth discussing, since the patients, who abandoned the programs are more prone to cardiac complications than those who complete them [26]. Also, a reduced mortality was documented after 14-years follow-up, with control of a dose-response. Beauchamp et al. showed that attendance at 10-24% of training sessions have a good correlation with the greater global cause of mortality if compared with an attendance rate of 75-100% [12]. In our study, the overall dropout rate was 36.8% (120 of 326). The literature data shows some variations in these values. Thus Turk-Adawi et al. observed 49.7% of dropout [27], Worcester et al. found 19.08% [28] and Pardaens et al. registered about 20% of overall dropout from CR program [29]. This difference should be due to the socio-cultural specificity of different samples as they belong obviously to different societies and countries.

Suaya et al. evaluated outpatient CR training effect on the patients that had acute MI or CABG surgery using a statistical sample which comprised 267427 post- hospitalisation patients aged ≥ 65-year-old [9]. They found CR and exercise training was prescribed to 13.9% of the patients hospitalised with acute MI and to 31.0% of the patients after CABG surgery [9]. Patients underwent this kind of surgery shown higher adherence due to the severity of the medical conditions. Worcester et al. found the attendance rates of 66%, 51% and 25% respectively for CABG, AMI, and PTCA. In several studies, the CABG surgery was found to be a variable which can serve a predictor for non-dropout. In the work of Yohannes et al. [30] the correlation between CABG cases and accomplishment of CR program was also established, however for AMI and PTCA such relation was not found. Similar results were observed in the work [28] where it was noted that patients after CABG less likely abandon CR program than patients after acute coronary syndrome or HF. High degree of adhesion of CABG patients to CR program was also shown in the article [27] (AOR: 1.54 [1.24-1.82]). In our study, 27% of all patients had CABG surgery and these cases were equally distributed between age groups. It may be noted that our results corroborate with the literature data establishing a correlation between CABG cases and adherence to CR program. The AOR showed that participants of CR program that never underwent a CABG surgery had almost three times more risk for dropout. These findings were not observed for AMI or PTCA patients. However, it is not associated with 'poor' adherence of AMI patients but rather with higher adherence to the program of CABG patients. This could be explained by the severity of CABG which serves as a motivator for changes in lifestyle [8].

The multicenter cohort study presented in [27] was performed in 39 CR services and analysed 4,442 patients. The authors noted that younger patients (aged <65 years) were more likely to abandon the program compared to older patients (aged ≥ 65 years). In this article, the notion of "adherent" (i.e. patients attended > 50% of sessions) was used as reciprocal to "drop out". Besides, this paper did not distinguish the old people and therefore our results should be compared with observations made in [27] with attention to the methodological differences. A prospective study, in turn, consisted of 556 patients eligible to participate in secondary prevention program of exercises. There a mean age was 64,9 years old and the overall dropout rate were 23.4% [28]. This paper studied predictors for the dropout and found no difference between patients < 70 and ≥ 70 years old. In another prospective research, the dropout rate of approximately 20% was documented for all cases where one or more sessions were analysed [21]. However, a quantitative analyst was carried out in this article using only the mean age, while partitioning by age group has not been made and therefore higher rates of dropout for elderly patients could not be noticed.

In turn, Yohannes coordinated a study which included 189 post-AMI patients. They were recruited from a consecutive series of outpatient referrals prior to a CRP consisted of 6 weeks [30]. The authors considered dropout those who abandon the CR program during first two weeks and completers were those who attended the entire program (12 sessions). The quantitative assessment made in [30] has shown a trend for younger patients to drop out (61.4 y vs. 58.7 y, $p = 0.08$). In another large cohort study, which sought to assess the rate and predictors for dropout of CR program, age was not used as a factor [21]. For a 12-week program overall dropout rate was found to be 12.9 %; however, there was no clear definition of what was considered "dropout" and what was "completer" in their study [21]. On the other hand, another cohort of 872 patients with mean age of 67 years, did not define dropout objectively and, in turn, used subjective parameters for such a concept. In this study, it was seen dropout rates greater in patients over 65 years; however, it is difficult to consider these findings since the fall of methods [31].

The notion of dropout (from CR program) is not strictly defined. Most often participation in < 50% of the training sessions is regarded as a dropout, but some other approaches, such as abandon during first weeks, also exist. At the same time, it seems that even participation in 50% of sessions may not be enough for adequate rehabilitation. So, the concept of dropout needs to be standardised, which is however not the goal of the present contribution. In any case, in the present research, it was shown that Young-Old (65-74 years) demonstrated the lowest rate of dropout (AOR 1.96 [1:16 to 3:29], $p = 0.01$) compared to non-elderly patients (< 65 years). On the other hand, the dropout rate of the Non-elderly group was close to that of the Middle-old group. It can be probably assumed that younger people are more often diverted from CR treatment by higher workload and family responsibilities [32], while elderly are more dependent and more often suffer greater disability, not necessarily from CVD but also from co- morbidities such as arthritis [33].

This study has some limitations which must be acknowledged. One of them is the nature of observational historic cohort study. Despite the broad spectrum of used variables, some potentially significant parameters are lacking, including for instance information on illness cognition (i.e. how people perceive the situation they experience). The latter has been recognised in the literature as a crucial determinant of health-promoting behaviour along with the financial situation [30, 34-36]. Besides the factors such as self-motivation [37] and work demands [32], also have been recognised as important in the

literature. Another limitation of the present study was exclusion the Oldest-Old group due to the inadequate number of patients in this age category. In addition, thirty-seven patients were not included in the study because they did not attend any session at all after being evaluated in CR centre. Further research could clarify the reasons of that dropout.

Besides, the electronic database used in the present study was built from medical records collected in a single CR centre and therefore some measurement bias cannot be excluded. Despite the mentioned limitations, the present study is the first attempt to differentiate age groups in the analysis of dropout rate of aged people in a Brazilian CR program. It has shown that there are important differences between dropout rates in different age groups. We also consider this contribution quite representative because even with omitted Oldest-Old patients the statistical samples can be considered moderate in size, compared to other, not numerous studies based on still smaller sample sizes. Our main outcome was achieved objectively based on the attendance lists. In spite of being carried out in single-site, the study covered rather a long period of time and was performed in a large CR service, in a university centre of reference of southern Brazil.

In conclusion, the dropout rate in a large Brazilian CR centre was approximately 39% during 48 sessions of exercise program. The Non-Old and the Middle-Old patients showed increased dropout rates compared to Young-Old. Thus, there is a difference of dropout rate at elderly categories. To ensure the best possible rehabilitation and to improve patients´ participation in CR, the CR programs should be customised to patients' needs in terms of their age. It was also established that CABG procedure is associated with better adherence to the program. The study also confirmed that despite reduced functional capacity of older people, it should not be considered an obstacle for CR and these patients remain admissible to CR program.

Due to limited literature data, more studies involving elder patients are needed, particularly those > 84 years, since the low presence of them in this type of service. This is necessary for analysis of the problems that came with ageing and could contribute to dropout.

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Influence of Early Intensive Rehabilitation on Functional Mobility after Low Back Surgery

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Abstract

AIM: The research aims to determine the influence of early goal-oriented physical therapy program in combination with educational booklet and standard physical therapy without written instructions on functional mobility outcomes in patients after low back surgery.

MATERIAL AND METHODS: Thirty patients with similar functional impairments were randomly divided into two groups, a control group (CG n = 10) and an experimental group (EG n = 20). The outcome measures include time to move from lying to sitting position, the TUG test and the 6-meter walk test. Rehabilitation program includes daily physical therapy with mild to moderate intensity, achieving sitting position and education sessions how to perform activities of daily living (ADL) from the first day after surgery.

RESULTS: There was a significant improvement from baseline in two groups for all performed tests ($p < 0.001$). Statistical significant differences between two groups for transfers in bed on discharge ($p < 0.05$), in one month ($p < 0.01$) and for TUG in one month ($p < 0.05$) were found.

CONCLUSION: The study revealed that early rehabilitation program consists of therapeutic exercises and written educational booklet after low back surgery improves transfer abilities and basic activities in one month.

Introduction

Degenerative spinal diseases are common disorders and they are a significant social problem. Spine surgery due to degenerative diseases is associated with prolonged hospital stay [1].

Low back pain is second to upper respiratory problems as a symptom- related reason for visits to a physician. There are wide variations in care, a fact that suggests there is professional uncertainty about the optimal approach. Magnetic resonance imaging has come to be widely used, the roles of exercise and bed rest have been clarified, and more information has been gained from clinical trials [2]. The integrated program of early rehabilitation improved the outcome and shortened the hospital stay without more complications, pain or dissatisfaction.

The aim of the research is to compare the influence of a physical therapy program that combines active exercises with written instructions (educational booklet) about activities of daily living (ADLs) based on guideline recommendations, or oral information on functional mobility in patients after low back surgery in one month after an operation.

Material and Methods

Thirty patients, voluntarily attended and were treated in the Department of Neurosurgery at University Hospital Sofiamed - Sofia was randomly divided into two groups, control group (CG n = 10) and

experimental group (EG n = 20). All patients had similar impairments and functional limitations. The mean age of EG is 55.9 ± 13.8, of CG is 58.3 ± 9.5. The mean length of stay for EG is 3.9 ± 0.9; for CG is 3.6 ± 0.7 days. There were no significant differences in the age and length of hospital stay in both groups.

Table 1: Overview of the physical program

Steps	Goals	Activities
Preventing complications	Increase respiratory capacity -improvement of peripheral circulation	Breathing exercises/ combined with movement of the upper limbs, diaphragmatic breathing Abdominal drawing in manoeuvre
Take a sitting position	Self-moving in bed	Transfers and bed mobility exercises for upper and lower limbs in bed
Strengthening the spinal muscles	To increase the strength of muscles forming lumbar muscle corset.	abdominal exercises, isometric exercises, PNF
Training in walking	Improve posture Improve gait	Exercises for posture Training of gait
Patient's education in ADL	Prevention and long-term self-care for the back	Home exercise program (HEP) Healthy posture Balanced position of the spine and extremities

Outcome measures include transfer from lying to sitting position, Timed Up and Go (TUG) test [3] and walking speed for a six-meter walk test [4]. Transfer time was assessed by instructions which were given to the patient to sit up with legs down. Time for independent sitting without touching the bed with hands was measured. All the tests were measured three times, on the first day after surgery, on the day of discharge and one month later.

Table 2: Exercises and Instructions

Exercises	Instructions	Time
Exercises in supine Neurodynamic glides Pivot does not twist with neutral spine with all transfers and bed mobility Posture principles for ADL	Training transfers to bed (until the drain is removed), The physical therapist helps patient to transfer from one position to the next, sit at the side of the bed without rotation (using techniques for convenience).	5-6 min. 2-3 times
Stabilisation exercises in supine-sit -standing with no weight	The first verticalization is with or without a belt according to the intervention.	3-4 times
Strengthening the muscles that support the lower back	Without pain	5-10 times
Training in ADL - lifting subjects, footwear, clothing - Golfer's lift and reacher	Patient's education focuses on the acquisition of information and technical skills and transition to self-fulfilling action, which facilitates patients, helping them to make decisions and take appropriate action when changes in their disease or condition [6].	10 min.
Walking to a place in countries of the right and left, Walking back, Skip the subject, Climbing down and up stairs	Reinforcement techniques transfers, sit and walking, training in walking, climbing down and upstairs and pick up the object from the floor [6]. The therapist helps with gait, balance and strengthening of lower and/or upper extremities.	10-15 min.

All patients performed daily physical therapy for 30 minutes with mild to moderate intensity achieving sitting position from the first day after surgery. Proprioceptive neuromuscular facilitation for transfer positions, gait training (Table 1, 2) and an educational booklet with instructions (based on guidelines recommendations) on how to perform exercises and ADLs at home for one month after surgery (Table 3) were given to the patients of EG. The CG was on the standard physical therapy program and instructions on how to continue exercise at home and to resume daily activities gradually.

Table 3: Instructions to patients after discharge from hospital

N	ADL	Instructions
1.	Lying	Put a pillow under knees when lying on the back, and between knees when lying on one side.
2.	Getting up from bed	Sit in and get up from bed by first turning to one side
3.	Putting on shoes [6]	- lying on the back, bend both legs and place one heel on the other knee - in standing - tread on the shoe rack, or tread with one knee on the floor
4.	Sitting in a chair	During prolonged sitting before you stand up, perform several forward and backwards movements of the pelvis. Do not sit in one place for more than 20 minutes
5.	Getting up from a chair	When getting up from a chair body should bow forward, hands push from the hips. Hands can be used for support in sitting, too. Avoid low and soft armchairs and sofas.
6.	Getting subject [6]	To get subject without reaching or bending, it has to be on the level between hip and shoulder. When you need to take products from the refrigerator or stove, while bending the shoulders, you must slightly outsource back leg back and up, so that the body is not excessive bent.
7.	Lifting	Lifting the weight from the floor is through the squat, keep the weight close to the body
8.	Getting the objects above the head	Getting the objects above the head should not be done with tightening the body, but standing on something that will bring the subject to the level of the head.
9.	Washing dishes [6]	In activities when we lower the upper part of the body, we can carry the pelvis slightly back, with a little step one foot back
10	Driving a car [6]	Avoid driving at least one week after surgery. While sitting in a car, do the seat back angle greater than 90 degrees. Avoid bending, especially when you get out of the car.
11.	Getting a shower	Place a rubber mat on the floor to avoid sliding. If necessary, lay handles a convenient location to help with getting up from the toilet seat bath
12.	Ironing	Ironing table should be at elbow level, you can use a chair to sit.
13.	Carrying	When carrying purchases, the weight is distributed evenly in both hands

Statistical analysis was performed using SPSS 19.00 for Windows. Independent sample t-test and chi-square test were used to examine the baseline characteristics of two groups for age and gender. Independent and paired sample t-tests were conducted to determine the effect of the intervention on and transfers in bed, TUG and six-meter walk speed. Statistical significance was set at $p < 0.05$.

Results

No significant differences in the groups' baseline characteristics were found. There was a significant improvement (Table 4) from baseline in two groups for all tests ($p < 0.001$). Statistical analysis found no significant differences between the two groups, except for transfers in bed on discharge $t(28) = 2.64$, $p < 0.05$, in one month $t(28) = 3.44$, $p < 0.01$ and for TUG in one month $t(28) = 2.74$, $p < 0.05$.

The walking speed for six meters during hospitalisation improves with 0.2 m/s for EG and CG. The time for transfers starts from 9.1 sec. for both groups and improves to 6.1 sec. for EG and 7.7 sec. for CG. The significant improvement in transfers and TUG in one month was 4.8 sec. and 11.3 sec. for EG.

Table 4: Outcomes for both groups

Tests	Group	1st assessment Mean ± SD	2nd assessment Mean ± SD	3rd assessment Mean ± SD
Transfer (sec)	EG	9.1 ± 2.7	6.1 ± 1.7	4.3 ± 0.7
	CG	9.1 ± 1.6	7.7 ± 1.7	5.5 ± 1.2
TUG (sec)	EG	20.1 ± 6.5	14.9 ± 5.3	8.8 ± 2.5
	CG	19.8 ± 6.3	16.5 ± 6.2	11.8 ± 3.5
6 meters walk (m/s)	EG	0.4 ± 0.1	0.6 ± 0.3	1.0 ± 0.3
	CG	0.4 ± 0.2	0.6 ± 0.2	0.8 ± 0.3

SD- standard deviation; EG – experimental group; CG – control group.

Discussion

The research aims to determine the influence of early goal-oriented physical therapy programs in combination with educational booklets compared to standard physical therapy without written instructions on functional mobility outcomes in patients after low back surgery. There are different views of surgeons in terms of the initial period of rehabilitation after surgery [5]. Seventy percent of surgeons prefer to mobilise their patients out of bed on the very first day after surgery, 20% on day 2 and 10% leave it until the patient feels able [6]. However, recent reviews on rehabilitation following lumbar disc surgery concluded that there was insufficient evidence that rehabilitation, in general, could lead to a faster decrease in pain and disability. The physiotherapy interventions still remain unclear [7]. Despite the lack of evidence, we consider that early physical therapy contributes to a better recovery of the patient and leads to a faster return to normal functional status. All our patients were mobilised as early as possible, according to surgeons' instructions. They took a sitting position immediately on the first day after intervention. Michael R, (2005) describes that on the day after surgery, the patient demonstrated an expected amount of post-operative stiffness, range of motion (ROM) limitations and functional deficits [8]. Therefore, the first thing we demonstrated and explained to the patient was: how to perform movements in bed as in pivoting, not twisting the spine in all transfers and bed mobility. Physical therapy is focused on functional mobility (getting in and out of bed, transfers, and walking). We consider that this contributes to a better recovery of the patients and leads to a faster return to a normal functional state.

In the last few years, the mean hospital stay has reduced from 6.6 days to 2.6 days (2011–2012), which means that the time for training the patients to perform proper ADLs is limited [9]. Since hospitalisation is just a few days, the patients performed the rehabilitation program at home. In our case, the mean hospital stay was 3.8 days. During this period, patients improved their transfer abilities and walking speed, but not sufficient to restore the possibility of normal functional mobility. At discharge, a significant difference between the two groups only in transfers in bed was achieved, probably due to the short time of hospitalisation. Our research shows that for a better recovery of functional mobility after spinal surgery, patients need at least one month.

The importance of written patient education as an integral component of postoperative rehabilitation is increasing [10]. This indicates the need for developing a program for home practice and self-performing exercises. Our opinion is that a written educational booklet contributed in the restoration of physical activity. The same opinion is shared by other researchers [8, 10-12].

There are many pieces of evidence for the effectiveness of outpatient physiotherapy post first lumbar discectomy [11]. No particular methodology for rehabilitation has proven to be the best yet. Engers A. et al., (2008) compared different types of individual education and did not find significant differences [13]. There is a wide variation in physical therapy practice with respect to proper post-operative management. A home exercise program “HEP” was given by Michael R, (2005) [7]. Leaflets with information about the behaviour after spinal surgery such as “Get Well Soon“ [14] and “Your Back Operation” [15] are available on the Internet. McGregor A. et al (2012), considers that the booklet was welcomed by patients and they valued the information [12]. In our “Instructions To Patients After Discharge From Hospital”, we have also tried to give the most important trends in the performance of ADLs in a way that is safe for the back and does not cause complications after surgery. Restrictions on lifting, sitting and driving showed considerable inconsistencies within the recommendations given by surgeons [5].

In his study, Danielsen J. et al. (2005) proved that the postoperative disability is reduced at least in the first six months. He recommended intensive, standardised exercise training that ignores the fear of provoking pain and begins four weeks after the surgery [16]. We started training in proper motion and performance of ADLs immediately after surgery. Also, we gave written explanations of the requirements of performance of ADLs, and added to the description, pictures for easy understanding. Thus, patients gained confidence and self-esteem in their movements even before the discharge from the hospital. Care after discharge shows greater variability. Proper implementation of the ADLs and avoiding bending and rotation, lead to faster recovery and increase the independence in daily living [5].

The monitored basic activities (TUG test) demonstrate that until the day of discharge from the hospital, patients still have limited abilities in functional mobility, which were normalised on the first month after surgery. TUG was used in the study of Michael R, (2005) and results showed significant improvements. He discussed that TUG is appropriate

for monitoring the recovery in patients after spinal surgery [8].

In conclusion, the survey revealed that early rehabilitation programs consisting of therapeutic exercises and written educational booklet after low back surgery improved transfer abilities and basic activities in one month.

Further investigations should be made to assure the number of baseline variables which may influence the effectiveness of a physical therapy program and written instructions on functional mobility.

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The Association between Urinary Incontinence and Low Back Pain and Radiculopathy in Women

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Abbreviations: Urinary Incontinence (UI); Low Back Pain (LBP); Radiculopathy (RP); Oswestry Disability Index (ODI).

Abstract

AIM: Urinary incontinence (UI) is a common dysfunction, affecting especially women of all ages. The terminology of low back pain (LBP) and radiculopathy (RP) may be misused interchangeably with each other. There are many reports of the association with LBP and incontinence but those involving compression of nerve root(as RP), has not been distinguished from isolated low back pain. This study was structured to analyse the association of UI, LBP and RP.

METHODS: One hundred twenty patients were included in the study. Patients with spinal or urinary infection, tumour (spinal or others), cauda equine, pelvic operation, spinal trauma, spinal surgery, urogenital pathology were not accepted for this study. Age and weight of all patients were determined. Oswestry Disability Index (ODI) was utilised for assessment of loss of function and SEAPI incontinence index was used for urinary incontinence. All patients were examined for neurological pathology to differentiate between the LBP and RP by department of neurosurgery. Student t-test and Mann-Whitney-U tests were used for statistical significance.

RESULTS: There was no statistical significance between low back pain with overall urinary incontinence (p = 0.131), urge (p = 0.103) or stress incontinence (p = 0.68), respectively. However; The statistical aspects were identified relationship between overall (p = 0.026) and urge (p = 0.001) urinary incontinence with radiculopathy. The association of urge incontinence and radiculopathy seems to show a more significant relationship. Yet there was no correlation between radiculopathy and stress incontinence (P = 0.062).

CONCLUSION: Low back pain should not be regarded as a predisposing factor for urinary incontinence; however, radiculopathy has a statistically positive correlation between overall incontinence and urge incontinence.

Introduction

Urinary incontinence (UI) is a common dysfunction, affecting especially women of all ages. The terminology of Low Back Pain and Radiculopathy may be misused interchangeably with each other. The terminology needs to be enlightened.

Low Back Pain (LBP); seems complicated and many individual, psychosocial and workplace associated factors may play a part [1-3]. LBP refers to a more wide description of pain patients feel on the dorsal aspect of the vertebral bodies which may be due to nerve involvement or simply dorsal muscle contractions. Reported lifetime prevalence varies from 49% to 70% and point prevalences from 12% to 30% are reported in Western countries. About 90% of all patients with LBP will have non-specific LBP, which, in essence, is a diagnosis based on the exclusion of specific pathology [4]. A recently published systematic review of prospective cohort studies found that distress, depressive mood and somatization are associated with an increased risk of chronic LBP [1-3, 5].

Radiculopathy (RP)*;* covers a more specific clinical picture describing a problem in which one or more nerves are affected and do not work properly, thus showing signs such as ischiatic pain or claudication. The most common symptom of radicular pain is sciatica pain that radiates along the sciatic

nerve; down the back of the thigh and calf into the foot. The nature of the patients pains its quality, intensity, location and profile over time is an important guide in the evaluation. A careful but directed physical examination is necessary for the clinical evaluation of patients with lumbar spine disease. Evaluation of the patient involves; inspection of the back and legs, palpation and observation. A careful neurological evaluation, examination of strength, deep tendon reflexes, sensation and muscular function is necessary. The most commonly involved nerve roots are L3, L4, L5 and S1. Lesions of each produce distinct symptoms and other conditions can mimic the radiculopathies. The specific investigation is necessary for an accurate diagnosis. Some of the major causes of acute and chronic LBP are associated with RP. However, RP is not a cause of LBP; rather, nerve root impingement, disc herniation, facet arthropathy and other conditions are causes of LBP.

Likewise, incontinence also covers a wide range of underlying pathology, all of which results in involuntary loss of urine. To appreciate the association between incontinence and LBP as well as RP, the types of incontinence that are relevant should also be established. In Western societies; approximately 40% of women have occasional incontinence and a further 8% have regular incontinence episodes. Risk factors for incontinence include multiparity and infection of the lower urinary tract, older age, obesity, previous surgery for incontinence and neurologic disorders [2, 6-9].

Urinary incontinence (UI): A useful framework for considering continence problems is to view them as being associated with either the urethra or the bladder. In the urethra, there can be a decrease in outlet resistance associated with urethral hypermobility, as occurs in stress urinary incontinence or a functional failure at the bladder neck-proximal urethra, which underlies intrinsic sphincter deficiency. Bladder problems most often resulting in incontinence include detrusor overactivity or poor bladder compliance [1, 10-13]. Spinal cord injury and any neurologic lesion are potential causes of severe incontinence. When a neurologic disorder is a basis for incontinence, management will probably require the care of a specialist. In this study overall, urge and stress type incontinence was analysed as different entities [13, 14].

The association between LBP and UI may not be explained by conventional neurologic or genitourinary pathology. There are reports of the association of LBP and UI but those involving a nerve root, so to say RP have not been distinguished from general LBP.

Neuropathophysiology: Innervation of the lower urinary tract with both somatic and autonomic nervous system takes place. Parasympathetic pelvic nerves, the spinal cord is divided into branches of the second and fourth sacral. Parasympathetic pelvic nerves are mainly responsible for bladder excitatory effect. Third and fourth sacral segments of the somatic nervous interests and provide innervation to the external sphincter and other pelvic floor muscles. Sympathetic nerves, the lower thoracic and upper lumbar segments of the interests. Sympathetic nerves have inhibitory effects on the bladder. The sacral segments in adults, the level of first and second lumbar vertebrae, 1-15% cases, and the resulting pressure on the central disc prolapse impairs parasympathetic and somatic innervation [4].

This study was structured to analyse the association of UI, LBP and RP. The results of this study will highlight the significance of proper neuro-urological evaluation of patients with LBP and co-exist UI.

Material and Methods

The study cohort was derived patients referred to our neurosurgery department for LBP and UI. Patients with spinal or urinary infection, tumour (spinal or others), cauda equine, pelvic operation, spinal trauma, spinal surgery, urogenital pathology were not accepted for this study. Age and weight of all patients were determined. After initial evaluation and physical examination 60 patients with RP and 60 with LBP were included in the study. The types of UI were stratified with a detailed history. The diagnosis of LBP and RP were made with history, neurological examination and neuroimaging when RP was suspected. Oswestry Disability Index (ODI) was utilised to assess pain and associated quality of life deterioration and SEAPI incontinence index for UI. Those patients with severe pain that affected the quality of life were included in the study. Student t-test, Mann-Whitney-U tests were used for statistical significance. Exclusion criteria were: *i) existing pregnancy; ii) presence of orthopaedic or neurological diseases that may affect the evaluation of the patients; and iii) treatment for psychological pathologies.*

In the group of patients with radiculopathy, those with pathology at the surgical border were removed.

Oswestry Disability Index (ODI): Consisted of the assessment of pain level, personal care, object lifting, walking, sitting, standing, sleeping, social activities, and travelling and Changing Degree of Pain (a total of 10 items). ODI is an extremely important tool that researchers and disability evaluators use to measure a patient's permanent functional disability and is considered the "gold standard" of pain functional outcome tools [15].

SEAPI Scoring: The SEAPI-QMM Incontinence Classification System [Raz and Erickson, 1992] was developed in the early 1990s as a system that could quantify UI and its impact without special equipment or time-consuming procedures [16]. It is a standardised classification system for incontinence. The system is analogous to the TNM system for tumour staging. Each letter of SEAPI QMM represents an aspect of incontinence, and each factor is assigned a number grade. SEAPI is the acronym for incontinence factors of (S) Stress-related leakage, (E) Emptying ability, (A) Anatomic, (P) Protection, (I) Instability. Zero represents no symptom, problem, or abnormality while 1, 2, and 3 represent mild, moderate, or severe problems, respectively. It is patient filled the questionnaire and has been validated in Turkish.

Statistical Analysis: Student t- test was used to for ODI and SEAPI test results. Mann-Whitney-U tests were used to compare different types of UI with LBP and RP. All statistical analyses were carried out using SPSS 11.5 software (SPSS İnc. USA). Calculated p-value was considered statistically significant if smaller than 0.05.

Results

The mean time from the initial onset of LBP and to diagnosis was 16.2 $\pm$ 2.4 months; whereas for RP corresponding mean time was only 4.1 $\pm$ 1.2 months. When the correlation of incontinence to the initial onset of LBP and RP was asked, the patients reported almost simultaneously with the RP and but 60% of the LBP patients reported having UI even before the LBP, 30% almost at the same time with LBP and 10% over the last couple of months.

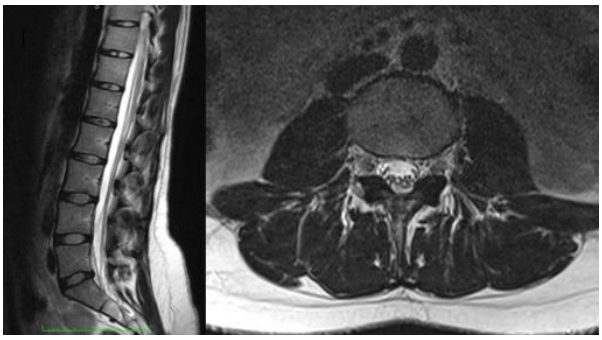

Figure 1: Lumbar MR images of the patient was evaluated by reason of LBP

The mean age and weight of the LBP and RP groups were 36.6 ± 12.8 years, 76.5 ± 10.2 kg and 38.50 ± 8.5 years and 77.67 ± 13 kg, respectively. There was no statistical difference between the two groups in terms of demographics.

There was no statistical difference between the two study groups in terms of ODI scores ($p > 0.05$); the mean ODI score for the LBP was 27.51 ± 18.31 and 26.33 ± 19.23 for the RP group. The mean SEAPI scores of the LBP and RP were 3.98 ± 6.621 and 5.28 ± 8.852, respectively.

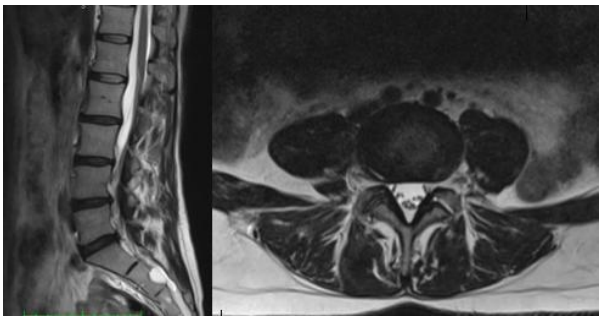

Figure 2: Lumbar MR images of patient was evaluated due to RP

The RP group was more compromised in terms of quality of life for urinary symptoms than the LBP group ($p < 0.05$) (Table 1).

Table 1: Mean age, mean weight, mean ODI score, mean SEAPI scores, mean time from the initial onset of low back pain and radiculopathy

	Low Back Pain	Radiculopathy
Duration Mean Time	16.2 $\pm$ 2.4	4.1 $\pm$ 1.2
Mean Age	36.6 ± 12.8	38.50 ± 8.5
Mean Weight	76.5 ± 10.2	77.67 ± 13
Mean ODI Score	27.51 ± 18.31	26.33 ± 19.23
Mean SAPI Scores	3.98 ± 6.621	5.28 ± 8.852

When stratified according to different types of UI using the Mann-Whitney U test analysis, p values of $p = 0.131$, $p = 0.103$, $p = 0.68$ are calculated between LBP and overall incontinence, incontinence due to overactive bladder (OAB) and stress urinary incontinence (SUI), respectively, showing no statistical correlation. When the same analysis was carried out for RP and different types of UI, there appears to be a strong correlation between overall incontinence and incontinence due to OAB ($p = 0.026$ and $p = 0.001$, respectively), but no correlation with SUI ($p = 0.62$). The correlation between RP and incontinence due to OAB seems to be stronger than overall incontinence rates (Table 2).

Table 2: When stratified according to different types of continence using the Mann-Whitney u test analysis of low back pain and radiculopathy

	Low Back Pain	Radiculopathy
Overall Incontinence	P = 0.131.	P = 0.026
Over Active Bladder	P = 0.103	P = 0.001
Stress Urinary Incontinence	P = 0.68	P = 0.62

Discussion

The terminology “LBP” is a very nonspecific symptom analogy. It should be differentiated from those pain conditions involving one or more nerve

roots. Nerve root involvement should be named as RP. LBP can be caused by a wide variety of factors. These include structural problems of the back, inflammation, muscle and soft tissue injury, a secondary response to other diseases or conditions, imbalances in body mechanics, and psychological/social factors, among others. There are the vast majority of data in the literature regarding the coexistence of LBP and UI [1, 2, 6-9]. Eliasson K et al reported; 77% of the women with LBP reported UI, of whom 73% occasionally, 23% several times and 4% often. 23% of the women could be classified as having ‘‘significant UI’’. Nineteen percent used sanitary pads because of the leakage. 32% percent were affected in their daily life, and 45% were psychologically affected [9]. 72% reported SUI, 1% UUI (Urge urinary incontinence) and 27% MUI (Mixed urinary incontinence). They have postulated that LBP increased the risk for UI almost three times for parous women and even more for nulliparous women [11].

However, Einstein *et al.*, in an afford to explain the relationship have concluded that the unusual association of LBP alone with UI, should be brought to the attention of clinicians, in the search for neurologic mechanisms to explain the phenomenon [17]. However, the term of LBP is a vague description of a symptom complex. In an effort to enlighten the association with lumbosacral pathologies and incontinence, the symptom spectrum should be stratified. Little is known about the relationship between UI and LBP. The relationship between UI and demographic factors such as age, weight and height is still controversial. Kim et al. acknowledged that little attention has been given to UI-related factors including LBP, static balance and demographic factors. Kim et al. hypothesised that a more severe UI condition results in more intense LBP and functional disability and in lower static balance ability may be relating the pelvic floor musculature. This logical approach could not be supported by evidence-based findings [12].

Our findings show that there is no correlation between lumbosacral pathologies and SUI. However, OAB symptoms and UUI is predominantly associated with lumbosacral pathologies. When subcategorized into those with LBP and those with RP, the pattern suggests that this relationship can only be established between RP. This actually provides a more explanation that relationship between LBP and UI.

There is actually no previous data that incorporates RP and UI in the literature. In a series by Einstein et al. reported that surgical approach for lumbosacral pathologies associated LBP has also cured urological symptoms of the patients [17]. In this report, one patient who’s RP did not respond to surgery due to pseudarthrosis in the fusion mass, continued to experience urinary symptoms. As a similar finding, De Riggo J. et al. reported degenerative spinal disease (LBP and RP) can result in acute or chronic UI. Surgical treatment improved or eliminated the symptoms of UI in more than half of the patients affected. They did not come up with an explanatory relationship [18]. Both results can be explained by our finding that only radicular involvement actually results in urinary symptoms and preoperatively may suggest possible improvement of UI.

This is a unique study that explains the possible neurological correlation between UI, LBP and RP. However, it is obvious that a larger study may yield other aspects of the correlation between UI types and pain syndromes. The surgical outcomes of RP in terms of UI would be another endpoint to explore.

In conclusion, not all lumbosacral pain syndromes are the same. Those patients, who report UI, should be carefully examined neurologically to stratify between LBP and RP as the lumbosacral surgery may be warranted for a cure. On the other side, neurologists, neurosurgeons and all specialities dealing with lumbosacral diseases should also be warned about possible co-existing UI since urinary symptoms are major factors in decreased quality of life.

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Diaphyseal Fractures of the Forearm in Adults, Plating Or Intramedullary Nailing Is a Better Option for the Treatment?

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Keywords: diaphyseal fractures of the forearm; plating; intramedullary nailing.

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Abstract

BACKGROUND: Fractures of the radius and ulna occupy a large field of the modern traumatology. Therefore, these fractures are a major subject in modern orthopaedics and traumatology. The study of the mechanisms of the trauma, and the pathophysiological changes that occur are of great importance for the development of ever more efficient and varied ways of the treatment and prophylactics of this type of fracture.

AIM: The aim of this paper was to study the pattern of the diaphyseal fractures of the forearm in adults, to decide the modalities of surgical management, to observe the period of fracture healing clinically and radiologically, as well to study the rehabilitation of the patients.

MATERIAL AND METHODS: The present study included 45 cases of diaphyseal fractures of both bones forearm in adults presenting to the orthopaedic outpatient department. For all the patients a detailed history was taken. A thorough clinical examination was carried out, required X-rays were taken, and initial treatment was given and admitted as in all patients. After careful pre-operative planning and evaluation for anaesthetic fitness, patients were operated for the fractures of both bone forearms. Twenty-three cases with 46 fractures were treated by open reduction and rigid fixation with DCP & Semi-tubular plates and 22 cases with 44 fractures were treated by closed reduction and fixation with “Talwarkar” intramedullary square nails.

RESULTS: United results were found in 100% of plating group vs. 86% in the nailing group. Delayed and non-union results were found in 9% of the nailing group only. Average time to union in weeks was 9.4 weeks in the plating group vs. 10.2 weeks in the nailing group.

CONCLUSION: Open reduction and internal fixation with compression plates with strict adherence to surgical technique is the gold standard method of treatment in both bones forearm fractures with excellent results than closed reduction, internal fixation with “Talwarkar” square nails which is also again a simple method with better results than conservative methods.

Introduction

Fractures of both radius and ulna are one of the most common fractures in adults in upper extremity [1]. In this era or active life, rapid industrialisation, increasing road traffic accidents, competitive sports, the incidence of fractures of forearm bones are increasing in frequency [2]. It is essential to regain length, apposition, axial alignment and normal rotational alignment while treating diaphyseal fractures of the radius and the ulna to gain good range of pronation and supination. The chances for the occurrence of malunion and non-union are greater because of the difficulties in reducing and maintaining the reduction of two parallel bones in the presence of the pronating and supinating muscles, which have regulatory as well as rotatory influences [3]. To obtain and hold an accurate reduction internal fixation is usually necessary.

The aim of this paper was to study the pattern of the diaphyseal fractures of the forearm in adults, to decide the modalities of surgical management, to observe the period of fracture healing clinically and radiologically, as well to study the rehabilitation of the patients.

Material and Methods

The present study included 45 cases of diaphyseal fractures of both bones forearm in adults presenting to the orthopedic outpatient department. For all the patients a detailed history was taken. A thorough clinical examination was carried out, required X-rays were taken, and initial treatment was given in all patients. After careful pre-operative planning and evaluation for anaesthetic fitness, patients were operated for the fractures of both bone forearms.

Twenty-three cases with 46 Fractures were treated by open reduction and rigid fixation with DCP & Semi-tubular plates and 22 cases with 44 fractures were treated by closed reduction and fixation with Talwarkar" square nails.

Postoperative management: Patients who were operated by compression plates or intramedullary nails are immobilised in the above elbow POP (plaster of Paris) slab immediately after the surgery, and the operated limb is elevated continuously and the distal neurovascular status is checked.

Antibiotics are given IV for the first 5 days and then replaced with oral antibiotics till the 12th day after the surgery. Anti-inflammatory agents, analgesics and other supplements were given.

The post-operative dressing of the surgical wound is done on the 2nd, 5th and 8th day after the surgery. Sutures are removed on the 12th day after the surgery, and in the case of suture line gapping they can be removed on the 15th day after the surgery. Appropriate active physiotherapy started.

Follow up: All patients followed up in outpatient department for a period of one month post-operatively for the clinical and radiological union, functional recovery and for the complications. All patients were evaluated based on the Anderson scoring system.

Elbow movements and wrist movements were noted and the union was assessed radiologically and clinically. Results were evaluated by the radiological outcome, functional outcome and postoperative complications in both groups according to the Saikia et al., 2011 (Table 1) [3].

Table 1: Radiological outcome, functional outcome and post-operative complications [3]

Results		Loss of flexion /extension	Loss of pronation/supination
Excellent	Union	<10	<25
Satisfactory	Union	<20	<50
Unsatisfactory	Union	>30	50
Failure		Non-union with/without loss of motion	

Results

Functional results by Anderson's Scoring system in both groups are presented in Table 2. We can see that excellent results in the plating group were 87% vs. 68% in the nailing group. Satisfactory results were found in 13% of plating group vs. nailing group. We found unsatisfactory and failure in 9% of nailing group only.

Table 2: Functional results by Anderson's Scoring system in both groups (plating and nailing)

Anderson's Scoring	No. of cases plating	%	No. of cases nailing	%
Excellent	20	87	15	68
Satisfactory	3	13	3	13.6
Unsatisfactory	0	0	2	9
Failure	0	0	2	9

Radiological findings are shown in Table 3: United results were found in 100% of plating group vs. 86% in the nailing group. Delayed and non-union results were found in 9% of the nailing group only. Average time to union in weeks was 9.4 weeks in the plating group vs. 10.2 weeks in the nailing group.

Table 3: Radiological Results of both groups

No.	Plating	%	Nailing	%
United	23	100	20	86
Delayed	0	0	2	9
Non union	0	0	2	9
Average time to union in weeks	9.4		10.2	

Complications were more associated with nailing group of which 2 cases had delayed union (2.2%), 2 cases developed non-union (2.2%), 1 case of malunion (1.1%), but in plating group only one patient developed superficial infection (2.2%), which was controlled with appropriate IV antibiotics after culture and sensitivity.

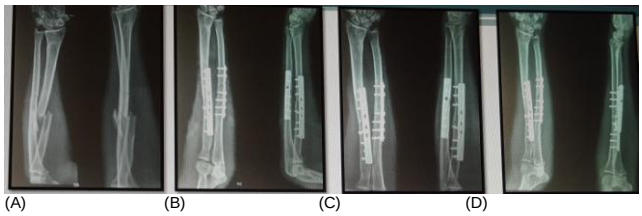


Figure 1: A- Pre-operative radiographic image. B,C and D- post-operative radiographic images after plating

Discussion

Fractures of both radius and ulna are one of the common fractures in adults in upper extremity [4]. Healing occurs relatively after closed treatment but mal-union with resultant decreased rotation of the forearm is common and has been associated with poop outcomes. Loss of rotation impedes the function

of the upper limb and activities of daily living [5].

The treatment of displaced fractures of shafts of radius and ulna is primarily operative [6]. The closed reduction and cast immobilisation for the displaced fractures should only be taken if there is a specific contraindication to operative treatment [7].

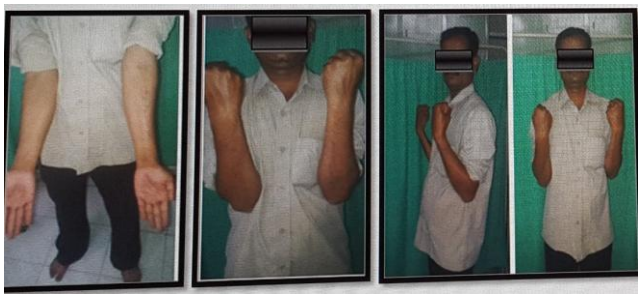
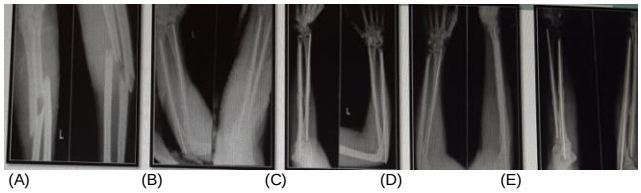


Figure 2: Postoperative results after plating

Open reduction and compression plate fixation have become the treatment of choice for diaphyseal fractures of forearm bones in adults. Compression-plate fixation gives a high rate of union, low rate of complications and the satisfactory return of rotation of the forearm. Thus excellent results of this mode of treatment have been reported in many series [8].

Figure 3: Postoperative results after plating

The AO- group has reported the successful use of compression plate and screws in the forearm shaft fractures. Since then it is one of the widely used and well-established methods of treating forearm bone fractures [8, 9].



(A) (B) (C) (D) (E)

Figure 4: A Preoperative radiographic image. B, C, D and E, Postoperative radiographic images after using intramedullary nail

The advantages of the plate and screw fixation are that the reduction is done under direct vision; the plates are applied so that there is compression at the fracture site. Bone grafting can be done if needed. The fixation is rigid, so postoperative immobilisation in a cast is not needed. The disadvantages being, the risks of any open surgical fixation, that is increased the chance of infection, disturbance of the soft tissues, periosteal stripping, and evacuation of fracture hematoma [10].

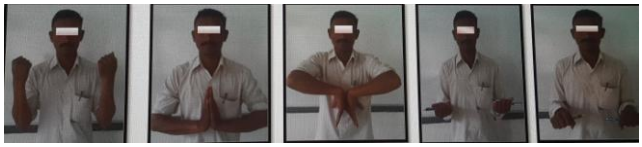


Figure 5: Postoperative results after using intramedullary nail

One important disadvantage is the risk of refracture after removal of the compression plate, which necessitates the forearm being protected in a splint for 6 weeks and from severe stress for 6 months [11].

Mechanically intramedullary nails offer several advantages over the plate and screw fixation. Intramedullary nails are subjected to smaller bending loads than plates and are least likely to fail by fatigue. The reason is that they are closed to the mechanical axis than usual plate position on the external surface of the bone [12].

Closed intramedullary nailing definitely has an advantage over the other modalities of treatment. It is minimally invasive procedure requiring shorter operating time. The biology of the fracture healing is not disturbed. Bone grafting is usually not needed. The risk of infection is minimal [13]. Intramedullary nails act as a load sharing devices in fractures with cortical contact. Stress shielding with resultant osteopenia commonly seen with plate and screws is minimised with intramedullary nails.

In conclusion, open reduction and internal fixation with compression plates with strict adherence to surgical technique is the gold standard method of treatment in both bones forearm fractures with excellent results than closed reduction, internal fixation with “Talwarkar” square nails which is also again a simple method with better results than conservative methods.

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The Role of the Velopharyngeal Sphincter in the Speech of Patients with Cleft Palate or Cleft Lip and Palate Using Perceptual Methods

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Keywords: cleft palate; velopharyngeal sphincter; velopharyngeal dysfunction; Czermak mirror fogging test; PWSS.

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Abstract

BACKGROUND: The velopharyngeal sphincter (VPS) plays the main role in speech formation. The cleft palate, due to the damage of the soft palate, leads to dysfunction of the velopharyngeal sphincter thus causing speech disorder.

AIM: To establish a link between the nasal air escape and the perceptual symptoms in the speech of patients with cleft palate or cleft lip and palate using auditory-visual perceptual procedures for determining the influence the velopharyngeal dysfunction has on speech.

MATERIAL AND METHODS: Twenty patients with speech disorders, out of which 10 have cleft palate or cleft lip and palate (experimental group), participated in the perceptual assessment by means of Czermak mirror fogging test for assessing the nasal air escape and Pittsburgh Weighted Speech Scale (PWSS) for assessing the probable nature of the velopharyngeal sphincter.

RESULTS: The respondents with a considerable nasal air escape have a higher velopharyngeal inability, that is, probably incompetent nature of the velopharyngeal sphincter. There is a strong correlation between the nasal air escape and the probable nature of the velopharyngeal sphincter (the coefficient of linear correlation r = 0.9756). The calculated p-value is p = 0.000002.

CONCLUSION: The perceptual speech symptoms and the nasal air escape provide unique insight into the state and role the velopharyngeal sphincter has in speech.

Introduction

The cleft palate and the velopharyngeal dysfunction can have great influence on the speech formation and the development of compensatory articulatory mechanisms [1].

In the case of cleft palate due to the damaging of the soft palate, dysfunction of the velopharyngeal sphincter (VPS) occurs, which is a three-dimensional muscle area that plays the most important role in speech formation. During speech production, the VPS separates the oral from the nasal cavity thus not allowing nasal air escape in the pronunciation of all sounds except for the nasal /M/, /N/ and /Nj/. Speech disorders are mainly characterised by hypernasality, nasal airflow, difficulties in phonation and compensatory misarticulation [2]. Velopharyngeal dysfunction (VPD) comprises a wide scope of speech disorders [3]. According to Trost-Cardamone [4], the term velopharyngeal inadequacy can be used as a generic term for all types of velopharyngeal dysfunction. Velopharyngeal insufficiency relates to the anatomic and structural defects, while the velopharyngeal incompetence refers to the neuromotor and physiological impairments. If there is mislearning of the articulatory schemes, then it is a case of velopharyngeal mislearning. Most authors suggest the

term velopharyngeal dysfunction as the most generic one.

The concept of velopharyngeal dysfunction exists theoretically; however, in clinical terms, velopharyngeal dysfunction is a diagnosis designed by perceptual symptoms in the process of speech production [5, 6]. The clinical examination of VPD begins with the evaluation of those perceptual symptoms appearing in speech production. It is important to establish the level of VPD on the qualitative and/or quantitative scale since this can offer some forecasting information and resources for following the changes through time.

This paper aims at establishing the correlation between nasal air escape and perceptual symptoms in the speech of patients with cleft palate or cleft lip and palate using auditory-visual perceptual procedures for evaluating the influence VPD has on speech. The focus of research is on the perceptual rating of the velopharyngeal function about the measurements of nasal air escape.

The most commonly used protocol for assessing the speech in velopharyngeal insufficiency among the experts from that field is that of perceptual assessment [7, 8]. Using perceptual assessment, various aspects of the speech formation are examined, including oral and nasal resonance, nasal airflow, consonantal strength/ oral air pressure and phonation in a specific context [9]. Due to their simplicity, noninvasiveness, non-technical nature and low costs of conduction, as well as fast and accurate diagnosis of VPD, auditory-perceptual examinations are of great importance for the further appropriate patient's treatment.

Material and Methods

Material

A total of 20 children between the age of 4 and seven were involved in this research, which was conducted in the period between September and December 2015. All the respondents have speech disorders (Dyslalia) and are divided into two groups regarding whether they have cleft palate or not. The first group (experimental group) comprises ten children with cleft palate (Palatoschisis) or cleft lip and palate (Cheilognatopalatoshisis) and speech disorders. The second, being the control group at the same time, comprises ten children without cleft palate or cleft lip and palate, but with speech disorders.

During the research period, all the respondents were given a speech therapy at the Centre for Rehabilitation of Hearing, Speech and Voice in Skopje according to the current protocol for that period. All the respondents were diagnosed with speech disorders.

Methods

In the research, two independent auditory-visual perceptual examinations were conducted for estimating the velopharyngeal function. The first, which falls within the category of the most relevant procedures for assessing speech disorders with cleft palate and velopharyngeal dysfunction, is the mirror fogging test [1, 10]; in our case Czermak mirror fogging test [11]. This test for nasal airflow is useful for assessing the function of the velopharyngeal mechanism [12]. In addition to this, auditory-perceptual testing was conducted by means of Pittsburgh Weighted Speech Scale (PWSS [13, 14], particularly standardised for assessing the velopharyngeal insufficiency [15] and also one of the most commonly used in practice [16, 17].

Czermak mirror fogging test

Often during the examination, speech disorders are first detected by perceptual assessment of speech quality, and one of the simpler methods used in this case is the mirror fogging test [18] (Figure 1). The technique used for administering the mirror fogging test which, at the same time, determines the level of nasal airflow is Czermak mirror fogging test [19].

For this test, a rectangular mirror with dimensions 10.5 cm x 17.5 cm was used. The mirror was not marked or graded. The mirror itself is used for visual indication of the nasal airflow.

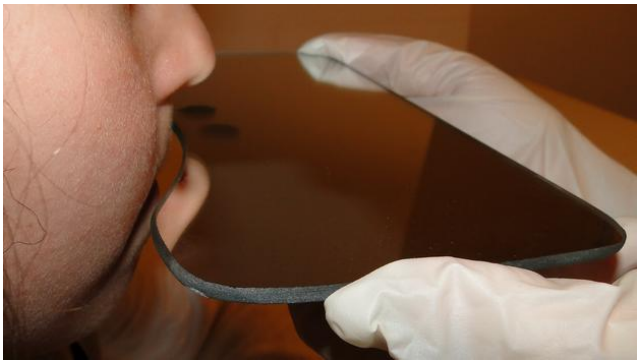

Figure 1: Administering the mirror fogging test

Practically, the procedure starts by placing the mirror horizontally under the patient's nose on the columella. Then, the patient pronounces test sounds, syllables and words. If fogged circles appear in the mirror, there is a sign of nasal airflow, thus implying velopharyngeal insufficiency which is considered as a positive result. According to the Czermak's test, depending on the size of the fogged circles appearing in the mirror, Figure 2, nasal air escape is ranged on a 4-grade scale, starting with 0 – no, 1 – small, 2 – medium up to 3 – large nasal air escape. When the result is a medium nasal air escape, the

velopharyngeal insufficiency is important, and hypernasality can be heard. If there is no fogging of the mirror, the result is marked as negative (normal result).

Figure 2: Rating the degree of the respondent's nasal air escape evaluated with the Czermak's test

The examination using Czermak's test practically consists of ranging assessment on eight items, Table 1. They are divided into items for testing hypernasality (non-nasalized and less nasalized vowels) and items for nasal airflow (the respondent is asked to blow, repeat words containing plosives and voiceless consonants, and say the fricative /S/ prolonged). The result for each item is determined by applying the semi-objective interpretation of the largeness of the fogged surface using Czermak's test. The final result of the patient's nasal air escape represents the highest score obtained with the rating.

Table 1: The items used for prospective ratings of hypernasality (1-3) and nasal airflow (4-8) using Czermak's test for assessing nasal air escape

	Item	Non-existent (0)	Small (1)	Medium (2)	Large (3)
1.	A				
2.	E				
3.	O				
4.	the respondent is asked to blow				
5.	Pa-Pa-Pa				
6.	Kapa				
7.	Kate kupi kaput.				
8.	prolonged /S/				
Result (highest grade)					

In this way, the results from a 4-graded rating can be compared with the results from confirmed speech analysis systems, such as the Pittsburgh Weighted Speech Scale (PWSS).

Pittsburgh Weighted Speech Scale (PWSS)

Pittsburgh Weighted Speech Scale (PWSS), Table 2, is a standardized method for auditory-perceptual assessment and one of the most commonly practiced methods used for rating the velopharyngeal insufficiency on a quantitative scale. This scale uses a standardised system of points that rate five speech components mainly noticeable in patients with velopharyngeal insufficiency: nasality, nasal emission, facial grimace, phonatory characteristics and compensatory misarticulations. Each component contains several items to which a varying weighted score has been ascribed. The overall score is a sum of the highest score for each component, except for the component articulation where the score represents a sum of all the scores for each separate item. The obtained score enables patients to be classified according to their velopharyngeal competency. If the established result is 0, there is a velopharyngeal competency; 1-2 means limited velopharyngeal competency, while 3-6 means limited velopharyngeal incompetency and 7 and above velopharyngeal incompetency.

Table 2: Pittsburgh Weighted Speech Scale (PWSS). The weighted score for speech symptoms connected to velopharyngeal insufficiency

Nasal air emission (0-3, highest score)	Right	Left
Not present	0	
Inconsistent, Visible	1	
Consistent, Visible	2	
Nasal escape on nasals appropriate	0	
Reduced	0	
Absent	0	
Audible	3	
Turbulent	3	
Facial grimace (0/2, presence)		
Absence of facial grimace	0	
Presence of facial grimace	2	
Nasality/ resonance (0-4, highest score)		
Normal	0	
Mild Hypernasality	1	
Moderate Hypernasality	2 (-3)	
Severe Hypernasality	4	
Mixed: Hyponasality - Hypernasality	2	
Cul de Sac	2	
Hyponasality	0	
Phonation / voice (0-3, highest score)		
Normal	0	
Hoarseness or Breathiness		
Mild	1	
Moderate	2	
Severe	3	
OR:		
Reduced Loudness	2	
Tension in System	3	
Other:		
Articulation (0-23, cumulative)		
Normal	0	
Developmental Errors	0	
Errors from other causes not related to VPI	0	
Errors related to anterior dentition	0	
Reduced intraoral pressure for the sibilants	1	
Reduced intraoral pressure for other fricatives	2	
Reduced intraoral pressure for plosives	3	
Omission of fricatives and plosives	2	
Omission of fricatives or plosives plus hard glottal attack for vowels	3	
Lingual Palatal sibilants	2	
Pharyngeal fricatives, plosives, backing, snorts, inhalations, or exhalation substitutions	3	
Glottal stops	3	
Nasal substitutions for pressure sounds	4	
	Total score	

Probable nature of the velopharyngeal sphincter	
- velopharyngeal competency	0
- borderline velopharyngeal competency	1-2
- borderline velopharyngeal incompetency	3-6
- velopharyngeal incompetency	7 and up

For the administration of the PWSS test, were used sounds (A; E; O), syllables (Ma-Ma-Ma-Ma; Na-Na-Na-Na; Pa-Pa-Pa-Pa; Ta-Ta-Ta-Ta; Ka-Ka-Ka-Ka), words (Saat; Shuma; Drvo; Fustan; Zhaba) and sentences (Simo se smee; Shana shie koshula; Rade pere motor; Kate kupi kapa; Tode vide dete).

Statistical analysis

The results from both conducted tests,

Czermak mirror fogging test and Pittsburgh Weighted Speech Scale (PWSS), were expressed in numbers. Afterwards, the results were statistically processed, graphically presented and descriptively analysed.

By using the data analysis software system STATISTICA version 7.1. [20], statistical analysis of the obtained results was carried out, and calculation of the coefficient of linear correlation r and p-value (probability value) was made.

Results

Twenty respondents with speech disorders, 10 of which comprised the experimental group and had cleft palate or cleft lip and palate (having previously undergone surgery for correcting the cleft), participated in conducting the auditory-visual perceptual assessment for determining the level of nasal air escape and probable nature of the velopharyngeal sphincter. A total number of 20 respondents underwent the examination, seven boys and 13 girls, with a mean age of 5 and a half years (between the age of 4 and 7). In the experimental group, made up of 10 participants, 6 participants had cleft palate – Palatoschisis, and four cleft lip and palate – Cheilognatopalatoshisis. The ten respondents in the control group had no cleft palate or cleft lip and palate at all.

The results from the assessment of the level of nasal air escape using Czermak's test are presented in Table 3. The mean score for all the respondents for that test is 1.1 (scope 0-3), thus indicating a small level of nasal air escape.

Table 3: Visual-perceptual assessment of nasal air escape – Czermak mirror fogging test

Respondent	Result (0-3, highest score)	Not present (0)	Small (1)	Medium (2)	Large (3)
Experimental group					
1	3			2	3
2	1	0	1		
3	3				3
4	3				3
5	1		1		
6	1	0	1		
7	3				3
8	2		1	2	
9	1		1		
10	2			2	
Control group					
11	0	0			
12	1	0	1		
13	0	0			
14	0	0			
15	0	0			
16	0	0			
17	1	0	1		
18	0	0			
19	0	0			
20	0	0			

Mean score	1.1
Scope	0-3

The mean score for the experimental group is 2 (scope 1-3), meaning there is a moderate level of nasal air escape, and what was noticeable for every respondent was mirror fogging, that is, the presence of nasal air escape during speech.

The mean score for the PWSS test is 5.8 (scope 0-22) which shows that for all the respondents the probable nature of the velopharyngeal sphincter is limited velopharyngeal incompetency, Table 4. The established mean score for the experimental group is 11.3 (scope 2-22) denoting that the probable nature of the velopharyngeal sphincter is velopharyngeal incompetency. 80% of the experimental group characterises with limited velopharyngeal incompetency and velopharyngeal incompetence, thus leading to more severe pathology in the verbal communication.

Table 4: Auditory-perceptual assessment of speech – Pittsburgh Weighted Speech Scale (PWSS)

Respondent	Total score	Nasal air emission (0-3, highest score)	Facial grimace (0/2, presence)	Nasality (0-4, highest score)	Phonation (0-3, highest score)	Articulation (0-23, cumulative)
Experimental group						
1	17	3	2	2	3	7
2	4	0	2	0	1	1
3	21	2	2	4	3	10
4	22	3	0	4	3	12
5	2	0	0	1	0	1
6	2	0	0	0	2	0
7	19	3	0	4	2	10
8	9	2	2	1	2	2
9	5	1	0	1	2	1
10	12	3	2	2	2	3
Control group						
11	0	0	0	0	0	0
12	0	0	0	0	0	0
13	1	0	0	0	1	0
14	1	0	0	0	0	1
15	0	0	0	0	0	0
16	1	0	0	0	0	1
17	0	0	0	0	0	0
18	0	0	0	0	0	0
19	0	0	0	0	0	0
20	0	0	0	0	0	0

Mean score	5.8
Scope	0-22

Pearson coefficient of correlation (the coefficient of linear correlation) is used for assessing the correlation between the results obtained from the Czermak mirror fogging test and PWSS test. The correlation was made only between the results obtained from assessing the experimental group, Figure 3, since the result for the control group is zero except two cases where it is 1.

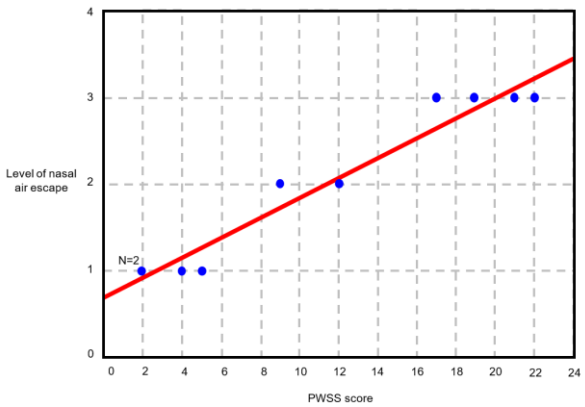

Figure 3: Correlation between the score from the PWSS and level of air nasal escape

The changes in the probable nature of the velopharyngeal sphincter are in close correlation with the changes in the level of nasal air escape, and there is a strong correlation between the two (r = 0.9756). Since there is a positive value for r, it can be concluded that when the first variable increases, so do the second. The calculated p-value is p = 0.000002.

Discussion

The children with cleft palate or cleft lip and palate show dysfunction of the velopharyngeal sphincter, and this leads to the pathology of verbal communication. Regardless of the size of the cleft, the articulatory speech is hindered due to the constant communication between the oral and nasal cavity. The core of the speech disorder lies in nasality that is, dragging part of the air through the nose while speaking due to the incomplete closure of the palatopharyngeal sphincter, which is insufficient contact between the soft palate and the rear wall of the pharynx. The situation can be more complicated and with hindered articulation. For diagnostic and therapeutic purposes, it is important to determine the nasal air emission and the level of dysfunction of the velopharyngeal sphincter. A suitable treatment of the velopharyngeal dysfunction depends on the precise interpretation of the perceptual and physiological characteristics the respondent possesses.

The results obtained from the two assessed variables in this paper, the level of nasal air escape and the probable nature of the velopharyngeal sphincter largely differ between the respondents with cleft palate or cleft lip and palate and speech disorders (experimental group) and the respondents with speech disorders only (control group). The higher values of the two assessed variables for the experimental group suggest bigger velopharyngeal opening. Looking in greater detail, it would be said that for the experimental group the overall PWSS score is in strong correlation with the level of nasal air escape. Clinically, this means that the respondent with severe perceptual speech symptoms, the stereotype of velopharyngeal insufficiency, shows a higher level of nasal air escape thus suggesting bigger velopharyngeal opening.

Because there is no a single study in literature which enables the results from the level of nasal air escape and probable nature of the velopharyngeal sphincter to be compared, the comparison with other studies was very difficult to be made, or it was limited. Still, a certain number of authors, one of which is Scarmagnani et al. [21], points out that there is a considerable correlation between the size of the velopharyngeal closure and the level of nasal air escape in patients with a corrected cleft palate which, in fact, overlaps with the result from our research. However, unlike this research, Scarmagnani uses various researching methods (aerodynamic speech assessment and audio-digital speech recording) and different statistical analysis (Spearman's rank correlation coefficient). On the other hand, the results obtained by Kummer et al. [22] suggest that hypernasality (with or without nasal emission) can be primarily connected with the relatively large velopharyngeal opening. In our research, the results revealed a direct positive connection between the level of nasal air escape and velopharyngeal dysfunction. Therefore, the greater the nasal air escape is, the bigger the velopharyngeal dysfunction is which is also acknowledged by Abou-Elsaad et al. [23].

There is also an overlap of the results from our research and those obtained from the research conducted by Gubrynowicz et al. [11] where the Czermak's test is used and reveals greater nasal emission due to the wide opening of the velopharyngeal opening. By experimenting with patients with a cleft who previously underwent palatoplasty, but in this case a larger and different age group than in our research, Dudas et al. [2] obtained results which show limited or completely incompetent velopharyngeal closure. There is an 80% overlap between those results and ours obtained from the PWSS test.

This research has a few limitations worth mentioning. First, further research should include a larger number of respondents so that obtained results would have greater relevance. Second limitation is that during the intraoral examination the presence of cleft (cleft palate or cleft lip and palate) was established, but not its size as well (for instance, by using the Veau Classification), nor how the size of the cleft affects speech. That is, how it affects the level of nasal emission or perceptual speech symptoms. Future research should include these aspects as well.

Knowing that certain aspect of speech are directly related to velopharyngeal anatomy, perceptual speech symptoms and nasal air escape provide unique insight into the status and role the velopharyngeal sphincter has in speech.

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Case Report

Treatment of Pediatric Open Supracondylar Humerus Fractures: Case Report

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Keywords: Humerus; Open Supracondylar Fractures; Radiography.

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Abstract

BACKGROUND: Open supracondylar fractures of the humerus are rare in children, and the treatment strategy for these fractures is yet to be standardised.

AIM: We present the case of a 7-year-old boy with open supracondylar humerus fracture that was managed with an external wrist fixator.

CASE PRESENTATION: A 7-year-boy was brought to our department with pain in the right arm after a fall from a height about 3 hours before admission. On examination, the elbow was found to be markedly swollen with restriction of movement of the right arm. A 4-cm-wide wound was also observed on the flexural aspect of the elbow, indicating severe contamination of the fractured site. Neurological examination revealed restriction of hand movement and decreased sensations, which suggested the possibility of nerve injuries.

CONCLUSION: A good clinical outcome was achieved in this case, without the development of any complications over a 6-month follow-up period.

Introduction

Supracondylar fractures of the humerus are common in children, accounting for 65% of elbow fractures and 3-16% of all paediatric fractures [1]. However, open supracondylar fractures of the humerus are rarely observed in the paediatric age group, and the treatment protocol for these fractures is yet to be standardised [2].

In this report, we present the case of a 7-year-old boy with an open supracondylar humerus fracture that was managed using an external wrist fixator. A good clinical outcome was achieved with this treatment method, without the occurrence of any complications.

Case report

A 7-year-boy was brought to our department with pain in the right arm after a fall from a height about 3 hours before admission. On examination, the elbow was found to be markedly swollen with restriction of movement of the right arm. A 4-cm-wide wound was also observed on the flexural aspect of the elbow, indicating severe contamination of the fracture site (Figure 1A). Neurological examination revealed restriction of hand movement and decreased sensations, which suggested the possibility of nerve injuries. Radiography performed at admission showed severe displacement of the right humerus fracture (Fig. 1B, 1C). The fracture was classified as Gartland type IIIb and Gustilo-Anderson type IIIa.

General anaesthesia was induced and wound cleaning and debridement were performed. Further, the fracture was reduced under C-arm X-ray guidance. Two lateral Schanz pins were inserted through the humerus and ulna. An external wrist fixator was attached to the Schanz pins. The right radial nerve was also found to be severely injured, and therefore, the nerve was anastomosed along with the fracture reduction. The patient was administered postoperative antibiotics.

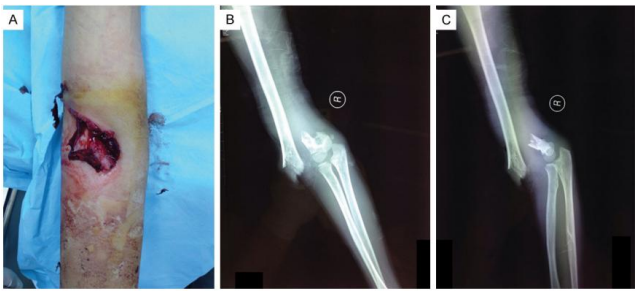

Figure 1: A) Preoperative view of the fracture. The right elbow was markedly swollen. A 4-cm wound is seen on the flexural aspect of the elbow, indicating severe contamination of the fracture site. B) and C) Preoperative radiography shows Gartland type IIIb supracondylar humerus fracture with severe displacement

Radiographic examination was performed every week after the surgery. The patient was encouraged to practice movement of the hand and arm with a static load. The elbow joint was alternatively fixed at the position of maximum flexion or extension after physical therapy every day. The external fixator lock was removed at postoperative week 3. At postoperative week 6, the external fixator was removed. At the follow-up examination, the wound showed signs of primary healing. No radiological or biochemical evidence of infection was obtained during the 6-month follow-up period (Fig. 2A, 2B). At the last follow-up examination, the patient showed radiographic evidence of good union (Fig. 2C, 2D); had a range of movement of 0°-145 at the elbow; and did not have any signs of osteomyelitis or varus or valgus deformity. Informed consent has been obtained from the patient or appropriate persons for publication, including photographs.

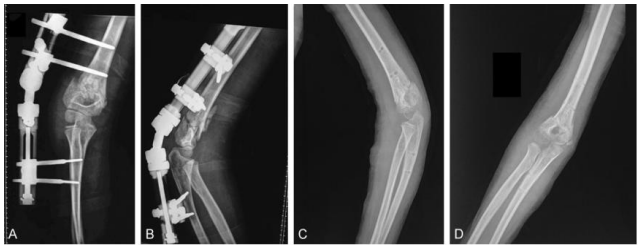

Figure 2: A) and B) Radiography at postoperative week 1 shows a good reduction of the fracture. C) and D) Radiography at postoperative week 6 shows the good union of the fractured ends without any deformities.

Discussion

The standard treatment protocol for the type I supracondylar humerus fractures in children is conservative management with closed reduction, percutaneous pin fixation, and the application of a long arm cast [2]. However, the treatment for Gartland type III supracondylar humerus fractures in children remains debatable. The American Academy of Orthopaedic Surgeons recommends closed reduction and pin fixation for the management of type II, type III, and displaced supracondylar humerus fractures in children [2]. However, the treatment protocol for open supracondylar humerus fractures in children is yet to be standardised.

Open supracondylar fractures are usually accompanied by severe displacement and extensive vascular and nerve injuries, which cannot be optimally managed with the application of a long arm cast alone. In addition, open fractures are often severely contaminated, whereby the risk of infection remains very high even after thorough wound cleaning and debridement. Therefore, the use of internal fixation in open supracondylar humerus fractures should be avoided because they are associated with an increased risk of infection [3]. Percutaneous pin fixation may also increase the risk of infection in cases of severely contaminated open fractures [4]. The Schanz pin used in external elbow fixators is large and can cause inadvertent iatrogenic fractures in children [5].

Therefore, instead of the external elbow fixator, we used an external wrist fixator, which has smaller Schanz pins that can provide adequate fixation without increasing the risk of infection. Further, since most external elbow fixators are hinged, they prevent the rotation of the forearm. On the other hand, the external wrist fixator used in our case enables movement in multiple planes, which stabilises the fractured humerus. The external wrist fixator offers several advantages to the treatment of open supracondylar humerus fractures in children, including stable fixation for bone union and promotion of the recovery of soft tissues, such as vessels, nerves, and muscles [6]. The use of this device precludes the use of K-wires, thereby minimising the risks of decreased circulation, infection, and osteomyelitis [7].

In addition, the external wrist fixator is light and does not cause much discomfort for the patient. One of the risks associated with the use of the external wrist fixator is a pin-tract infection, which can be managed well with Betadine or povidone-iodine [8]. Another possible complication is fracture displacement because the bone is not directly fixed by the external wrist fixator. Therefore, the weekly radiological examination is recommended to monitor the patient for possible displacement.

In conclusion, we presented a case of paediatric open supracondylar humerus fracture in

which a good clinical outcome was achieved with the use of an external wrist fixator. The patient did not develop any postoperative complications such as osteomyelitis, nonunion, and varus/ valgus deformity and the elbow joint showed a good range of movement. On the basis of our experience in the case, we recommend the use of the external wrist fixator in the management of open supracondylar humerus fracture in children. Further large-scale studies need to be conducted to confirm its safety and efficacy.

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Case Report

Goodpasture Syndrome Diagnosed One Year And A Half after the Appearance of the First Symptoms (Case Report)

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Keywords: alveolar haemorrhage; glomerulonephritis; immune system; anti-glomerular basement membrane (anti-GBM) antibodies; Goodpasture syndrome.

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Abstract

BACKGROUND: Goodpasture syndrome was originally described as an association of alveolar haemorrhage and glomerulonephritis. It occurs when the immune system attacks and destroys healthy body tissue.

AIM: We are presenting a patient with a clinical picture of pulmonary haemorrhage and glomerulonephritis, which is diagnosed by renal biopsy.

CASE PRESENTATION: His illness began a year and a half before being diagnosed. In that period he had occasional exacerbations. He was received at our Clinic in extremely serious condition, and after stabilisation of his medical condition, there was made a biopsy of the kidney. The p-ANCA was 8.93 U/ml (neg < 3, poz > 5 U/ml). Histopathological diagnosis of biopsy of the kidney was: Glomerulonephritis extra capillaries focalis, segmentalis et globalis. Based on this he was diagnosed with Goodpasture syndrome. He received corticosteroid therapy and cyclophosphamide, with good response to treatment, and he is currently in a stable condition, receiving only corticosteroid therapy.

CONCLUSION: Goodpasture syndrome is a severe illness caused by the formation of antibodies to the glomerular basement membrane and alveolus with consequential damage to renal and pulmonary function. With current therapy, long-term survival is more than 50%.

Introduction

Goodpasture syndrome was originally described as an association of alveolar haemorrhage and glomerulonephritis. It occurs when the immune system attacks and destroys healthy body tissue, so it is defined as an autoimmune disease. Gender distribution is reported differently in different studies, according to some dates it is twice more in men than in women, and the age at presentation can range from the first to the ninth decade. Goodpasture syndrome is manufactured by rapidly progressive glomerulonephritis and alveolar haemorrhage in association with the presence of anti-glomerular basement membrane (anti-GBM) antibodies and another capillary basement membrane. Although it is rare, it is very severe immunological disease [1, 2].

Like other autoimmune conditions, the anti-GBM disease is thought to result from an environmental insult in a person with genetic susceptibility. The human leukocyte antigen (HLA) serotype HLA-DR15 has been strongly associated with the anti-GBM disease. An initial insult to the pulmonary vasculature is required for exposure of the alveolar capillaries to the anti-GBM antibodies. Environmental factors that may lead to such exposure include the following: exposure to organic solvents or hydrocarbons, smoking, infection (eg, influenza A2),

cocaine inhalation, exposure to metal dust, lymphocyte depletion therapy, such as alemtuzumab, extracorporeal shock wave lithotripsy [3-5].

Symptoms: constitutional symptoms (eg, malaise, chills and fever, arthralgias) may precede or develop concurrently with pulmonary or renal manifestations; hemoptysis is the presenting symptom when the disease affects the lungs. The level of hemoptysis may vary and, in a small percentage of patients, may be absent. Other pulmonary symptoms include a cough, dyspnea, and shortness of breath. Massive pulmonary haemorrhage leading to respiratory failure may occur. Chest pain is present in less than half of the patients. Renal manifestations include hematuria, oedema, high blood pressure and eventually uremia. Significant anaemia may result from persistent intrapulmonary bleeding [6].

Physical examination findings in patients with the anti-GBM disease include the following: tachypnea, inspiratory crackles over lung bases, cyanosis, hepatosplenomegaly (may be present), hypertension (present in 20% of cases), rash, oedema [6].

Diagnosis can be established the bay presence of pulmonary haemorrhage, pulmonary radiography, kidney biopsy and positive resultants of anti-GBM antibodies. The treatment of this syndrome should be initiated as soon as possible using a combination of corticosteroid therapy, cytostatics and plasmapheresis [7].

Case Report

The patient DS, male, born in 1967, has been admitted to the Clinic of Pulmonology and Allergology on May 15th, 2016. On inspection severely ill, presenting with a productive cough, expectoration mixed with blood, chills, and fever, which has been present for three days and measured increased body temperature up to 39^0C. The patient has complained of nausea, fatigue, and chest tightness, as well. Formerly he had worked as a driver; presently he works with automobile lacquers and has a 10 pack-year smoking history.

He has sought medical advice at his primary care health practitioner since 2014 when he was referred to the Pulmonology Outpatient's Clinic, whereas he has been prescribed antibiotics. He has been treated at the outpatient's clinic on several occasions because of fever, nausea, and cough with bloody expectoration, malaise and sideropenic anaemia. These symptoms and signs have been repetitive the last year. He has been investigated at the Clinic of Rheumatology, suspected of the diagnosis of Gilbert syndrome, as well. Later he has been referred to a nephrologist because of the occurrence of proteinuria, hematuria and increased values of serum urea and serum creatinine. Therefore, a kidney biopsy has been indicated, for which the patient has not given his consent to be performed (April, the 14th, 2016). A computer tomography of the chest has been performed using intravenous contrast, with a series of scans during the arterial phase, with the following findings: interstitial fibrous changes bilaterally "ground-glass" opacities of the lung parenchyma, bilaterally with centrilobular emphysema in the proximal parts (April, the 28th, 2016).

The last admission has been at the department of infectious diseases of the clinical hospital in Kumanovo, and because of the severity of the general condition he has been referred to the University Clinic of Pulmonology and Allergy in May, the 15th, 2016. He has been admitted in a severely ill condition, presenting with fever (39^0C) the last three days, headaches, vertigo, cough accompanied by hemoptysis, tachypneic, tachycardia, severe dyspnoea and malaise. The patient has shown decreased oxygen saturation ($SatO_2$ = 50%) and hypoxemia (PaO_2 = 6.35 kPa), anemia (Hgb = 7.9 g/L; Hct = 23.2%, RBC = 2.7 x 10^12/L; WBC = 16.9 x 10^9/L), ESR = 60 mm/h, urea = 13.9 mmol/L, creatinine = 206 mmol/L, total proteins = 56 g/L, albumins = 33 g/L, Fe in serum = 8.1 mmol/L, CRP = 134 U/L, troponins = 777 U/L, CK = 144 U/L, CK-MB = 29 U/L, LDH = 686 U/L, proteinuria, hematuria. Chest X-ray found a massive, diffuse lung consolidation bilaterally, with reactive hila (Fig. 1, 2).

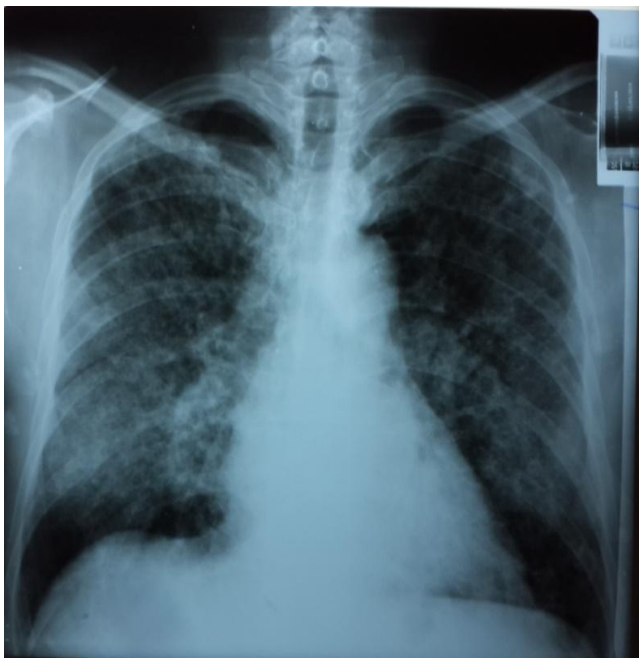

Figure 1: RTG Posteranterior: Chest X-ray found a massive, diffuse lung consolidation bilaterally, with reactive hila

Because of suspected Wegener's disease,

parenteral corticosteroids, antibiotics and oxygen supplementation has been administered, with consequent improvement of the patient's condition, when the patient has been referred to a kidney biopsy at a private clinic in Skopje. Anti-dsDNA = 2.32 U/ml (neg < 40, pos > 60 U/ml), Anti-MPO p-ANCA = 8.93 U/ml (neg < 3, pos > 5 U/ml).

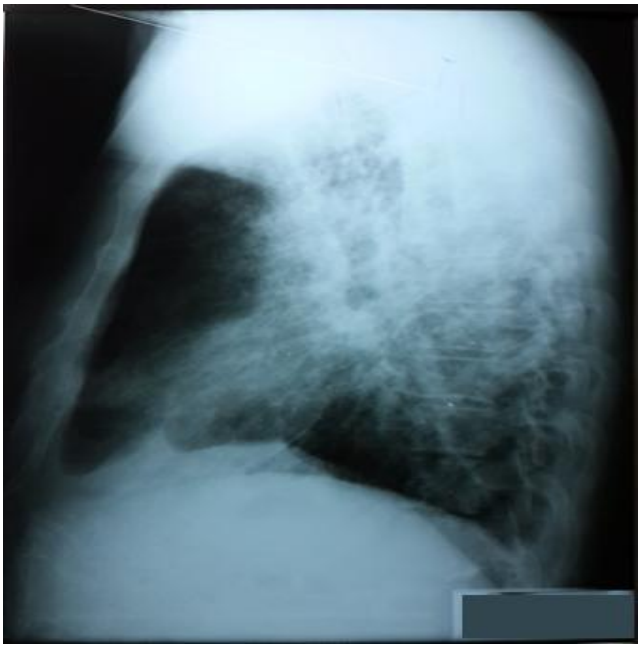

Figure 2: RTG lateral view: Chest X-ray found a massive, diffuse lung consolidation, with reactive hila

The histopathological findings of the kidney biopsy, performed in May, the 15th, 2016, where as follows: Microscopically the sections of the samples have revealed 11 glomeruli with surrounding tubular interstitium. Ten out of eleven glomeruli have had relatively preserved architectonics, without increased cellularity, endocapillary and extra capillary, whereas in one-to-two glomeruli the GBM (glomerular basement membrane) is slightly is chemically wrinkled.

In one of the glomeruli, there has been found a segmental lesion of GBM collapse with synechiae in between the visceral and parietal epithelium. In the surrounding tubular interstitium, there are focuses of tubular atrophy and interstitial fibrosis (< 25%). The immunofluorescence technique carried out has shown linear IgG deposits along the GBM in eight out of eight glomeruli, whereas in two of the glomeruli there is globally collapsed GBM, and in another two there can be seen segmental epithelial proliferations with a slight impression of GBM.

The diagnosis made is Extracapillary Glomerulonephritis, focal and segmental (2/19) and global (2/19). According to the above, the diagnosis of Goodpasture´s syndrome has been made.

The patient has been admitted for the second time at the University Clinic of Nephrology, on June, the 7th, 20116, with the following values of the blood and biochemistry analyses: WBC = 15.5 x 10^9/L; serum urea = 11.6 mmol/L; creatinine = 137 mmol/L; urine: specific weight = 1014; pH = 6, proteins (+), glucose (-), blood (+), RBC = 4-5 in the sediment, WBC = 3-4 in the sediment. Anti GBM antibodies were negative during this hospitalization.

Urinary tract ultrasound (June, the 9th, 2016): the kidneys, bilaterally are with proper size and shape, right: 110 x 52 mm, on the left 113 x 54 mm, with a granular parenchyma – 19 mm diameter, with slightly increased echogenicity from where the hypoechogenic medulla is noticeable. There is no stasis. The urinary bladder was without any changes. The prostate is oval, homogenous, weighing approximately 21 gramme.

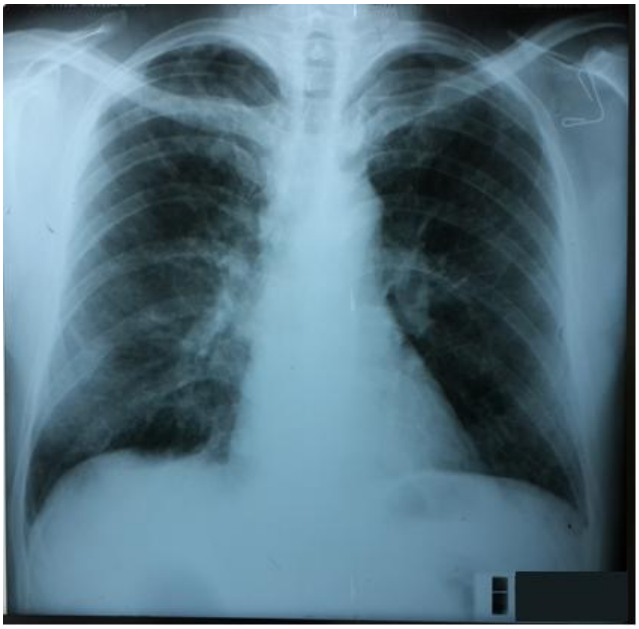

Figure 3: RTG Posteranterior: Chest X-ray has shown bilaterally pleura-diaphragmatic adhesions, other findings are normal

During the hospital admission the patient has been treated with immunosuppressive therapy, initially with high-dose methylprednisolone, 500 mg per day, three days in a row, with tapering the dosage, and using cyclophosphamide in one occasion. During the hospital admission, he had not had any respiratory symptoms, and the other parameters have been improving. Hence, plasmapheresis has not been indicated. Blood pressure measurements, as follows: 115/80 – 126/89 mmHg, with no need of anti-hypertensive therapy. There were noted slight decreases in the serum creatinine and the proteinuria measurements. The diuresis noted 2000 ml. The patient has been discharged with an improved general condition and prescribed the following drugs: Decortin 60 mg, one tablet per day; Ranitidine 150 mg, twice per day. The follow-up examination at the University Clinic of Pulmonology and Allergy, performed in August, the 1st, 2016 has revealed a patient in good general condition. The blood gas analyses, as follows:

SaO_2 = 96.4%; PaO_2 = 9.46 kPa; $PaCO_2$ = 3.67 kPa, normal spirometry measurements. The chest X-ray has shown bilaterally pleura-diaphragmatic adhesions, other findings were normal (Fig. 3, 4). At the moment the patient is stable and he is being treated with oral corticosteroids.

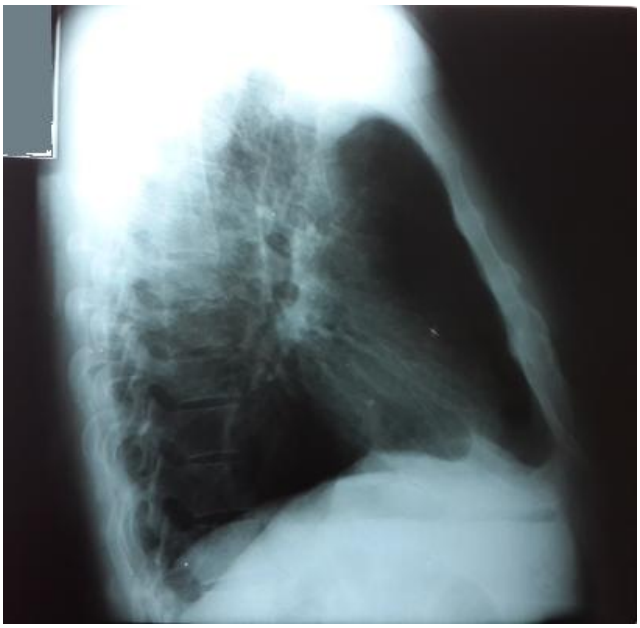

Figure 4: RTG lateral view: Chest X-ray has shown pleura-diaphragmatic adhesions, other findings are normal

Discussion

Ernest Goodpasture first described the disorder in 1919 [8]. He reported a case of pulmonary haemorrhage and glomerulonephritis during an influenza epidemic. In the 1950s, Krakower and Greenspon identified GBM as the antigen [9]. In 1967, Lerner, Glassock, and Dixon confirmed that the antibodies taken from the diseased kidneys produced nephritis in experimental animals [10]. The discovery of anti-GBM antibodies led to the understanding of the pathogenesis of Goodpasture syndrome. Anti-GBM disease is an uncommon disorder [11].

Careful attention to the medical history, physical examination, and targeted laboratory evaluation often suggests the underlying cause [12].

Our patient has been diagnosed almost after one year and half, after his symptoms appeared for the first time, when his condition became very severe, with breathlessness, massive haemopthises, renal failure, anemia, hematuria. Formerly he had worked as a driver; presently he works with automobile lacquers, and has a 10 pack-year smoking history. Anti-MPO p-ANCA was 8.93 U/ml (neg < 3, pos > 5 U/ml). The diagnosis was made as an extracapillary glomerulonephritis, focal and segmental (2/19) and global (2/19). The patient on his admission on Clinic of pulmology and allergology has shown decreased oxygen saturation ($SatO_2$ = 50%) and hypoxemia (PaO_2 = 6.35 kPa), anemia (Hgb = 7.9 g/L; Hct = 23.2%, RBC = 2.7 x 10^12/L; WBC = 16.9 x 10^9/L), ESR = 60mm/h, urea = 13.9 mmol/L, creatinine = 206 mmol/L, total proteins = 56 g/L, albumins = 33 g/L, Fe in серум 8.1mmol/L, CRP = 134 U/L, troponins = 777 U/L, CK = 144 U/L, CK-MB = 29 U/L, LDH = 686 U/L, proteinuria, hematuria. Chest X-ray found a massive, diffuse lung consolidation bilaterally, with reactive hila.

The incidence of the anti-GBM disease is estimated to be 0.5-1.8 cases per million per year in both European white and Asian populations. It is responsible for 1-5% of all types of glomerulonephritis and for 10-20% of crescentic glomerulonephritis [13].

From 60-80% of patients have clinically apparent manifestations of pulmonary and renal disease, 20-40% has renal disease alone, and less than 10% have a disease that is limited to the lungs. Anti-GBM disease occurs more commonly in white people than in black people, but it also may be more common in certain ethnic groups, such as the Maoris of New Zealand. The age distribution is bimodal, 20-30 years and 60-70 years. The prevalence of the disease is higher in men in the younger age group and women in the older age subgroup [13, 14].

A subgroup of patients is double-positive for anti-GBM antibodies and antineutrophilic cytoplasmic antibodies. The peak age incidence for this subgroup is 60-70 years, with a male predominance [15].

Patients presenting with serum creatinine levels greater than 4 mg/dL, oliguria, and more than 50% crescents on renal biopsy rarely recover. They usually progress to end-stage renal failure that requires long-term dialysis. In a retrospective analysis of patients with the anti-GBM disease who started renal replacement therapy for end-stage renal disease (ESRD) in Australia and New Zealand (ANZDATA Registry), the median survival rate was 5.93 years with death predicted by older age and history of pulmonary haemorrhage. Conditions that affect the lung and kidney (pulmonary-renal syndromes) are important in the differential diagnosis. These include granulomatosis with polyangiitis (Wegener granulomatosis), systemic lupus erythematosus, microscopic polyangiitis, and other forms of systemic vasculitides. Distinguishing granulomatosis with polyangiitis from Goodpasture syndrome is particularly important. Interestingly, some patients with Goodpasture syndrome may present with antineutrophilic cytoplasmic antibodies (ANCAs), which are predominantly observed in patients with granulomatosis with polyangiitis. Pulmonary-renal syndromes are less commonly a manifestation of IgA-mediated disorders (eg, IgA nephropathy or Henoch-Schönlein purpura) and of immune complex–mediated

renal disease (eg, essential mixed cryoglobulinemia). Rarely, rapidly progressive glomerulonephritis alone can cause pulmonary-renal syndromes through a mechanism involving renal failure, volume overload, and pulmonary oedema with hemoptysis [16-18].

In the past, Goodpasture syndrome was usually fatal. Aggressive therapy with plasmapheresis, corticosteroids, and immunosuppressive agents has dramatically improved prognosis. With this approach, the 5-year survival rate exceeds 80% and fewer than 30% of patients require long-term dialysis. In a study of patients admitted to intensive care units for the acute manifestation of small-vessel vasculitis, including anti-GBM disease, delayed administration of cyclophosphamide was associated with a higher mortality rate [19, 20].

On the Clinic of nephrology during the hospital admission our patient has been treated with immunosuppressive therapy, initially with high-dose methylprednisolone, 500 mg per day, three days in a row, with tapering the dosage, and using cyclophosphamide in one occasion. In this moment the patient is in remission with only “in one occasion use” of cyclophosphamide and plasmapheresis. He is being treated only with oral corticosteroids. The prognosis of this case, for now, is not so bad although delayed diagnose.

In conclusion, Goodpasture syndrome is a severe illness caused by the formation of antibodies to the glomerular basement membrane and alveolus with consequential damage to renal and pulmonary function. With current therapy, long-term survival is more than 50%. Before, the mortality was higher than 90%. The treatment involved corticosteroid therapy, cytostatic therapy and plasmapheresis. Very rarely it appears only with pulmonary involvement, so it can be concluded all cases of repeated pulmonary haemorrhage should raise suspicion of this syndrome.

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Case Report

Scapular Fractures in Blunt Chest Trauma – Self-Experience Study

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Keywords: fractured scapula; thoracic trauma; associated injuries; rib fractures; pulmonary contusion.

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Abstract

AIM: The aim of this retrospective study was to report the scapular fractures in patients with blunt chest trauma and to present the type and the frequency of associated thoracic injuries.

MATERIAL AND METHODS: Nine patients with fractures of the scapula were included in the study. The mechanisms of the injury, the type of scapular fractures and associated thoracic injuries were analysed.

RESULTS: Scapular fractures were caused by high-energy blunt chest trauma. The body of the scapula was fractured in all scapular fractures. In all cases, scapular fractures were associated with other thoracic injuries (average 3.25/per case). Rib fractures were present in eight patients, fractured clavicula - in four cases, the affection of pleural cavity - in eight of the patients and pulmonary contusion in all nine cases. Eight patients were discharged from the hospital up to the 15th day. One patient had died on the 3rd day because of postconcussional lung oedema.

CONCLUSIONS: The study confirms the role of scapular fractures as a marker for the severity of the chest trauma (based on the number of associated thoracic injuries), but doesn't present scapular fractures as an indicator for high mortality in blunt chest trauma patients.

Introduction

Scapular fractures are one of the most unusual bony fractures in hospitalised patients. Mainly, they result of the major direct trauma to the thorax, such as motor vehicle accidents, falls from heights and crushing injuries [1-6]. The importance of the scapular fractures in blunt chest trauma lies not just in the injury of the scapula but in the close association with other thoracic injuries (associated injuries), some of them being life-threatening.

The aim of this retrospective study was to report on scapular fractures in blunt chest trauma patients and to present the type and the frequency of associated thoracic injuries.

Material and Methods

Nine patients (eight men and one woman) with fractures of the scapula, hospitalised at Department of Orthopaedic & Traumatology, Belhoul European Hospital –Dubai from June 2013 to August 2015, were included in this study. They age of the patients ranged from 32 to 72 years (mean – 49.3 /SD – 14.6 years). The injury mechanisms, fracture type of scapula and associated thoracic injuries were analysed.

Computed tomography (CT) examination of the thorax was performed in all nine patients at the time of their admission, in six of them in combination with 3D CT reconstruction. Conventional chest radiography examinations were used to follow the

patients 'conditions. The treatment results weren't the subject of this study.

Results

Mechanism blunt chest trauma of injury in eight of patients was motor vehicle accident: car accident - two cases; motorcycle accident - four cases; ATV (all terrain vehicles) accident – one patient and one patient as a pedestrian (Table 1). One patient was injured by severe assault.

The total number of scapular fractures was eleven as two of patients (motorcyclist and pedestrian) had fractures on both scapulas (Fig. 1). There were six fractures on the right scapula and five fractures on the left scapula. In all the eleven scapular fractures the body of the scapula was fractured. Fractures of the acromion were established in two of the cases; scapular spine fractures - in two cases, and fracture of the glenoid (glenoid rim) - in one case.

None of the reported cases of blunt chest trauma had isolated scapular fractures (Table 1). In all cases, scapular fractures were associated with other thoracic injuries (average 3.25/per case).

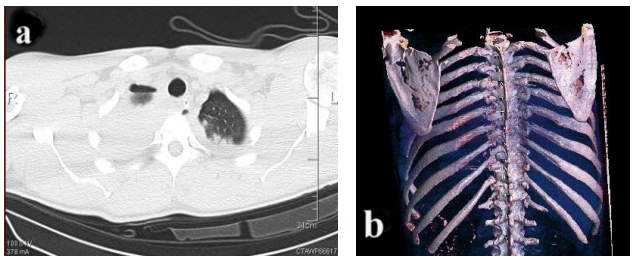

Figure 1: Patients 8: a – axial computed tomography showing: bilateral scapular fractures, bilateral pulmonary contusion, left haemothorax, right pneumothorax; b – 3D computed tomography reconstruction showing bilateral scapular body fractures

Associated chest bone fractures were established in all patients. Rib fractures were seen in eight patients - ipsilateral fractures in all eight patients and in one of them (with right scapular fracture) additionally fracture of the contralateral left 12th rib (Table 1). Multiple rib fractures (3 or more fractured ribs) were established in seven of patients. The fracture of the first rib was present in two patients (Fig. 2 & 3) – on the ipsilateral side. Four of the patients had ipsilateral fractures of the clavicula (Fig. 2 &3). Fractures of the thoracic spine were seen in two patients (one with a fracture of posterior part of the body of Th10-vertebra, one with fractures of spinous processes of the Th4 and Th5-vertebra), none of whom had neurological damage (Fig. 3).

The pulmonary contusion was established in all of the patients (bilateral in two cases)/ (Table 1). The ipsilateral pleural cavity was affected in eight of the patients: haemopneumothorax was seen in four cases, haemothorax – in three; and pneumothorax – in two cases (Table 1).

The patient with a fracture of the glenoid rim was admitted in the Departments of Trauma and Orthopedics at 11th day for immobilisation of the shoulder. Operative treatment was performed in patients with associated pleural injuries (haemothorax, pneumothorax and team-pneumothorax). Pleural drainage was performed in these cases.

Eight patients were discharged from the hospital up to a 15th day (mean hospital stay – 8.8/ SD – 3.9 days). One patient died on the 3rd day after chest trauma (severe assault case) from the associated injuries (postconcussional lung oedema with pulmonary sepsis) (Table 1).

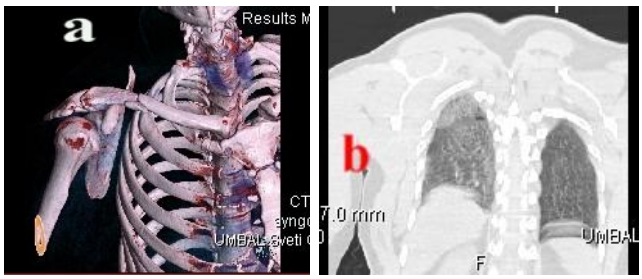

Figure 2: Patient 9: a - 3D computed tomography reconstruction (anterior view) showing fractures of the right clavicular and the first rib; b – sagittal computed tomography showing right pneumothorax and lung contusion

Table 1: Case summary of the reported nine patients

n	Age (years)	Gender	Injury mechanism	Side of scapular fracture	Type of scapular fracture	Associated injuries	Stay (in days)
1	34	m	Car accident	Right	Body	right clavicular fr.; lung contusion - right	5
2	51	m	Motorcycle	Right	Body	ribs fr. – 2-4th right; lung contusion - right	10
3	68	m	Motorcycle	Left	Body; Acromion	ribs fr. – 7 -10th left; lung contusion- left; haemothorax - left	5
4	49	f	Pedestrian	Bilateral	Right: Body Left: Body	ribs fr.: 4 -8th left; haemothorax – right; haemopneumothorax left; lung contusion - bilateral	8
5	72	m	Assault	Left	Body	ribs fr.: 5 - 10th left; left clavicula fr.; haemopneumothorax – left; lung contusion - left	3 (death)
6	50	m	Motorcycle	Right	Body; Glenoid	ribs fr.: 1st , 3-10th right; 12th left; clavicula fr.- right; Th4 and Th5- spinous processes fr.; haemopneumothorax – right; lung contusion - bilateral	11
7	55	m	Car accident	Left	Body; Spine; Acromion	ribs fr.: 4 - 11th left; Th10- body fr.; haemopneumothorax - left; lung contusion - left	10
8	33	m	Motorcycle	Bilateral	Right: Body Left: Body; Spine	ribs fr.: 4 - 6th left, 6th right; haemothorax – left; pneumothorax – right; lung contusion - bilateral	15
9	32	m	Atv accident	Right	Body	clavicula fr.- right; ribs fr. - 1st rib right; pneumothorax – right; lung contusion - right	13

Abbreviations: m = man; f = female; fr. = fracture; n = case number; Atv = all terrain vehicles accident.

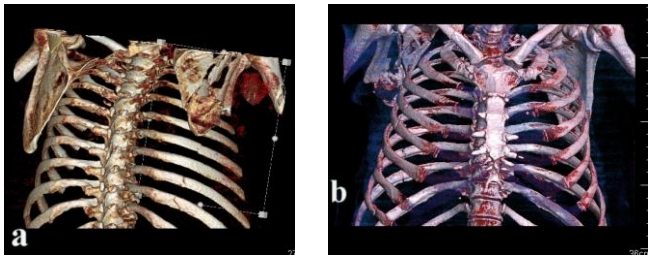

Figure 3: Patient 6. 3D computed tomography reconstruction: a – posterior view showing fractures of the body of the right scapula and Th4, Th5 spinous process fractures; b – anterior view showing right clavicular and first rib fractures

Discussion

Scapular fractures are uncommon (accounting for only 0.4 - 1% of all bone fractures and 3 to 5% of all shoulder girdle injuries) due to the muscular coverage of the scapula and as a rule, they are the result of the marked force applied in the course of high-velocity chest trauma [7]. According to the literature data, 32 to 80% of scapular fractures are a result of traffic accidents [2, 3, 6-8]. We report road accidents to be the cause of 88.7% of the scapular fractures and confirm that fractures of the scapula have a huge impact on the thoracic cage.

Other publications claim that patients with scapular fractures are predominately male gender, an average age of 35 years (range, 25-50 years) [6-10]. Our patient population's average age of 44 years and range of 32-72 years, with only one woman, are similar to the data reported in the literature. The age and sex distribution in the frequency of the scapular fractures can be explained by differences in behaviour and physical activity.

Several authors have presented the sequence of the scapular fractures in declining order: fractures of the scapular body followed by those of scapular neck, glenoid, acromion and coracoid process of the scapula [1, 5, 6, 11]. Because of the relatively small number of patients, we couldn't present a declining order in the frequency of the type of scapular fractures. The type of scapular fractures, according to the affected part of the scapula depends on the mechanism of trauma – direct or indirect. It is a rule that direct blunt chest trauma leads to fractures of the scapular body, spine, or the acromion. Indirect trauma that pulls the arm or compresses the shoulder causes fracture of the scapular neck and/or glenoid. Our reported patients were exposed to direct chest trauma. That explains why the body of the scapula was broken in all of the reported nine cases, as well as why the glenoid was fractured only in one case and there were no fractures of the scapular neck.

Although the treatment isn't a subject of this study, we notice that scapular body fractures, irrespective of the number of the fragments, usually are managed non-operatively, with favourable results. Conservative treatment was successfully applied to all of our patients, with pain relief medication and only in one case shoulder immobilisation.

Two of our patients had bilateral scapular fractures. These were cases with severe direct high–energy impact on the thoracic cage. According to the data in the literature bilateral scapular fracture is a very rare injury complex [12-16]. We found published several single case reports and only one study with reported six cases with bilateral scapular fractures. Two mechanisms of bilateral scapular fractures were described: by direct violence (as in our cases) or by muscle spasms due to epileptic seizure or electrical shock. It is expected that the increasing of the number of chest trauma patients with high-energy impact will increase the potential for bilateral scapular fractures.

Isolated fractures of the scapula are very rare and they are usually treated in the outpatient clinics. In contrast with the isolated scapular fractures, these with other associated thoracic injuries are admitted to the hospital, as we managed with the reported nine cases. Associated thoracic injuries determine the importance of the scapular fractures, being an indicator of the severity of blunt chest trauma [2, 3]. We report the number of associated injuries of the 3.25/per case, that are similar to the other published data. At this point, we confirm the scapular fractures as a marker of the severity of the chest trauma.

The review of the literature established rib fractures as the most common associated chest injury in patients with a fractured scapula. We reported 88.9 % fractured ribs and confirmed that damaged ipsilateral ribs in the lower thoracic cage are more frequent since they are relatively less protected there. The injury complex fractures of the scapula and the first rib are quite rare. It is a rule, that fractures of the first rib require high energy injuries due to its profound location and good protection by overlying soft tissues, clavicle and scapula [12, 17, 18]. In cases with injury complex scapular and first rib fractures, there is a high risk of other thoracic injuries like an injury of the subclavian artery, brachial plexus injury, rupture of the apex of the lung, pneumothorax. Our reported two cases had no injury of the subclavian artery and neurological deficiency. We established pleural involvement, without knowing the right cause of the pleural injury: fracture of the first rib or fracture of another chest bone.

In a declining order, we found that clavicular fractures were the second type of associated chest bone fractures. We established that the clavicula was broken in its lateral third, close to the shoulder joint, which can be explained by the direction of the impact

to the shoulder girdle. As in cases with first rib fractures, there was a similar risk of injury of the subclavian artery, brachial plexus, as well as rupture of the apex of the lung. Fortunately, the only pleural injury was established in three of our reported cases with associated clavicular fractures.

We reported associated pleural injuries (pneumothorax, haemothorax and haemopneumothorax) in 66.7 % of the cases with scapular fractures. Although the review of the literature established rib fractures as the most common associated chest injury, we found that pulmonary contusion was present in 100% of the reported patients. We explain that to be a result of the severe mechanical impact on the thoracic cage. The severity of lung contusion was determined on the basis of the result of imagining methods (chest X-ray and CT) but this wasn't the subject of this study. Pulmonary contusion, with postconcussional lung oedema, is considered as the most common cause of the death in hospitalised blunt thoracic trauma patients that was found in the only death case we report. The mortality in scapular fractures in our study was lower than the one reported in the literature. Therefore the results of our study allow us to conclude that scapular fractures couldn't be accepted as a marker of the mortality in blunt chest trauma patients.

In conclusion, this retrospective study presents one of the relatively rare injuries in blunt chest trauma patients – scapular fractures. The study presents the effects of direct high energy impact on the thoracic cage: (1) causing of fractures of the scapular body and (2) causing of other associated thoracic injuries, which are more common - pulmonary contusion. The study confirms the role of scapular fractures as a marker of the severity of the chest trauma (based on the number of associated thoracic injuries) but doesn't present scapular fractures as an indicator of high mortality in blunt chest trauma patients.

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Case Report

Right Hemispheric Leukoencephalopathy as an Incidental Finding Following a Lightning Strike

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Keywords: lightning injuries; electrical discharge; leukoencephalopathy; neurologic sequelae.

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Abstract

BACKGROUND: Lightning injuries may produce a variety of medical conditions, and specific neurological complications have been identified, with the character of immediate aftershock effects or even long-term consequences.

AIM: The authors describe the incidental finding following a routine unenhanced brain MRI performed to a young female patient, suffering from a headache.

CASE REPORT: Diffuse white matter changes with the character of a leukoencephalopathy were seen, which strictly interested only the right cerebral hemisphere. The parents referred that she suffered from an indoor lightning strike at age of seven months, although she survived with almost no external burns or signs, and recovered uneventfully at that time. A discussion over the effects of electrocution and lightning strike on the human body in general, and over the nervous system, is made. Particular attention must be shown when making the differential diagnosis of leukoencephalopathies with a strictly one-hemisphere extension since several other conditions might resemble each other under the radiological aspect, here including brain viral infections, genetic disorders, and so on.

CONCLUSION: The particularity of the long-term aftershock effects of the lightning strike on the central nervous system raise again the necessity of collecting data and duly reporting every electrical accident, lightning events included.

Introduction

Electrical discharge, electrocution and lightning, in particular, represent accidental events whose aftermath might be really serious to humans. Contrarily to the lay opinion which comprehensively is related to the fearfulness of the phenomenon, as much as two third of the lightning sufferers will survive the occurrence and will be delivered to specialised trauma centres for further treatment [1].

Lightning will inflict serious injuries, primarily related to the high amperage of the electrical current produced, that might lead to myocardial asystole, highly lethal if not resuscitated promptly; and secondarily due to thermal effects (burns) and the blast itself, rendering the accident a highly traumatic one. Neurological complications of the lightning injuries have as well widely reported and studied; when present, these complications generally are already competence of specialised centres, and not of emergency teams. Cherington et al. have made several authoritative contributions toward classifying and explaining the nature of lightning-related neurological damage [2, 3].

Under a topographic point of view, the author has divided three groups of patients with regard to the level of the lesions: (a) lesions above the foramen magnum, (b) cranial nerve palsy / palsies and (c) spinal cord lesions [2]. In an attempt to classify the lesions under a temporal perspective as well, Cherington suggests following groups of syndromic value:

1. Immediate and transient symptoms (such as

loss of consciousness, amnesia, weakness etc.);

2. Immediate and prolonged or permanent lesions (post-hypoxic encephalopathy, brain infarction, cranial nerve palsies);
3. Possible delayed neurologic syndromes (such as motor neurone disease, movement disorders etc.);
4. Lightning-linked secondary trauma from falls or blast (epidural haemorrhage, tympanic membrane rupture) [3].

Overall, authors report a diversity of neurologic complications that cover almost the entire spectrum of the clinical neurology: there is clear evidence of damage to the central nervous system (cerebral and cerebellar lesions, spinal cord injuries), to the peripheral nervous system (neuropathy, plexopathy), to the sensorial systems (auditory and visual systems), as well as to the skeletal muscle itself (cramps, rhabdomyolysis and compartment syndromes) [4].

Little is known about the mechanisms for delayed neurological damage following lightning injuries, and the explanations are made much more hypothetical when there is no evidence of structural damage, as in cases when imaging studies reveal no abnormalities at all [5, 6]. Free radicals resulting from oxidative stress might explain the demyelination that frequently precedes the death of spinal neurones [5, 7]. The electroporation hypothesis, namely the formation of additional pores in neurones following electrical injury, is another intriguing assumption, provided that sufficient clinical data will be supportive to it [8].

Case Report

A nine-year-old female child was referred from her paediatrician for a routine brain MRI. Her general health status and growth were normal, but during the last two months she complained of daily non-specific headaches, mainly focused on the frontal region, infrequently accompanied by nausea.

The neurological status was within normality; she had brisk tendon reflexes bilaterally but no pyramidal signs or pathological reflexes. The cognition was considered as adequate, and the educational achievements of the young girl were satisfactory.

Her mother suffered from a migraine, and the family paediatrician had the suspicion of a similar medical occurrence. The MRI was performed unenhanced and detected diffuse white matter changes strictly confined to the right cerebral hemisphere (Figures 1 and 2).

The parents have interviewed a new and recalled only an episode of a lightning strike, which after all was not mentioned in the medical files when she was seven months old. At that time, the girl was lying down on the right side of the head next to the window when the lightning struck; the baby was found crying but conscious, with no signs of burn or other external trauma. Apart from some sustained material damage inside the room, parents could not remember any further injury to their daughter, in relation with the event of eight years before.

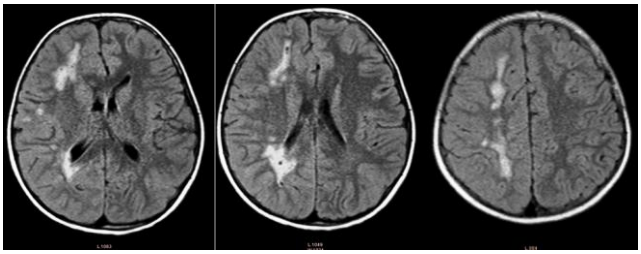

Figure 1: T2-weighted Flair (Fluid-Attenuated Inversion Recovery) images, unenhanced brain MRI

The girl was dismissed without any specific neurological therapy for a headache she complained of. A six-month follow-up medical examination showed no symptoms and signs as well, in spite of the persisting of isolated episodes of headaches. One year after performing the MRI described above, another imaging study showed no significant changes, and the radiologist concluded in favour of some 'non-progressive leukoencephalopathy changes' in the right cerebral hemisphere, with no evidence of any involvement of the contralateral hemisphere.

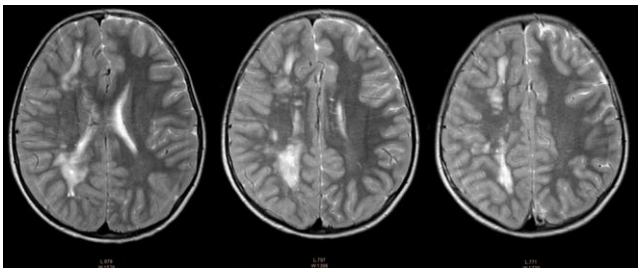

Figure 2: T2-weighted TSE (Turbo Spin Echo) images, unenhanced brain MRI

Discussion

Lightning strikes might be lethal, but under extraordinary circumstances, their thermal effects over the victim can be minimal, or confined to the contact wound, when present [9]. One hundred and twenty years after that Franklin elucidated the physical aspects of the lightning, it was the time of medical doctors to summarise and publish relevant experiences [10]. Of course, the spectrum of neurological disorders in the aftermath of a lightning

injury is immensely wide, and ad hoc terminological novelties have been coined, such as 'kerauno-paralysis' (from the Greek '*keratinous*, κεραυνός' – lightning), depicting the clinical image of a transient motor deficit mainly in the lower limbs following the occurrence [11].

The presence of an incidental finding on the brain MRI such a hemispheric leukoencephalopathy clinically silent will obviously raise important questions vis-à-vis the aetiology. In our case, the significant time delay separating the lightning strike with the brain imaging will even further endorse scepticism; nevertheless, specific MRI changes have been found in the aftermath of the lightning [12]. On the other hand, similar cases of leukoencephalopathy confined to a single hemisphere have been described, but authors have already identified respective etiologies [13-15].

The case we described above was probably related to an indoor lightning strike; this is not the first case of an indoor injury from our experience, albeit the other publication of ours dealt with a fatality [16]. However, particular attention must be shown with regard to long-term and subtle neurological consequences and sequelae of any electrical injury, lightning strikes included.

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Stomatology

A Twofold Comparison between Dual Cure Resin Modified Cement and Glass Ionomer Cement for Orthodontic Band Cementation

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Keywords: dual cure resin; glass Ionomer; orthodontic band; band cementation; solubility.

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Abstract

AIM: To test the solubility of dual cure resin modified resin cement in a food simulating solution and the shear bond strength compared to conventional Glass ionomer cement.

MATERIALS AND METHOD: The materials tested were self-adhesive dual cure resin modified cement and Glass Ionomer (GIC). Twenty Teflon moulds were divided into two groups of tens. The first group was injected and packed with the modified resin cement, the second group was packed with GIC. To test the solubility, each mould was weighed before and after being placed in an analytical reagent for 30 days. The solubility was measured as the difference between the initial and final drying mass. To measure the Shear bond strength, 20 freshly extracted wisdom teeth were equally divided into two groups and embedded in self-cure acrylic resin. Four mm sections of stainless steel bands were cemented to the exposed buccal surfaces of teeth under a constant load of 500 g. Shear bond strength was measured using a computer controlled materials testing machine and the load required to deband the samples was recorded in Newtons.

RESULTS: GIC showed significantly higher mean weight loss and an insignificant lower Shear bond strength, compared to dual cure resin Cement.

CONCLUSION: It was found that dual cure resin modified cement was less soluble than glass ionomer cement and of comparable bond strength rendering it more useful clinically for orthodontic band cementation.

Introduction

Traditional systems for bonding orthodontic brackets and bands require multiple steps including etching, rinsing, drying and application of primer before using adhesive resin. This was time-consuming and may affect the bond strength of the brackets or bands [1]. Self-adhesive resin cements were introduced to the dental market in 2002. They did not require pre-treatment of the tooth surface, and they are fluoride releasing cements similar to glass ionomer [2], hence they were thought to be easier to handle, had shorter chair-time and decreased technique sensitivity [3].

Self-adhesive resin cements have better mechanical properties than resin modified glass ionomer [4]. This may render it as a good candidate for cementing orthodontic bands which are subjected to a great number of forces in the mouth and adequate bond strength is important for the long treatment period needed with the bands in the patient's mouth [5].

The importance of cements lies in their versatility in clinical use; not only they are used in cementing orthodontic bands, but as luting agents, and for cementing different fixed partial prostheses as

well. However, this variability requires the presence of different physical properties and manipulative characteristics [6].

The solubility of the dental cement in saliva may be a serious cause of the development of enamel demineralization and even caries under orthodontic bands and fixed prostheses [7]. Furthermore, it results in debanding of orthodontic bands as it affects the flexural strength, Vichers hardness and mechanical stability [7]. However, cements are not only exposed to saliva in the oral environment, there are multiple other media in food and beverages that can seriously affect the degradation process of the cement [8].

Hence, this study was conducted to test the solubility of dual cure resin modified resin cement in a food simulating solution and the shear bond strength compared to conventional Glass ionomer cement.

Materials and Methods

Materials

The materials tested were self-adhesive dual cure resin modified cement (G-CEM capsule, GC corporation, Tokyo, Japan), Table 1, and Glass Ionomer (Ketac^{TM} cem, 3M ESPE, Deutschland GmbH Germany), Table 2.

Table 1: G-CEM capsule chemical composition

4-Methacryloxyethyltrimellitate anhydride (4- META)	6 - 10%
Urethane Dimethacrylate (UDMA)	1.5 - 3%
Alumino-silicate glass	65 - 70%
Pigment	< 1%
Dime thacrylate	15 - 20%
Distilled water	1.5 - 3%
Phosphoric ester monomer	1-2%
Initiator	<1%
Camphorquinone	< 1%

Table 2: Ketac^{TM}-cem powder/liquid composition

Powder	Liquid
Glass powder	Water
Polycarboxylic acid	Tartaric acid
Pigments	Conservation agents

Methods

The study was a twofold study that investigated and compared the solubility of the materials as well as the shear bond strength and failure mode.

The solubility investigation

Twenty cylindrical Teflon moulds of size 1.2 mm (diameter) x 5 mm (height); measured using a digital calliper to the nearest 0.01 mm, were used. The moulds were divided into two groups; in the first group ten moulds were injected with the modified resin cement and packed using 1mm diameter condenser (Thomson, Tactile Tone SS, OREGON # 2). The specimens were left to set for 5 minutes according to the manufacturer's instructions. In the second group, Glass Ionomer cement powder and liquid were mixed and packed in 10 moulds and left to set for 5 minutes according to the manufacturer's instructions. Glass slabs lined with Mylar strips were used to cover the moulds in both groups until they completely set.

Each mould was weighed using an analytical balance to an accuracy of ± 0.1 mg and inserted in numbered light-proof sealed tubes containing 10 ml n-heptane 95% analytical reagent, (chosen according to the FDA Food and Drug Administration guide lines 1976, USA) [9], at 37^{0}C. The reagent was changed on daily basis, and it simulated butter, fatty meats, and vegetable oils. The specimens were aged in the solution for 30 days [9] then they were removed from the solution, washed in running water, wiped with a soft absorbent paper [10] and reweighed. The solubility was measured as the difference between the initial and final drying mass.

Shear bond strength

For this part of the study, 20 wisdom teeth freshly extracted for surgical reasons were used. The teeth were embedded in self-cure acrylic resin moulds, the buccal surface directed upwards to allow cementation and testing. The teeth were scaled and polished.

The study consisted of two groups each comprised of 10 teeth. The tooth blocks were randomly distributed in dark numbered envelopes. Using the online computer program Random sequence generator; random.org, each tooth was assigned to one of the two groups. This randomization was performed by a researcher who didn't participate in the rest of the study.

The stainless steel bands were sectioned into equal 4mm sections for the sake of standardisation. In group one; the study group, the band sections were cemented to the tooth surface under a constant load of 500 g [4] using dual cure modified resin cement and left to set for 5 minutes according to the manufacturer's instructions. In group two, the control group, the sectioned bands were cemented to the teeth using Glass Ionomer cements under a constant load of 500 g [4] for 5 minutes according to manufacturer's instructions.

The shear test was designed to evaluate the bond strength. All samples were individually and horizontally mounted on a computer controlled materials testing machine (Model LRX-plus; Lloyd Instruments Ltd., Fareham, UK) with a loadcell of 5 kN and data were recorded using computer software

(Nexygen-MT; Lloyd Instruments). Samples were secured to the lower fixed compartment of the testing machine by tightening screws. Shearing test was done by compressive mode of the load applied at the band-tooth interface using a mono-beveled chisel shaped metallic rod attached to the upper movable compartment of testing machine travelling at a cross-head speed of 0.5 mm/min. The load required to deband the samples was recorded in Newtons [11]. The load at failure was divided by bonding area to express the bond strength in MPa: τ = P/ A; where τ = shear bond strength (MPa), P = load at failure (N) and A = interfacial area (mm^2).

Statistical analysis

Statistical analysis was performed with IBM® SPSS® (SPSS Inc., IBM Corporation, NY, USA) Statistics Version 22 for Windows. The significance level was set at P ≤ 0.05.

Results

Data was explored for normality using D'Agostino-Pearson test for the Normal distribution. Shear bond strength (MPa) and Weight loss (%) showed nonparametric distribution, hence Mann-Whitney U-test was used to compare between different tested cement.

Means and standard deviations (SD) of weight loss (%) and Shear bond strength (MPa) for different Types of cement were presented in Table 3 and Figures 1 & 2.

Table 3: Mean and standard deviation (SD) of Shear bond strength (MPa) and weight loss (%) for different Types of cement

	Cements								p-value
	Glass ionomer				Dual-cure resin				
	Mean	SD	Min.	Max.	Mean	SD	Min.	Max.	
Shear Bond Strength (MPa)	1.15	.36	.57	1.43	2.44	1.42	1.21	4.58	0.056
Weight loss (%)	13.21	5.83	7.84	22.64	6.36	1.72	3.85	8.00	0.016*

** = Statistically significant.*

Weight loss (%)

Glass-ionomer Cement (13.21 ± 5.83 %) showed the highest significant mean weight Loss (%) Compared to dual cure resin Cement (6.36 ± 1.72 MPa), p = 0.016 (Table 3, Figure 1).

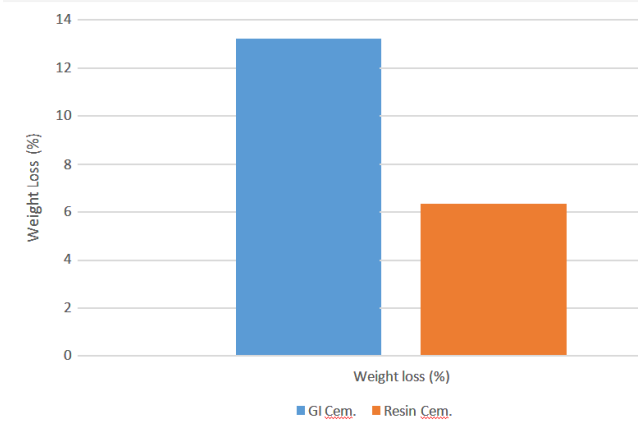

Figure 1: Histogram showing the mean Weight loss (%) for Different Types of cement

Shear Bond Strength (MPa)

Glass-ionomer Cement (1.15 ± 0.36 MPa) showed the lowest insignificant mean Shear bond strength (MPa) Compared to dual cure resin Cement (2.44 ± 1.42 MPa) p=0.056. (Table3, Figure 2).

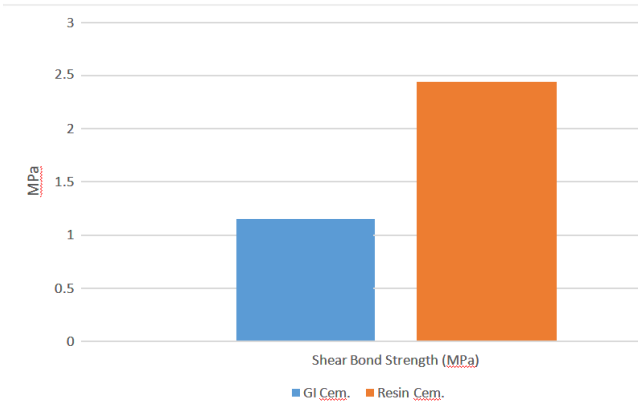

Figure 2: Histogram showing the mean Shear bond strength (MPa) for Different Types of cement

Discussion

The current research was a twofold study to assess the solubility and the shear bond strength of Glass Ionomer cement and Dual-cure resin modified cement. It was argued that resin modified cement had better mechanical properties and less water sorption [3] than conventional cement and that its bond strength to dentin was equivalent to conventional resin cement [12, 13]. Since Glass Ionomer cement are conventionally used in orthodontic banding, it was necessary to test their sustainability in the oral environment and whether there was a more superior material or not. This is important to ensure clinical efficiency as orthodontic treatment, normally and unlike any dental treatment, takes a long time.

The solubility of the Glass Ionomer cement was significantly more than that of the dual cure resin cement as was shown by the weight loss percentage. This may favour rapid degradation of the cement [10] under the orthodontic bands predisposing to their mechanical debanding, and hence delays or interferes with orthodontic treatment mechanics, alongside with the possibility of the occurrence of secondary caries [14, 15]. Those findings were similar to other studies which also reported that the solubility of cements had a potential effect on their mechanical stability [10, 14, 15]. The increased solubility of Glass Ionomer could be attributed to the plasticizing effect of the solvent used which resulted in erosion and degradation of the material [14-16]. On the other hand, the presence of resin network in the resin modified cement reduced the solvent diffusion into the cement [17], which reduced the dissolution of the cement.

Multiple factors affect the rate and amount of dissolution of materials like time, concentration and pH of the dissolving medium, specimen thickness, and powder/liquid ratio of the cement [18]. Ideally, the solutions used must simulate the oral environment complexity, however, static solubility tests are only made because it's impossible to simulate the oral environment as it varies from person to person and within the same person [19]. The organic solution n-heptane, used in this study, simulated butter, fatty meats, and vegetable oils [20], which are materials that accelerate the chemical ageing process [9].

To be able to evaluate the full mechanical performance of the cement in a clinical setting, it was also necessary to investigate their bond strength in which the bonding was done under a constant load of 500 g [4]. Hattar et al [2] reported that adhesive cements should set under pressure [21, 22] to facilitate the intimate adaptation of the relatively highly viscous cement [23]. The results of the study revealed an insignificantly lower bond strength of the Glass Ionomer cement than the Dual-cure resin modified cement. Nakamura et al [4] reported that dual cure resin modified cement had both physical properties and chemical composition similar to resin modified glass ionomer; which could offer a possible explanation for this insignificant difference. Furthermore, when the dual cure resin modified cement was compared to other resin adhesive systems used for bracket adhesion; the bond strength was found to be significantly less [11, 24] which further highlighted that it’s more comparable to resin modified glass ionomer cement than to other resin adhesives. On the other hand, bands cemented with dual cure resin modified cement showed superior tensile strength when compared to those cemented with glass ionomer cement [5]. These literature reports showed that the results obtained were not only influenced by the type of the material, but also the various types of the tests used, indicating that further evidence-based studies unifying the variables should be conducted to obtain more comparable results.

In conclusion, within the limitations of this in-vitro study, it was found that dual cure resin modified cement was less soluble than glass ionomer cement and of comparable bond strength rendering it more useful clinically for orthodontic band cementation.

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Stomatology

Buccal Corridors: A Fact or a Myth in the Eyes of Laymen?

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Keywords: buccal corridor; smile; esthetics; laymen.

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Abstract

AIM: This study aimed to investigate laymen knowledge of the existence of the buccal corridor and whether it was an important factor for them in judging smile attractiveness and the effect of introducing the knowledge to them on their further judgment.

MATERIALS AND METHODS: Nine subjects were randomly selected with variable buccal corridor percentages. They were coached to smile in a posed fashion and full face smile photographs were taken from a standardised distance. The photographs were randomly arranged in a power point presentation and displayed to a panel of thirty-nine randomly selected laymen judges. The judges made their beauty judgment on a visual analogue scale (VAS) and filled a questionnaire. After their education about the buccal corridor, they were asked to make a second judgment on a second sheet with VAS and with a different random sequence of the photographs.

RESULTS: Intra-class correlation agreement for all the judges between the first and second scores was 0.713. The Spearman's rho Correlation coefficient indicated a positive correlation for all the photos. For the male judges, the agreement between the ratings was 0.839, and the correlation was positive for all the photos. For the female judges, the agreement between the ratings was 0.510, and the correlation was positive for all the photographs. Hundred percent of the judges were not familiar with the buccal corridor. Eighty percent of the female judges and 44.4% of the male judges mentioned that it would affect their further judgment.

CONCLUSION: Laymen build their esthetic judgments on what we teach them, and modifying treatment plans to include corrections of buccal corridors for esthetic reasons only is a myth.

Introduction

Beauty, in general, has often been defined as the harmony of proportions; which largely depends on upon subjective feelings and interpretations of observers. Cultural factors, which change over time, play a significant role. In addition, the diversity of ethnic backgrounds [1-3] and education affect the esthetic judgment of different populations.

Smile attractiveness was the point of research for scientists seeking to create a marriage of science and emotion in their orthodontic or esthetic treatment plans. They investigated many smile traits; some of which were the smile arc [4, 5], the smile line [6], the amount of gingival display and gingival smile line [7-9].

The buccal corridor gained special attention in the past and was described by Frush and Fisher [10] as the space between the buccal surfaces of posterior teeth and the corners of the lips on smiling. Frush and Fisher argued that proper size buccal corridors were important to prevent the "denture" appearance created by broad smiles. With the passage of time, the buccal corridor remained as an important point of investigation for many researchers owing to the internationally growing demand for high esthetic standards. The fact that the perception of what was attractive or natural looking had changed over time led to the general belief that the display of more teeth during smiling was more attractive, and that broader smile were much more preferred [11-14].

Throughout the years, the main interest of researchers was confined to finding the proper size of the buccal corridors perceived by laymen [3, 13, 15-18] and/ or orthodontists [2, 12, 14, 16, 18], without questioning whether people noticed it in the first place

or not.

This study investigated, for the first time, the attractiveness of the smile twice; once before introducing laymen to the buccal corridor and another time after it. Accordingly, the aim was to investigate laymen knowledge of the existence of the buccal corridor and whether it was an important factor for them in judging smile attractiveness and the effect of introducing the knowledge to them on their further judgment.

Materials and Methods

The study included nine subjects which were randomly selected by a participant in the research other than the one conducting it. They all had complete natural dentition with no rotations, no gingival inflammation or gingival recession, no previous orthodontic treatment, no spacing, and straight teeth or minimal crowding 1-2 mm.

The subjects had variable buccal corridor percentages. The percentage was calculated as the ratio between the measurement of the visible maxillary teeth and the width of the inner lip commissure multiplied by 100 [12-14, 18]. Buccal corridor of 28% was considered wide, 15% was considered a medium buccal corridor, and 0-2% was considered as a broad smile with no buccal corridors [12-14, 18]. Three subjects were chosen for each percentage (Figure 1).

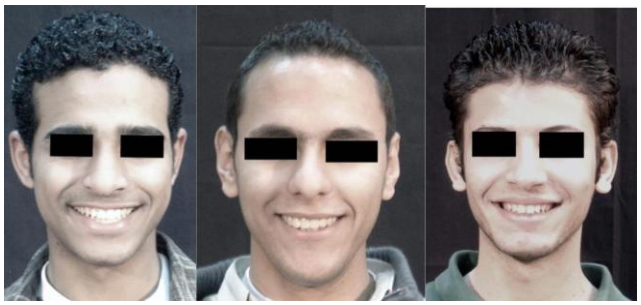


Figure 1: Different buccal corridor percentages

The subjects were coached to smile in a "posed" fashion without laughing or straining [19] and to achieve the same lip configuration at least twice successively before any photographs were taken [8, 20]. Full face photographs, in the natural head position [21], were taken with a 35 mm digital camera and 100mm focal length, mounted on a tripod at a standardised distance of 120 cm for all subjects.

The natural head position was determined as follows [21]:

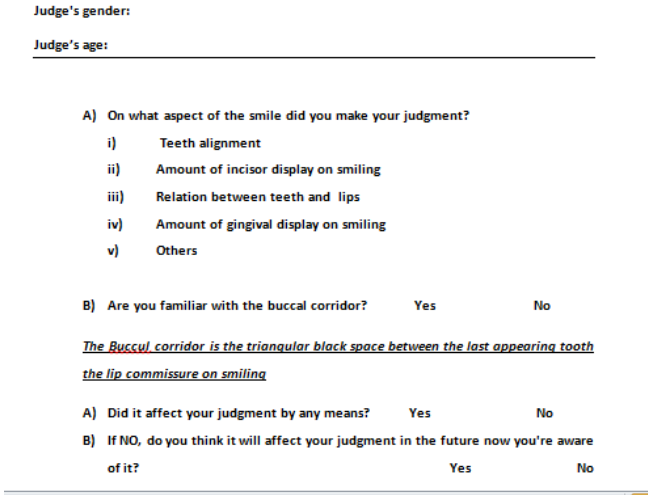
1. The interpupillary line was parallel to the horizontal plane.
2. The distance from the outer canthus of the eye to the hairline was equal on each side.
3. The line from the outer canthus of the eye to the superior attachment of the ear (C-SA line) parallel to the horizontal plane was used to prevent tilting of the head.

Ethical approval from the National Research Centre, Medical Research Ethics Committee, and registration number 16025 was obtained.

All the subjects' eyes were covered using Adobe Photoshop 7.0 (Adobe systems, San Jose, Calif). They all signed an informed consent that their photographs would be used for research purposes.

For generating a concealed allocation sequence, each photograph was saved in a separate computer folder and numbered. Using Random sequence generator online software (www.random.org), the sequence of the photographs was determined. They were included in a power point presentation, (Microsoft office 2010), in the same determined random sequence. The whole process was executed by the participant who randomly selected the nine subjects in the first place.

A questionnaire (Figure 2) and a 10 mm visual analogue scale (VAS) were formulated to serve the study aim. The anchors of the VAS were: attractive, and unattractive [18].


Judge's gender:

Judge's age:

A) On what aspect of the smile did you make your judgment?

i) Teeth alignment

ii) Amount of incisor display on smiling

iii) Relation between teeth and lips

iv) Amount of gingival display on smiling

v) Others

B) Are you familiar with the buccal corridor? Yes No

The Buccal corridor is the triangular black space between the last appearing tooth the lip commissure on smiling

A) Did it affect your judgment by any means? Yes No

B) If NO, do you think it will affect your judgment in the future now you're aware of it? Yes No

Figure 2: Questionnaire

Thirty-nine laymen judges (18 males, 21 females) were randomly selected on the basis of their lack of dental or esthetic education. Their age ranged from 20 to 30 years. They all were university graduates who worked in the educational or business fields.

To calculate the sample size, a pilot study was conducted on 10 laymen judges other than those participating in the research. A minimum of 16 males and 16 females were found to be sufficient to detect the correlation between the two judgment scores, with

a power of 90 % and error = 0.05; using G*power version 3.1.9.2.

The presentation was displayed for the judges to rate the photographs by the VAS; each photograph was displayed for 15 seconds and returning back to previous photographs was not an option [18]. Each judge was then handed a questionnaire to answer, they were all asked to indicate their age and gender on the questionnaire paper (Figure 2).

The questionnaire was designed such that to find out on what aspect of the smile they based their judgment and whether they were familiar with the buccal corridor or not. Then, they were introduced to the term "buccal corridor", and were asked if it affected their judgment, and whether it would affect any future smile attractiveness judgment now that they became aware of it.

After the questionnaires were filled another sheet with a visual analogue scale was handed to the panel of judges for a second smile attractiveness judgement, provided that the photographs displayed in the second presentation were randomly re-ordered using Random sequence generator (www.random.org) to ensure the absence of bias. This second rating was done to confirm or negate their answers in the questionnaire.

Intra-class correlation (ICC) used to quantify the reliability between the two ratings for the same Candidate, and Spearman's rho Correlation coefficient was calculated for the total judgment panel and for the male and female judges. Frequency and percentages for the Answers and comparisons between male and female judges were done. The difference between males and females for questionnaire results were assessed using Kruskal-Wallis for the first question and Chi Square for the other three questions. Statistical significance was set at $P < 0.05$. Statistical analysis was performed using IBM® SPSS® (SPSS Inc., IBM Corporation, NY, USA) Statistics Version 22 for Windows.

Results

The intra-class correlation between the first and second ratings for the whole judgment committee was 0.713. There was a positive and significant Spearman's correlation for all the photographs (Table 1). For the male judges, the agreement between the first and second ratings was 0.8395, and the correlation was positive and statistically significant for all the photographs (Table 2). For the female judges, the agreement was 0.50975 while the correlation was also positive for all the photographs and statistically significant for most of the photographs (Table 3).

Table 1: Intra-class correlation (ICC) between the 2 scores for all the judges and Spearman's rho Correlation coefficient

Photographs	ICC	95% Confidence Interval		Spearman's rho Correlation coefficient	P-value
		Lower Bound	Upper Bound		
1	0.825	0.666	0.908	0.721	≤0.001*
2	0.797	0.613	0.893	0.083	≤0.001*
3	0.755	0.534	0.871	0.644	≤0.001*
4	0.621	0.285	0.800	0.518	0.001*
5	0.654	0.348	0.817	0.513	0.001*
6	0.588	0.215	0.784	0.477	0.002*
7	0.686	0.394	0.837	0.466	0.003*
8	0.779	0.575	0.885	0.598	≤0.001*
Mean	0.713				

* = significant. P-value = $p < 0.05$.

There was an insignificant difference between the answers of the first question; p=0.967. In addition, 100% of the judges were not familiar with the buccal corridor. 71.4% of the female judges mentioned that the buccal corridor didn't affect their judgment, initially, compared to 50% of the male judges.

Table 2: Intra-class correlation (ICC) between the 2 scores for the male judges and Spearman's rho Correlation coefficient

Photographs	ICC for males	95% Confidence Interval		Spearman's rho Correlation coefficient	P-value
		Lower Bound	Upper Bound		
1	0.938	0.836	0.977	0.919	≤0.001*
2	0.927	0.804	0.973	0.851	≤0.001*
3	0.919	0.785	0.97	0.810	≤0.001*
4	0.878	0.672	0.955	0.877	0.001*
5	0.562	-0.194	0.837	0.498	0.035*
6	0.791	0.444	0.922	0.688	0.002*
7	0.791	0.420	0.925	0.562	0.019*
8	0.910	0.749	0.967	0.808	≤0.001*
Mean	0.839				

Only 44.4% of the male judges mentioned that their introduced knowledge about the buccal corridor would affect their future judgment compared to 80% of the female judges (Table 4).

Table 3: Intra-class correlation (ICC) between the 2 scores for the female judges and Spearman's rho Correlation coefficient

Photographs	ICC for females	95% Confidence Interval		Spearman's rho Correlation coefficient	P-value
		Lower Bound	Upper Bound		
1	0.710	0.269	0.883	0.550	0.01*
2	0.515	-0.094	0.796	0.559	0.008*
3	0.447	-0.323	0.773	0.455	0.038*
4	0.193	-0.918	0.667	0.179	0.437
5	0.734	0.364	0.891	0.512	0.018*
6	0.392	-0.342	0.745	0.477	0.002*
7	0.553	-0.130	0.82	0.364	0.114
8	0.534	-0.166	0.812	0.332	0.141
Mean	0.510				

* = significant. P-value= $p<0.05$.

Discussion

In order to provide a totally non-biased environment for conducting the study, full face photographs were taken in accordance to validation [22] who suggested that laymen assessment of the buccal corridor differed on judging full faces rather than just the smiles. Moore et al [13] also suggested that when full faces were taken into consideration the

sizes of the buccal corridor affected smile attractiveness. Further, different subjects were used for the photographs in this study in contrast to manipulating a single photograph as in other studies [13, 18, 22]. This was done to provide real life simulation which enhanced the judges' precision.

Table 4: Frequency and percentage for the Answers and comparison between Male and Female judges

		Gender of judges				p-value
		N	Male %	N	Female %	
1. On What Aspect of the smile did you make your judgment?	i) Teeth alignment	5	27.8%	2	9.5%	0.967
	ii) Amount of incisor display on smiling	1	5.6%	4	19.0%	
	iii) Relation Between teeth and Lips	4	22.2%	7	33.3%	
	iv) Amount of the gingival display on smiling	1	5.6%	2	9.5%	
	v) Other's	7	38.9%	6	28.6%	
2. Are you Familiar with the buccal corridor?	Yes	0	0.0%	0	0.0%	NA
	No	18	100.0%	21	100.0%	
3. Did it affect your judgment by any means?	Yes	9	50.0%	6	28.6%	0.170
	No	9	50.0%	15	71.4%	
4. If No, do you think it will affect your judgment in the future now you're aware of it?	Yes	4	44.4%	12	80.0%	0.074
	No	5	55.6%	3	20.0%	

* = significant. NA=not applicable. P-value= p<0.05.

When the research was concluded and the data was analysed statistically, one of the nine subjects chosen to be judged had an extreme variation in his scores among all the judges, and thus was discarded from the analysis. This extreme variation in scores was suggested to be due to the presence of a beard on the male subject in the photograph, which affected people's judgment variably.

From the results, it was found that laymen lacked any knowledge of what is a buccal corridor and were not familiar with its esthetic impact in all the subjects.

Of the whole judgment committee, 71.4% of the female judges answered that the buccal corridor didn't affect their judgment by any means, even though they had no knowledge of it, while only 50% of the males mentioned it didn't affect their judgment at all.

When the first and second ratings given to the photographs were compared, the total judgment committee showed no agreement between the two scores; 0.713. This indicated that after they were introduced to the knowledge their judgement had changed and that knowledge had an impact on them; which was a positive impact with an increase in overall ratings regardless of the size of the buccal corridor. This incidence demonstrated that even with their introduced new knowledge laymen were not able to distinguish the impact of different buccal corridor sizes on smile esthetics. This was in contrast to Moore et al [13], Martin et al [16], and Zange et al [18], and similar to Hulsey [4], and Parekh et al [14], however, they made their judgment regarding smile arc in conjunction to buccal corridors so the judgment regarding buccal corridors solely was compromised.

Furthermore, Hulsey [4] didn't consider the buccal corridor as mentioned by Frush and Fischer [10]; they only considered it as the ratio of the distance between the canines and the width of the smile, which was not a true buccal corridor. Besides, Hulsey [4] conducted his study using photographs limited to the mouth region while in this study full face photographs were used.

All the previously conducted studies determined the proper size of the buccal corridor without mentioning how the laymen were introduced to it and whether they were instructed to differentiate between the different sizes or it was left for their ratings to determine it [1, 11, 13-18].

The male judges showed a good agreement; 0.8395, between the first and second ratings. However, still, the correlation was positive with a significant increase in overall ratings, despite that only 55.6% of the males mentioned that the knowledge of the buccal corridor would not affect their judgment. This was an indication that male judges were somehow not capable of harmonising their subconscious and conscious opinions.

On the other hand, in the female judges, there was no agreement at all between the two ratings; 0.50975, with a significant increase in ratings for most of the photographs and 80% of them mentioned that the knowledge of the term would affect their judgment. Hence, the female judges showed more reliability in their answers and were both consciously and subconsciously sure of their judgment.

The frequency and percentage calculations of the smile aspects, chosen in the questionnaire, that were supposed to affect the smile attractiveness rating; showed no statistically significant difference. This denoted that the esthetic judgment was based on multiple factors combined together. That was in contrast to the findings of Lukez et al [23] in which the esthetic judgment was mainly based on malposition of teeth.

The results of this study showed that laymen lacked all knowledge of the buccal corridor and that it didn't affect their judgment at all, initially. The introduced knowledge affected laymen's further judgment positively with no discrimination among the different sizes of buccal corridors. Female judges showed more reliability in their opinions than male judges.

In conclusion, laymen build their esthetic judgments on what we teach them, and modifying treatment plans to include corrections of buccal

corridors for esthetic reasons only is a myth.

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Stomatology

The Effect of Orthognathic Surgery on Osteoprotegerin as Immunological Caliper of Bone Healing

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BACKGROUND: Osteoprotegerin (OPG) is considered to be the cytokine that plays an important role in the healing process. OPG regulates bone cell biology, osteoblast–osteoclast, bone-immune cross-talk and maintenance of bone mass. It plays an important role in the development, induction, and repair of bone. Orthognathic surgery as multiples segmental osteotomies has been taken as a model surgery to assess the changes in osteoprotegerin levels in the post-operative bone healing period.

AIM: The aim of the study was to evaluate OPG as immunological caliper of bone healing.

MATERIAL AND METHODS: OPG was evaluated in nine patients seeking orthognathic surgery. Patients were examined and checked to be medically and immunologically free prior to surgery. Blood samples were collected immediate pre-operative as control group and for six weeks post-operative as study group.

RESULTS: Data were collected from nine consecutive patients. The results showed higher levels of OPG. it showed significant increase in the immediate post-operative value ($p = 0.001$) which started to increase gradually during the six weeks ($p < 0.001$).

CONCLUSIONS: Significant higher levels of OPG during the healing period of orthognathic surgery suggest the its use as immunological caliper of bone healing.

Abstract

Introduction

Osteoprotegerin (OPG) is an antiresorptive cytokine and an osteoblast-secreted decoy receptor; it is considered a key regulator of osteoclastogenesis. It specifically binds to osteoclast differentiation factor and inhibits osteoclast maturation. OPG is involved in bone remodelling and in its healing system since it regulates bone cell biology, osteoblast-osteoclast, immune cross-talk and maintenance of bone mass [1].

The RANKL-RANK-OPG cytokine system is one of the principal mediators in the maintenance of bone cell function and activation of bone remodelling by the Basic Multicellular Unit (BMU) which carries out remodelling. The balance of the trimolecular control factor complex composed of OPG, RANKL { receptor activator of nuclear factor kappa beta (NFkB ligand)}, It is also commonly referred to as osteoprotegerin ligand (OPGL) or osteoclast differentiation factor (ODF) and RANK (receptor activator of NFkB) maintains physiologic bone remodeling and renewal of the bone matrix [2, 3].

Osteosynthesis of the bone matrix is achieved by osteoblasts and coordinated with resorption of extracellular bone matrix performed by osteoclasts. The mismatch between the activities of osteoblasts and osteoclasts has immunopathologic implications associated with either a decrease or increase of bone mass mineral density. The OPG–RANKL complex counterbalances the effect of the RANK–RANKL complex, thus playing the most important role in bone homoeostasis [4, 5]. Circulating levels of OPG were found to be a predictor of delayed bone healing and non-union of bone suggesting the active relationship of its circulating levels to healing process. OPG was found to be of value as the prognostic marker of bone activity, wound healing activity and the therapeutic progress following surgery [6-8]. Orthognathic surgery as an elective surgery is considered the most convenient in the maxillofacial surgeries to study the

immunological effect of surgical trauma on patients and changes detected in OPG levels. Patients could be carefully chosen intact preoperatively and not suffering from any systemic or immunological diseases, as these factors will affect the preoperative immunological status of the patient.

Patients and Methods

The study was conducted on nine patients suffering from dentofacial deformity and seeking correction by orthodontic-orthognathic surgery. The patients were chosen randomly from those attending the outpatient clinic of Oral and Maxillofacial Surgery Department at Faculty of Oral and Dental Medicine Cairo University. Eight patients had undergone bimaxillary osteotomy and case number nine had mandibular surgery only. All patients were healthy not suffering from any systemic or bone diseases and the age range between 16-30.

Each patient was subjected to the following: clinical diagnosis (history, clinical evaluation, photographs and radiographs), pre- operative orthodontic treatment and patient preparation before surgery. All patients were informed about the surgical procedure and the schedule of blood samples and an informed consent was assigned. OPG levels were evaluated by taking blood samples according to the following schedule; immediate preoperative, immediate post-operative, three days, one week, two weeks, four weeks and six weeks post-operative. A Serum separator tube (SST) was used to allow samples to clot for two hours at room temperature or overnight at 4°C before centrifugation for 15 minutes at 1000 × g. Aliquot was removed immediately and the samples stored at -80°C. Human Osteoprotegerin (OPG) ELISA Kit (catalogue noCSB-E04692h was used to detect OPG levels. The assay employed the quantitative sandwich enzyme immunoassay technique with a detection range 0.312 ng/ml-20 ng/ml.

Statistical analysis

Data were fed to the computer and analysed using IBM SPSS software package version 20.0 [9]. Quantitative data were described using range (minimum and maximum) mean, standard deviation and median [10].

Results

The post-operative course was eventful expect; limited mouth opening was seen in the patient with single jaw surgery (BSSO) and managed by physical therapy. One case showed periodontal abscess in the lower right molars; it appeared one-month post-operatively and treated in a routine manner. All patients experienced periods of tolerable pain that decreased gradually after surgery and remained from 3 to 4 weeks. Oedema was observed in all patients and resolved gradually after 1 to three months (Fig. 1).

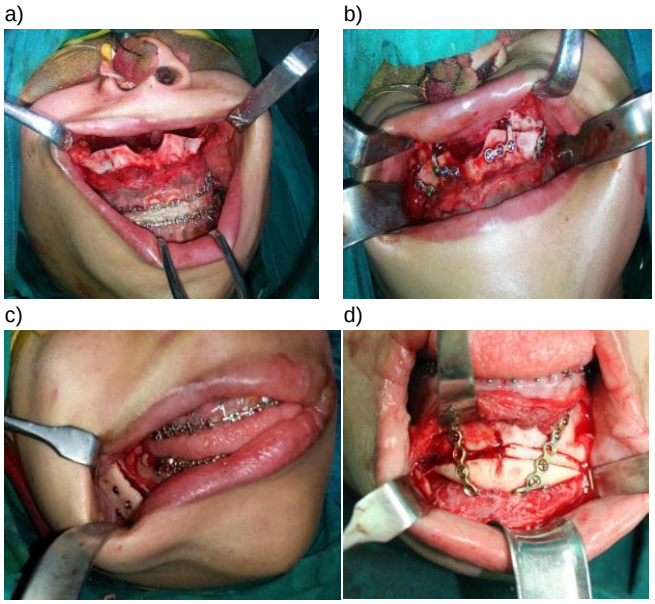

Figure 1: Fixation of case number 8. a) Maxilla and mandible wired together in maxillomandibular fixation. b) Stabilisation of the maxilla by two plates on each side, one at piriform region and one at the maxillary buttress. c) Proximal segment passively seated and fixed to the distal segment by 3 screws. d) Bone graft and rigid fixation of sliding genioplasty

The changes in OPG in the whole group in the predetermined period immediate postoperative, after three days, after one week, two weeks, four weeks and six weeks in comparison to the preoperative level was investigated.

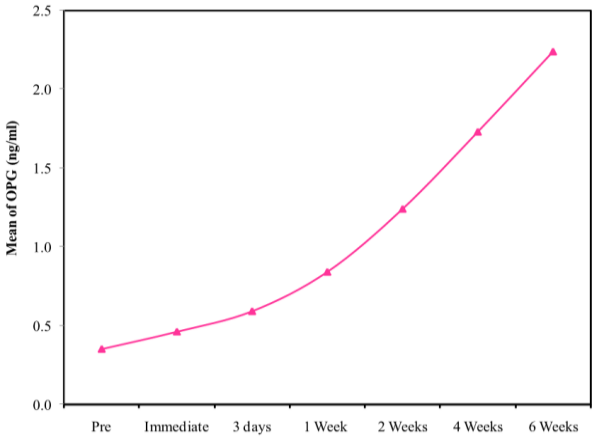

Figure 2: Comparison between the different periods according to OPG ng/ml

It was detected that there is a significant increase in the level of OPG immediate post-operative at (p = 0.001). the concentration of OPG was started to increase gradually and it was significant at the following periods (3 days post-operative, one week, two weeks, four weeks and six weeks post-operative) at p-value <0.001. The increase in OPG level in six weeks postoperatively was found to be significant regarding the immediate post-operative value p < 0.001 (Fig. 2).

Discussion

Osteoprotegerin (OPG) has been chosen in this study as the new most recent calliper. It is sensitive to hard tissue changes and a key regulator of both physiologic and pathologic osteoclast formation that regulates bone resorption and remodelling. An elevated level of serum OPG was connected with high bone turnover and administration to humans has also demonstrated marked decreases in bone resorption. These opinions were supported by the findings reported from several studies.

In the present study Osteoprotegrin (OPG) showed a significant increase throughout the post-operative period in a steady way till six weeks post-operative. This finding is concordant with Giganti MG et al, [11] and Lee et al, [12] as OPG showed significant increase immediately after fracture. The results of the present study are not in agreement with Clombini et al, [13] he investigated the role of OPG in humerus fracture healing at different time points before and after surgery and observed higher OPG, but it was not significant.

OPG can be used as marker for post-operative complications, it is not bone-specific as elevated levels may be accompanied by post-operative complications, this opinion was supported by several studies [14-16]. Post-operative pain could be attributed to the elevated level of OPG as it is known to be involved in a complex cytokine network that causes immune activation and may contribute to nociceptive sensitization. This was in agreement with the findings that showed the relation of the significant increase OPG and the incidence of complex regional pain syndrome (CRPS) [17].

The assessment of OPG in the current study was planned for a period of six weeks post-operatively. The follow-up period of OPG was concordant with previous studies, [18-20] OPG was assessed for 12 weeks and the increase was found to be significant at 4 weeks post-operative and turned to be not significant at 8 and 12 weeks post-operative. For the previous finding six weeks follow-up period is an appropriate duration. The significant increase without remission in the six weeks post-operative period was attributed to the process of bone healing following the osteotomies. Bone healing begins immediately after fracture or osteotomy by hematoma formation and release of cytokines, mesenchymal cells and growth factor, followed by the extended period of bone remodelling.

Orthognathic surgery has been selected as a model surgery in this study. Patients are young, intact, non-traumatized and immunocompetent. Candidates for orthognathic surgery are considered systemically and immunologically free, so for the reliability of the results pre-operative levels of the two callipers are considered control. This category of surgery being an elective procedure, so blood loss is easily estimated, operative time pre-determined and the surgical trauma is the planned and any adverse effect will be attributed to the effect of surgery. Patients incorporated in the present study were demanding surgery and has no psychological hindrance like patients of trauma or neoplasm. Recalls for follow-up was accomplished easily as orthognathic patients are committed to the surgeon and orthodontist.

Significant higher levels of OPG following orthognathic surgery suggest the use of OPG as a good calliper for the assessment of bone healing and regeneration of hard tissue. Further investigations with wide scale sample and for the extended period of time is needed to emphasise the application of OPG as a pre and postoperative immunological calliper predicting the post-operative healing course.

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Dental Implantation of Atrophic Jaws Reconstructed with Iliac Bone Graft Crest - Outcome of Seven Cases

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Keywords: Albania; alveolar ridge; augmentation; edentulism; iliac bone graft.

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Abstract

BACKGROUND: Iliac bone grafts are used to augment alveolar ridges followed by subsequent dental implants in completely edentulous patients. In Albania the information about these issues is scarce.

AIM: To describe the procedure of iliac bone grafts augmentation of alveolar ridges and evaluate the survival rate of dental implants in completely edentulous patients in Albania.

SUBJECTS AND METHODS: Seven totally edentulous patients (three males, average age 45.9 years) presenting at Durrës Regional Hospital during 2008-2015 and seeking a solution to their problem through implantation procedures were included in the study. Patients were thoroughly examined, evaluated and the best augmentation procedure, using iliac crest bone grafts, and dental implantation technique was chosen. The number of dental implants placed was recorded and their survival rate was calculated.

RESULTS: The most common intervention site was maxillae (in 71.4% of cases). Dental implants were installed six months after augmentation, all fixed on the very stable augmented alveolar ridge. On average between 20%-30% of bone grafts, volume was resorbed. Of 37 implants settled, 36 of them or 97.3% survived.

CONCLUSION: Iliac bone grafts are a suitable augmentation source of bone in a patient suffering from complete edentulism in Albania. The survival rate of dental implants is very satisfactory.

Introduction

Complete lack of teeth (edentulism) is rather frequent among human beings, varying considerably according to the age-group of the population studied and ethnicity [1]. Peltzer and colleagues found that the prevalence of edentulism varied from a minimum of 0% to a peak rate of 72% and being higher among older populations [1]. The most common factors associated with increased risk of edentulism, besides age, include disadvantageous socio-demographic and socioeconomic variables (female gender, rural residence, low education, low economic status and health security), some chronic conditions such as diabetes, asthma, high blood pressure and obesity; a range of risky health behaviors such as smoking, low consumption of fruits and vegetables, inadequate dental care and factors associated with disadvantageous general health, mental health and social support [1].

There are different classifications of edentulous patients such as those offered by Cawood and Howell [2], McGarry and colleagues [3] etc. Cawood and Howell classify the changes in the shape of the alveolar processes of maxillae and mandible ranging from Class I (dentate) to Class VI (depressed rigid form, with some basilar loss) [2]. Class V and VI represent the most severe forms of the condition.

After tooth loss, the alveolar bone reacts through resorptive processes that finally cause loss of

tissue affecting virtually all spatial entities in this region, affecting mostly the structures of mandible than maxillary ones [4]. Bone resorption is attributed to the diminished functional loading at the edentulous ridges even though this theory is not completely proven [5]. General and local factors affect the resorption process including osteoporosis, endocrine disorders, nutritional deficiencies, local vascular changes, diseases of the soft tissue of the mouth, jaw surgeries, traumatic tooth removals, etc. [6,7]. The role of these factors may vary from individual to individual, but anyway, the determinant factor remains the tooth loss for a prolonged period of time.

Loss of teeth could lead to a series of undesirable aesthetic and functional effects, including reduction of total height of face (prognathic face), extreme lowering of labial angle, lips thinning, deepening of nasolabial sulcus, ptosis of mental muscles, collapse of mimic muscles, deepening of columella-filtrum angle, etc. [7]. Substitution or repairing of missing teeth is important as it has a positive effect on the general well-being of the patients, quality of life and reducing health care costs [8-10]. Besides classical removable denture, recent developments can offer edentulous patients fixed implant-based solutions to their problem [11]. Adaptive dental implants techniques have shown to be relatively successful with high survival rates [12, 13]. A review of fixed implant prostheses success rates reported an implant survival rate ranging from 93.4% to 100% and prosthodontic survival rates ranging from 96.3% to 100% [14]. However, the selection of these techniques does not always restore the aesthetic dimensions of the lower third of the face.

Autologous bone grafts from the iliac bone have proven successful for augmentation purposes prior to dental implants in patients with atrophic alveolar ridges [15, 16]. The total atrophic maxilla needs to be reconstructed with a bone block graft to re-establish the facial size and shape and to create the opportunity for dental implant placement. This is because the tonus of the upper lip does not depend on the teeth arch but on the volume of the alveolar process. The right evaluation of these defects caused by bone resorption is very important in order to have an optimal aesthetic result. The anterior iliac crest is a reasonably safe extra oral bone source, which can yield relatively large bone volumes ranging between 70-140 cc. In Albania the information about pre-implant augmentation of alveolar ridges using autologous bone grafts in completely edentulous patients is scarce.

In this context, the purpose of this study was to describe the reconstruction of atrophic jaws with iliac bone graft crest in a series of seven complete edentulous patients and evaluate the success of dental implants following augmentation procedure.

Subjects and Methods

This is a cross-sectional survey including seven participants. The study subjects showed up in the Durrës Regional Hospital, the second largest hospital in Albania during 2008-2015.

Five patients presented with a severe resorption of maxillae whereas two other participants had a totally atrophic mandible. For the five patients presenting with severe resorption fixed solutions for the upper jaw were taking into consideration. All patients belonged to Class V or VI of edentulism according to the classification by Cawood and Howell [2].

During the clinical examination of the patients, it was noted that they had a considerable loss of the mandible contour, loss of volume of the lower third of the face, prognathic face, significant lowering of a labial angel, deepening of the nasolabial sulcus, ptosis of chin muscles and thinning of the upper lip. As a result, a collapse of mimic muscles had occurred. Two out of seven participants had been carrying removable prostheses for more than ten years.

After clinical examination, it was decided that simple implantation would not be able to restore normal aesthetics of the lower third of the patients' face and therefore the adequate maxillary bone volume had to be restored. The selection of the type of bone graft was done based on the aesthetic and functional evaluation of the lower third of the face. The x-ray examination enabled the specification of the implant position. Using the Cawood and Howell classification criteria [2] the necessary bone volume to achieve the desired augmentation of alveolar processes was determined.

In all cases, the source of the bone was the bio cortical part of the anterior iliac crest maintaining the prior margin in order to avoid any aesthetic defect. For obtaining the iliac bone graft we followed the same technique as described by Grillon and colleagues [17]: "raising the osteoperiosteal flap from the iliac crest and removing a medial cortico-cancellous bone block". The graft was then modelled and split in half leaving the cortical bone in the vestibular side and the cancellous bone in contact with the alveolar process. The alveolar process was decorticated prior the installation of the graft. Graft installation was done in the anterior area until reaching the level of upper first molar teeth. The iliac bone graft was fixed within the alveolar process using titanium screws. A high volume of the iliac bone graft was used and fixed to take into consideration the subsequent relatively high rates of bone resorption when using this type of bone graft. Ultimately, the wound closure was done without causing any tension and releasing flaps until deep into fornix. Dental

implants were installed six months after the augmentation procedure.

The volume of bone loss after augmentation was approximately measured using the 3D panoramic x-ray, by comparing the volume of augmented bone to the volume of bone before implantation.

Statistical analysis

Absolute numbers and respective percentages for categorical variables (gender and location of augmentation) as well as mean values and standard deviation for numerical variables (age) were reported.

The number of the dental implants was also reported as well as the number of dental implants that survived.

Results

In total seven patients participated in the survey. Three participants were males and the remaining four subjects were females. The average age of the patients was 45.9 years ± 6.5 years and ranging from 35 years old to 54 years old (Table 1)

Table 1: Baseline characteristics of study subjects

Study variable	Absolute number	Frequency
Age (years) - mean ± SD	45.9 ± 6.5	
Gender		
Male	3	42.9
Female	4	57.1
Total	7	(100.0)

In two patients (or 28.6% of study participants) the intervention location was the mandible, whereas in the five remaining cases it was intervened in the maxilla (Table 2).

Table 2: Number of dental implants and implant survival among study participants

No.	Age (years)	Gender	Place of augmentation	Number of implants	Number of implants survived	Dental implants survival rate
1	54	Female	Maxillae	4	4	100%
2	52	Female	Maxillae	6	5	83.3%
3	41	Male	Maxillae	7	7	100%
4	35	Female	Maxillae	5	5	100%
5	48	Male	Mandible	3	3	100%
6	46	Male	Mandible	7	7	100%
7	45	Female	Maxillae	5	5	100%
Total				37	36	97.3%

The installation of dental implants was done six months after the augmentation procedure. In all cases, the dental implants were fixed on a very stable (augmented) alveolar ridge. After the opening of the first flap, it was noted that bone grafts had lost about 20% to 30% of their initial volume but their integrity and healing process was excellent.

In total, there were implanted 37 implants, and only one implant failed. Thus, the overall survival rate of implants was 97.3% (Table 2). In every study participants the success rate of dental implants was 100% and in only one subject (a 52 year old female) the success rate was 83.3% since one out of 6 maxillary implants installed failed (Table 2). In this patient, the procedure of dental implantation was postponed for another three months. In some patients fixed bridges were placed whereas in other patients prosthesis was placed.

Long-term monitoring and check-up of these patients showed very good stability and little bone resorption in all the studied patients. Figure 1 shows a male patient with complete edentulous maxilla followed during intervention, augmentation, implantation and recovery.

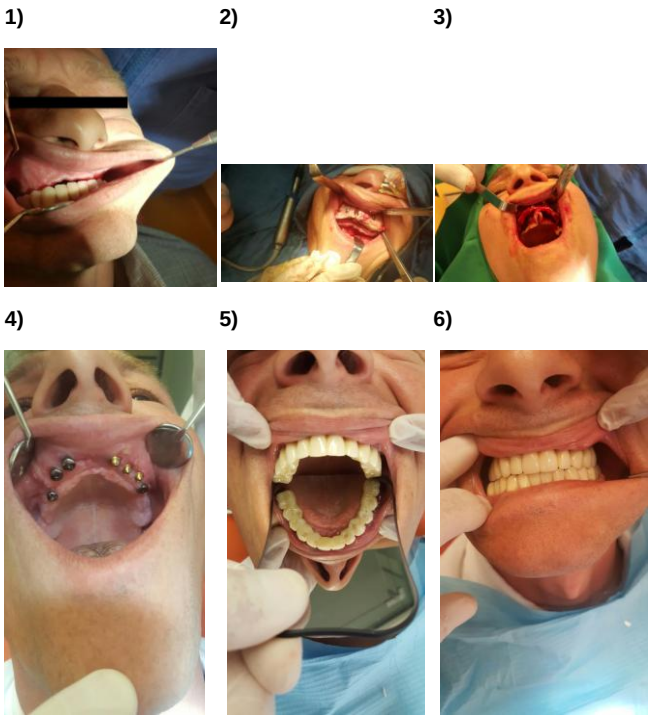

Figure 1: A male patient with complete edentulous maxilla (1); during intervention (2, 3); after implantation (4); recovered (5, 6)

Discussion

The present study, including 7 patients suffering from complete edentulism in Albania and showing up at the premises of Durrës Regional Hospital during 2008-2015 for specialised treatment of their condition, aimed to assess the procedures of alveolar augmentation and evaluate the success rate of dental implants. This is the first study scientifically reporting about augmentation procedures in completely edentulous patients in Albania and the

survival rates of dental implants following augmentation procedures. The results of this study suggest that the average age of completely edentulous patients in Albania is about 46 years and the most affected region is the upper jaw (maxilla). In addition, iliac crest bone grafts are suitable for the augmentation of alveolar ridges creating a stable and adequate base for the installation of dental implants. The survival rate of dental implants following augmentation with iliac bone grafts in Albania was high: 97.3%.

The average age of the patients in our study was relatively low. Age is a strong determinant of edentulism and the combination with poor socio-economic conditions [1] could increase the risk of complete tooth loss further. Albania is a developing country with considerable rates of poverty [18] and disadvantageous health care seeking indicators and therefore this could be a risk factor for tooth loss as well. There are no studies in Albania to measure the prevalence of partial or complete tooth loss. In addition, the procedures of bone augmentation and dental implantation are very expensive and unaffordable for the majority of Albanians experiencing partial or complete edentulism. Stomatology procedures are not part of health insurance scheme in Albania and all the associated costs are paid out-of-the-pocket by the patients. This could be a reason for the very few patients presenting with this condition in our hospital resulting in a relatively very small study population. In this context, it is very difficult to reach a definite conclusion and to compare our data with those reported in international literature. In general, higher percentages of males than females seek treatment for edentulism [19]. In our study, there was more female than male patients. Such discrepancy could be due to the very small study population in our survey including only seven patients.

The use of iliac crest bone for the augmentation of large defects of alveolar processes is thoroughly described in the literature [15, 16, 20, 21]. However, autologous grafts from iliac bone exhibit higher bone resorption rate compared to other types of bone grafts or grafts [22], which can be a problem usually requiring augmentation of a larger bone volume in order to take into account the resorption process. In our study, the resorption rate of the bone graft was 20% to 30% compared to the initial augmented volume. These results are similar to those reported in the literature that suggests a resorption rate ranging from 12% to 60% for iliac bone grafts [15]. Bone grafts originating from other locations, such as calvarial bone and chin bone grafts show a lower resorption rate [15]. However, in cases where the alveolar ridge defect or atrophy is severe, a high volume of bone is required and in these cases, the iliac bone offers a better solution. Nevertheless, it should be kept in mind that iliac bone grafts often require extensive surgical interventions requiring general anaesthesia and bearing the risk of various complications, factors that must be weighed against the benefits of iliac bone grafts compared with other solutions.

The survival rate of dental implants among seven participants included in this study was relatively high, at about 97%. Only one out of 37 implants failed. This result is comparable with success rates of dental implants following augmentation procedures in the international arena. In general, the survival rate of dental implants varies from 95% to 97.5% [23-26]. A study of 24 consecutive patients was 56 implants were installed after augmentation of alveolar ridges with autogenous mandibular bone graft reported that 54 of them (or 96.4%) survived [25]. Adell et al suggested that dental implant survival rates ranged between 89%-98% in the mandible and between 81-82% in maxilla [11]. Several factors could increase the risk of failing of dental implants, including age over 60 years, smoking, diabetes mellitus, neck radiation, post-menopausal oestrogen therapy among females, etc. [27]. The higher the frequency of these factors in the studied populations the higher the likelihood of dental implant failure. However, in our study we couldn't retrieve any information regarding the existing diseases our patients might be suffering from and as a result, due also to the limited number of participants, it was not possible to reach any definite conclusion linking dental implant failure with such conditions.

The average number of implants per patient in our study was 5.3 (37 implants / 7 patients) whereas other international studies reported on average 6.6 implants per patient among totally edentulous patients [11]. The average number of implants among complete edentulous patients is clearly much higher compared with partial edentulous patients. For example, an international study among partial edentulous patients reported on average 1.2 implants per patient [19].

Our study has several limitations. Its cross-sectional nature does not allow drawing definite conclusions about the temporality of events. Furthermore, the small study population and the non-random selection of participants does not allow to generalise the present findings to larger population groups. Therefore, our study might suffer from selection bias. However, the advantage of the present survey is that for the first time it reports on augmentation procedures among completely edentulous patients in Albania and survival rates of dental implants, contributing to highlighting these under-researched topics in this Balkan country. Despite this, future research is needed to be conducted employing larger target populations in order to better understand these processes and to confirm our findings in Albanian settings.

In conclusion, iliac crest bone grafts are suitable for the augmentation of severely damaged or atrophic alveolar ridges in completely edentulous

patients in Albania. If the procedure is carried out carefully and following standard procedures it could create a solid stable bone base on which dental implant could be fixed. The survival rate of dental implants is quite high contributing to the overall satisfaction of patients and probably an improvement of their self-esteem and quality of life.

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The Use of Different Irrigation Techniques to Decrease Bacterial Loads in Healthy and Diabetic Patients with Asymptomatic Apical Periodontitis

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Abstract

BACKGROUND: Diabetes mellitus is a multisystem disease which weakens the human's immunity. Subsequently, it worsens the sequelae of apical periodontitis by raising a fierce bacterial trait due to the impaired host response.

AIM: This study aimed to estimate bacterial reduction after using different irrigation techniques in systemically healthy and diabetic patients with asymptomatic apical periodontitis.

MATERIAL AND METHODS: *Enterococcus faecalis, Peptostreptococcus micros*, and *Fusobacterium necleatum* bacteria were chosen, as they are the most common and prevailing strains found in periodontitis. Bacterial samples were retrieved from necrotic root canals of systemically healthy and diabetic patients, before and after endodontic cleaning and shaping by using two different irrigation techniques; the conventional one and the EndoVac system. Quantitive polymerase chain reaction (qPCR) was utilised to detect the reduction in the bacterial count.

RESULTS: The EndoVac irrigation system was effective in reducing bacteria, especially *Peptostreptococcus micros* in the diabetic group when compared to conventional irrigation technique with a statistically significant difference.

CONCLUSION: The EndoVac can be considered as a promising tool in combination with irrigant solution to defeat the bacterial colonies living in the root canal system. Additional studies ought to be done to improve the means of bacterial clearance mainly in immune-compromised individuals.

Introduction

Apical periodontitis (AP) is viewed as a provocative procedure that happens around the apex of a tooth. Inflammation is caused by the ingress of bacteria from an infected pulp canal system. Extension of the periradicular lesions causing bone destruction is a sequel resulting from the coexistence of polymicrobial irritants from the diseased root [1].

Diabetes mellitus (DM) is an assembly of complex multisystem metabolic disorders which has a direct influence on the functions of the immune system which leads to delayed healing and affected immune responses. Diabetes mellitus may act as a precursor for inducing pulp necrosis and successive periapical lesions and failed endodontic treatment cases due to altered wound repair, immune and vascular functions [1, 2].

It was known that root canal treated teeth showing apical periodontitis have decreased success rate when compared with teeth with no apical disease which may end by endodontic failure [3]. An intimate noteworthy link between an increased incidence of apical periodontitis and diabetes mellitus was noticed. Moreover, when cases with preoperative periradicular lesions were investigated, diabetics had lower successful outcomes when compared with non-diabetics' patients preoperatively [4].

To overcome the restrictions of culture

techniques, molecular analysis have been agreed for invading the microbial world. The advantages of molecular tests are the detection of uncultivable bacteria in diseased root canals, given the chance to obtain definite and detailed new evidence on the endodontic microbial field. However, the differentiation between living and dead organisms was still questionable and impossible [5]. The intervention of qPCR, with new advancement process by using propidium monoazide (PMA), permits quantitative distinguish between viable and non-viable cells [6].

The good prognosis of endodontic treatment depends mainly on the efficient eradication of co-existed bacterial biofilms and their end products from the affected canal by using required cleaning and shaping means. The agitation of irrigant used is mandatory during filing to eradicate debris and bacteria from root canal system. To improve the flow and distribution of irrigating solution various techniques and devices should be introduced [7]. The EndoVac System is considered as negative pressure alternating devices safely used in debris removal from the working length without causing their extrusion into the periapical area [8].

This study aimed at estimating bacterial reduction after using different irrigation techniques in systemically healthy and diabetic patients with asymptomatic apical periodontitis.

Materials and Methods

The study protocol was approved by the ethical committees of Faculty of dentistry, Ain Shams University, Cairo, Egypt and the National Research Center, Cairo, Egypt (protocol number 15/026). All patients included in the study signed an informed consent.

Forty samples retrieved from single rooted single canaled lower premolars. Teeth were collected from 20 healthy and 20 diabetic patients. Patients were recruited from the Endodontic Department, Faculty of Dentistry, Ain Shams University, and from the Dental Clinic at the Diabetes Institute, Cairo, Egypt. Patients were divided into two main groups according to health condition being systemically normal (Group A) or diabetic (Group B), then they were subdivided according to irrigation methods used during cleaning and shaping to conventional syringe groups (Micro-Mega, Besancon, France) A1 & B1 and EndoVac groups (Discus Dental, Culver City, CA) to A2 & B2. The patients included were chosen to have pulp necrosis and infected root canals with asymptomatic apical periodontitis confirmed by vitality pulp testing and radiographic examination. The only systemic disease accepted in selection criteria was controlled type 2 Diabetes Mellitus based on a range of glycosylated haemoglobin (HbA1c) [9].

Selected teeth (n=40) received no prior endodontic treatment. Subjects who received antibiotic treatment within the preceding three months, teeth with periodontal probing depth greater than 4 mm, teeth had pain on palpation or percussion or had swelling, any multi-rooted tooth, non-restorable tooth, with root fractured tooth, or canal communicated with oral cavity were not included in the study. In addition, any chronic systemic diseases other than type 2 diabetes mellitus were excluded from the study.

After determining the provisional working length, complete teeth isolation and disinfection protocols were performed to avoid any field contamination. Strictly stuck to aseptic conditions, an appropriate access cavity was done; the canal was filled with sterile saline solution, and then introduced by a sterile #15k file one mm short of the root apex. The pre-operative microbiological sample was taken by four sterile paper points with a size compatible with the root canals anatomic diameter for 60 seconds. Then, the paper points were immediately placed in sterile 1.5 ml labelled tubes containing 500 μl of sterile phosphate buffered saline (PBS) solution, transported to the microbiological laboratory and frozen -70°C until quantitative real-time polymerase chain reaction (qPCR) analysis.

Cleaning and shaping were started using a #k-file of size 10 or 15 put to the full length of the root canal. Canal preparation was completed with one shape file system. According to subgroups classification, in groups A1 & B1, the root canals were irrigated with 5.25% NaOCL aided with side vented needle gauges 30 (Micro-Mega, Besancon, France) whilst groups A2 and B2 were treated by 5.25% NaOCL using EndoVac irrigating device (Discus Dental, Culver City, CA). The working time for the chemo-mechanical procedure was established at 15 minutes for all teeth. All canals were temporised using reinforced glass ionomer as coronal restoration for the next appointment after 48 hours to inhibit the degrading action of NaOCL on DNA amplicons. Post-instrumentation sampling in next visit followed the same aseptic conditions and same sample taking steps. Finally, all canals were obturated with gutta-percha points using lateral condensation technique. Coronal portion of the tooth was restored using composite resin.

Bacterial culturing and DNA Extraction

The positive control was settled by choosing *E. faecalis* because it contains four copies of the 16S rRNA gene covering almost the DNA sequence of most known endodontic bacteria which helps in drawing the standard curve for the bacterial comparative template. *Enterococcus faecalis* were cultured on trypticase soya broth media overnight. Once, the black colonies specific for the bacterial

strains appeared 100 colony forming units up to 10^8 CFU/μl were used for DNA extraction. Quantification of total bacteria levels for each sample was performed using a standard curve made off known concentrations of genomic DNA extracted from *Enterococcus faecalis* [10].

At room temperature, clinical samples were thawed, vortexed vigorously, and centrifuged at 8,000 x g for 5 minutes. The pellets were used for DNA extraction. The DNA was extracted and purified with a Qiamp DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Using enzymatic extraction method, DNA from both Gram-positive and Gram-negative bacteria was retrieved with no apparent discrimination against either bacterial group [11].

Quantitative Real-time polymerase chain reaction procedures (qPCR)

The primers (forward & reverse) and probes designed for the *E. faecalis* were (CGCTTCTTTCCTCCCGAGT), (GCCATGCGGCATAAACTG) and (CAATTGGAAAGAGGAGTGGCGGACG) [10]. While for *Peptostreptococcus micros* (AAACGACGATTAATACCACATGAGAC), (ACTGCTGCCTCCCGTAGGA), and (TCAAAGATTTATCGGTGTAAGAAGGGCTCGC) [12], and for *Fusobacterium nucleatum* (AAAGTAGCG GCGAGCGAAATGG), (TGGTCCTCACTGATTCACACAGA), and (ACTTTGCTCCCAAGT AACATGGAACACGAG), respectively [13].

The PCR primers and TaqMan probe were based on species-specific highly conserved regions from the 16S rRNA gene. qPCR amplification and detection were performed with the ABI-PRISM 7500 Sequence Detection System using a 96-well format. qPCR reaction conditions for the three different bacteria included in this study were 95°C for 15 min for initial heat activation, followed by 40 cycles of 95°C for 15 seconds for denaturation, 95°C for 30 seconds for primer annealing and 60°C for one min for an extension. Cycle threshold (CT) values were calculated using the Sequence Detection Software and compared to an *E. faecalis* standard curve generated in parallel with quantification of target DNA from clinical test samples.

Statistical analysis

The mean and standard deviation values were calculated for each group. Data were explored for normality using Kolmogorov-Smirnov and Shapiro-Wilk tests and showed non-parametric distribution, while Mann–Whitney U-test was used to compare the difference between the two groups. The significance level was set at $P \leq 0.05$. Statistical analysis was performed with *IBM® SPSS® Statistics Version 20 for Windows.

Results

Effect of different irrigation techniques on bacterial reduction by qPCR

F. nucleatum and *E. faecalis* number were reduced in healthy and diabetic individuals when using EndoVac technique compared to using the conventional syringe technique without significant difference. Regarding the effect of the health condition of the patients on the bacterial reduction**,** there was no significant difference in *F. Nucleatum, P. Micros* and *E. faecalis* count between healthy and diabetic patients, regardless of the method of irrigation used. On the other hand, *P. micros* count was reduced upon irrigation with EndoVac at a higher rate when compared to the conventional syringe method without significant difference in the healthy group; While there was a significant difference in the diabetic group (P = 0.05).

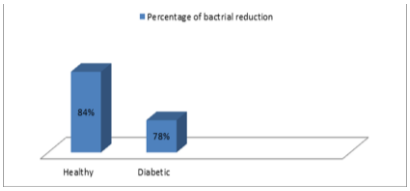

Figure 1: Column chart of percentages of overall bacterial reduction according to patient`s health status

The assessment of the health condition of the patients could not be ignored. It was observed that the overall bacterial reduction was higher in healthy population collectively when comparing with the diabetic group without statistically significant difference.

Table 1: Mean, standard deviation values and percentages of *Fusobacterium nucleatum*, *Peptostreptococcus micros* and *Enterococcus faecalis* reduction of the experimental groups

Variables	*Fusobacterium nucleatum*					*Peptostreptococcus micros*					*Enterococcus faecalis*				
	Conventional Syringe (1)		Endovac (2)			Conventional Syringe (1)		Endovac (2)			Conventional Syringe (1)		Endovac (2)		
	Reduction (Mean ± SD)	% of Reduction	Reduction (Mean ± SD)	% of Reduction	*p-value*	Reduction (Mean ± SD)	% of Reduction	Reduction (Mean ± SD)	% of Reduction	*P-value*	Reduction (Mean ± SD)	% of Reduction	Reduction (Mean ± SD)	% of Reduction	*p-value*
Healthy (A)	$2.15 \times 10^7 \pm 5.93 \times 10^{7\ a}$	81.82%	$3.34 \times 10^7 \pm 7.70 \times 10^{7\ a}$	99.6%	0.31	$5.92 \times 10^6 \pm 1.84 \times 10^{6a}$	88.02%	$5.14 \times 10^4 \pm 9.64 \times 10^{4a}$	97.40%	0.68	$1.30 \times 10^5 \pm 2.78 \times 10^{5\ a}$	49.64%	$5.66 \times 10^4 \pm 1.02 \times 10^{4\ a}$	57.80%	0.75
Diabetic (B)	$6.17 \times 10^4 \pm 1.66 \times 10^{5\ a}$	90.06%	$7.56 \times 10^4 \pm 2.00 \times 10^{5\ a}$	98.41%	0.57	$1.38 \times 10^5 \pm 2.53 \times 10^{5a}$	82.61%	$1.36 \times 10^6 \pm 2.14 \times 10^{6b}$	99.62%	0.05	$2.78 \times 10^2 \pm 3.93 \times 10^{2\ a}$	26 %	$3.44 \times 10^4 \pm 7.58 \times 10^{4\ a}$	40.00%	0.91
P-value	0.68		0.19			0.43		0.01*			0.34		0.84		

According to the present study, the EndoVac as negative pressure device was found to be more effective than conventional side vented syringe in the bacterial reduction in both groups

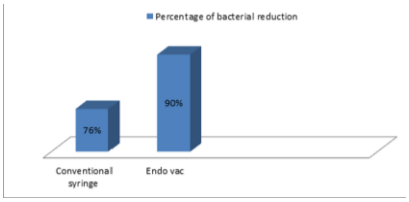

Figure 2: Column chart of percentages of overall bacterial reduction by using different irrigation techniques

Discussion

The main objective of endodontic treatment is getting a bacteria free canal to get an optimum successful outcome. Despite, the rapid evolution in irrigating materials, devices, and tools, the persistence of bacteria remains questionable. The diversity of root canal anatomy and the organisation of microorganisms hindered in the dentinal tubules, isthmuses and ramifications complicate the complete bacterial eradication from root canal which usually ends with apical periodontitis [2, 14]. It was confirmed that there is an actual relationship between the presence of specific bacterial taxa in filled root canal and treatment failure, which suggested that some taxa, such as streptococci, *Olsenella uli, Propionibacterium acnes*, and *Fusobacterium nucleatum*, might have the potentiality to be the initiator of risk factors and cause periapical diseases [15].

Diabetes mellitus is considered a modulator disease for impaired immunity, which may have a direct influence on the severity of periodontitis, the spread of periradicular lesions, endodontic flare-ups and endodontic treatment failure. Diabetics showed the least percentages of successful outcomes compared with healthy individuals [16]. *F. nucleatum*, *P. micros,* and *Streptococcus* spp. were the most prevalent pathogenic microorganisms retrieved from diabetic and non-diabetic specimens [17–19]. The selected bacterial species in this study were chosen because they are commonly present in the two studied groups. DM may trigger variations in dental pulp tissue which promotes pulp necrosis [20].

The regular trials to overcome the limitations of culturing techniques evolved the appearance of molecular technology for bacterial detection, being more accurate with greater sensitivity to provide a mean for distinguishing between the living and dead cells. Moreover, the new technologies have the ability to analyse DNA and RNA for more specificity. The elucidation of the obscure enigma of root canal microbiology was recently clarified by using different types of PCR [21, 22]. qPCR for quantification was used in this study to give an accurate survey about bacterial reduction and realise the reliability of the work.

For achieving the goal of endodontic treatment, the irrigant solutions, and the delivery devices played a very critical role in the final outcomes. After being the magical antibacterial irrigant over the years, NaOCL is the best choice when the bacterial reduction is required [23, 24]. However, its cytotoxic effects restrict its use in certain biological experiences; efforts have been made to find alternatives [25, 26]. The traditional irrigation approaches are efficient in cleaning root canals coronally, but less effective apically [27]. So for effective irrigation, an enhanced delivery system is highly desired. The EndoVac with negative pressure promoted better cleaning of main and simulated lateral canals, consequently, it helps in reducing bacterial contamination when used [28–30]. According to the results obtained from this research, irrigation with EndoVac was highly effective than conventional syringe irrigation in all groups with no statistically significant difference as described previously [29, 31]. On the contrary, two studies proved that there is no difference in bacterial reduction between different delivery devices [32, 33]. The discrepancies in results between the studies were due to the difference methods of culturing, type of bacteria selected and the systemic conditions of the patients.

To our knowledge, our study is the first to report a statistically significant difference in the reduction of *P. micros* after irrigation with Endovac in diabetic patients when compared to healthy ones. Only one study reported higher efficacy in microbiological reduction with Endovac when compared to the positive pressure with a statistically significant difference but still the group of interest is systemically healthy [34].

This study has its own limitations. Only single rooted and single-canalled teeth were included, for an easier accomplishment of the aseptic condition in this group of teeth and the chances of taking a good representative sample from the main large root canal are allegedly increased when compared with narrow canals. However, it is likely that in molars with more complex canal anatomy or in teeth with oval canals, the magnitude of bacterial reduction might have been different, even though it is reasonable to assume that not to the point of affecting the comparison between the two instrumentation techniques. Also, the recognised limited ability of paper points to collect a representative sample from the root canal system makes the information on bacterial counts restricted to the main canal [35].

In conclusion, the negative pressure irrigating

devices (EndoVac) can be considered as a promising tool in combination with irrigant solution to defeat the bacterial colonies living in the root canal system. Further studies are needed to test a wider range of endodontic microbiological species mainly in patients with systemic diseases to expand horizons to new areas of learning. It will be highly recommended to correlate the cruelty of pathogenic microorganisms with uncontrolled diabetes. The question always remains; the available irrigating devices are efficient in bacterial clearance mainly in immune-compromised individuals or not.

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Stomatology

Can Low Level Laser Therapy Benefit Bone Regeneration in Localized Maxillary Cystic Defects? - A Prospective Randomized Control Trial

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Keywords: LLLT; low intensity pulsed ultrasound; cystic lesion; digital radiograph.

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Abstract

AIM: The aim of the study was to evaluate the effect of Low-Level Laser Therapy (LLLT) on bone formation in cystic defects following cyst enucleation.

PATIENTS AND METHODS: The sample was composed of sixteen patients with enucleated maxillary bony cystic lesions. With an age range from 20 - 44 grouped as eight Laser and eight Control patients. Laser group was subjected to low intensity diode laser immediately after surgery and then for three times per week for two weeks using a therapeutic laser irradiation. Group B (control group): patients were not subjected laser therapy.

RESULTS: The predictor variable was exposure of bone defect to LLLT or none. The outcome variable was bone density changes measured by digital radiographs at day 1 and days 90 postoperatively. Descriptive and bivariate statistics were computed. There were no statistically significant differences between the 2 groups for the bone density at day 1. There was a statistically significant difference in bone density changes in each group at day 90: Significant at $P \leq 0.05$. After adjusting for differences in day 1 for bone density, the estimated mean change in bone density changes at day 90 was significantly larger for Laser compared with control.

CONCLUSION: The results of this study suggested that LLLT can enhance bone healing in maxillary cystic defects. This can serve as an adjunct method in preventing possible delayed healing and pathological fractures This also will be helpful for more researchers in early loading in case of dental implants to accelerate osseointegration.

Introduction

A standout among well-known issues confronted by oral and maxillofacial specialists are the osseous defects and dead spaces and how can be obliterated and augmented in the facial regions.unerupted tooth and bone loss after its removal,remaining roots and enucleation of cysts.all of these makes prosthetic rehabilitation and implant application more complicated [1].

Bone is undergoes remodelling via cycles of bone resorption and bone formation which is considered a mineralized connective tissue [2], an inflammatory immune reaction which is triggered by local injury which is thought to highly influence the outcome of the bone healing process [3]. Low-Level Laser Therapy (LLLT) is a form of phototherapy that involves the application of low power monochromatic and coherent light to areas of injuries and lesions. It has been shown to induce wound healing in non-healing bone defects [4].

The bone healing is a multidimensional process of reconstruction of the bone tissue with an overlapping timeline. Because of the regeneration ability of the bone, bone defects can heal spontaneously under suitable physiological environmental conditions. The healing process of the bone defect is time-consuming, and new bone generation takes place slowly because of diminished

of blood supply to the fracture site and insufficiency of calcium and phosphorus to strengthen and harden new bone [5].

The low-level laser therapy (LLLT) has a positive effect on bone tissue metabolism and on fracture consolidation [6, 7]. 980-nm GaAlAs low-intensity diode laser irradiation is beneficial for the initial stages of alveolar bone healing and for further calcification In both diabetic and normal rats under Histological observations and gene expression analyses when applied every day at a dose of 13.95 J/cm2 for 60 sec [8].

The purpose of this study was to evaluate the effect of LLLT on bone healing. The investigators hypothesised that LLLT will have an effect on stimulation of bone healing, more than in control group. The specific aims of the study were: measuring the bone density by assessing the bone density changes after applying LLLT using direct digital radiography (Diogra).

Patients and Methods

This research was approved by National Research Center (Medical Research ethical Committee).

The study included sixteen healthy patients with an age range of 20-44 years presenting with intrabody maxillary cystic lesions that were blindly divided into two equal groups, eight patients each; Laser group, where patients were subjected to low-intensity diode laser application after surgery to the area of the enucleated lesion and Control group where patients were not subjected to low-level laser therapy.

Patients were assessed radiographically for changes in bone density using direct digital images that were obtained by means of size 2 photostimulable plate (PSP) using the Digora Optime imaging system (Soredex, Tuusula, Finland) at day 1 postoperative and at day 90.

The study population was composed of all patients presenting to National Research Center, Giza, Egypt for evaluation and management of painful maxillary anterior teeth with a cystic lesion to be included in the study sample cystic lesion with maximum size 3 x 4 cm. Patients were excluded as study subjects if they have any systemic disease that interferes with bone healing.

Following a thorough preoperative assessment, patients were scheduled for enucleation of the cystic lesions (Fig. 1) and were randomly divided into 2 equal groups.

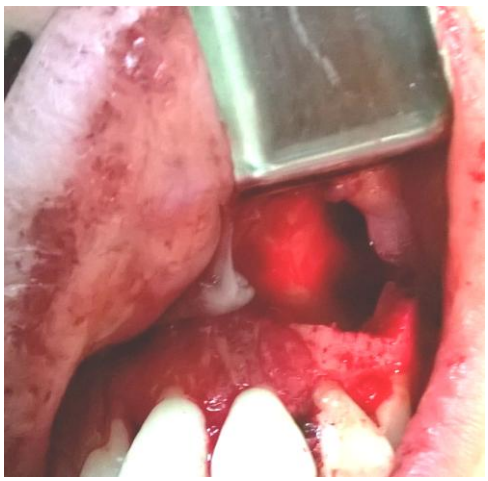


Figure 1: Photograph showing the bony cavity after removal of the cyst

Group A (Laser group): Patients were subjected to low intensity diode laser "soft-laser-SL202" ("PERTO LASER", pr. Stachek, 47, Saint-Petersburg, 198097, Russia) immediately after surgery and then for three times per week for two weeks. The method of irradiation is scanning uniformly over a surface of the lesion. the spot diameter 2mm.with the intensity 1591 mW/cm^2 and the dosage 24 J/cm^2, that corresponds to action by continuously modulated radiation (CW mode) of power 50 mW with an 870nm wavelength and exposure time 60 sec (Fig. 2).

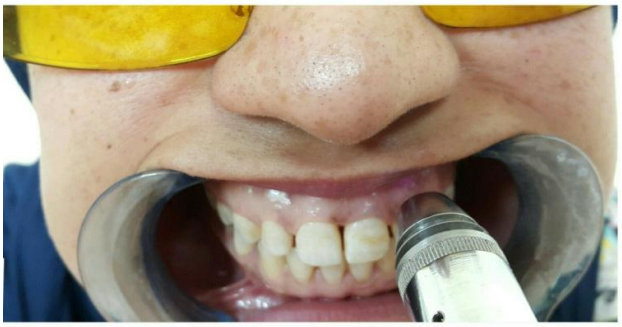


Figure 2: Photograph showing application of LLLT

Group B (control group): patients were not subjected to low-level laser therapy.

Data was analysed by Descriptive and Method of Density Measurement with Area measurement (area density index) (Fig. 3).

A rectangle area was marked included the area of enucleated cyst and apices of the affected teeth to take the mean of density in the bone area, but digora software does not allow hands-free measurements - only rectangle measurements.

Patients were evaluated at day 1 and day 90 for changes in bone density using digital radiography (Soredex, Tuusula, Finland) operating with tube voltage 70 kVp and tube current 7 mA at 0.08 second.

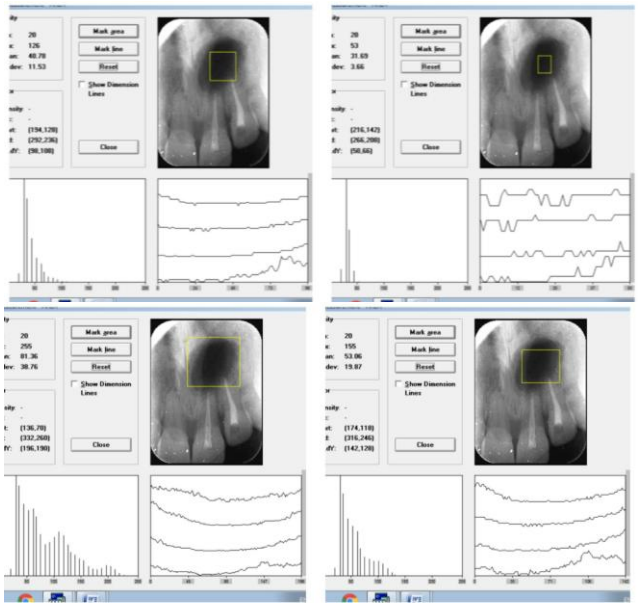


Figure 3: A digital radiograph of one of the investigated cases demonstrating rectangles drawn to calculate the mean of area density measurement

Results

Descriptive statistics of (bone density) for different experimental factors (time and treatment groups)

The results of bone density for different experimental factors are shown in Table 1.

Table 1: Descriptive statistics for the primary outcome variable (bone density) for different experimental factors (time and treatment option)

Treatment option	Time	Mean	SD	SE	CI	Max	Min
Laser	Day 1	52.559	8.562	1.211	2.433	65	30.85
	Day 90	98.384	10.956	1.549	3.114	132	87
Control	Day 1	52.99	7.566	1.07	2.15	69.45	40
	Day 90	55.764	7.437	1.052	2.114	71.3	45

SD: Standard deviation; SE=standard error of mean; CI =confidence interval of the mean; Max=Maximum recorded value; Min = Minimum recorded value.

Laser (A) group: The highest Bone density mean value was recorded at (day 90) (98.384). While the least Bone density mean value was recorded within (day 1) (52.559). There was a statistically significant difference between (Day1) and (day 90), 45.825 where p-value < 0.001.

For Control (B) groups: The highest Bone density mean value was recorded at (day 90) (55.764) and the least bone density on day 1 (52.99). There was a statistical significance difference between (Day 1) and day 90, 2.775 where (p = 0.067).

Comparison of (bone density) for different experimental factors (time and treatment option)

The results of comparison for bone density for different experimental factors (time and treatment option) are shown in Table 2.

Table 2: Comparison of (bone density) for different experimental factors (time and treatment option)

	Laser		Control		F- value	P-value
	Mean	SD	Mean	SD		
Day 1	52.559	8.562	52.99	7.566	1.956	0.145
Day 90	98.384	10.956^{a} <0.001	55.764	7.437^{c} <0.001	332.872	<0.001*
Difference	45.825		2.775			
t–value	23.304		1.849			
P-value	<0.001*		0.067			

SD = standard deviation; Different small letters indicate significant difference according to Tukey test; the subscribed value is the P-value for the Tukey test, * is Significant at P ≤ 0.05.

At day 1 there was no statistical significance difference between (Laser) (52.559) and (Control) (52.99), where p = 0.145.

Table 3: The mean, standard deviation (SD) and percentage of bone density change after different methods of treatments

Variables	Change in bone density		% of change	
Groups	Mean	± SD	Mean	± SD
Laser (A)	43.85	$\pm 11.06^{b}$	77.94 %	±17.94%
Control (B)	13.64	$\pm 1.34^{a}$	24.18 %	±5.98%
P-value	0.001*			

At day 90 there was a statistically significant in favour of the laser group compared to control group (Table 3).

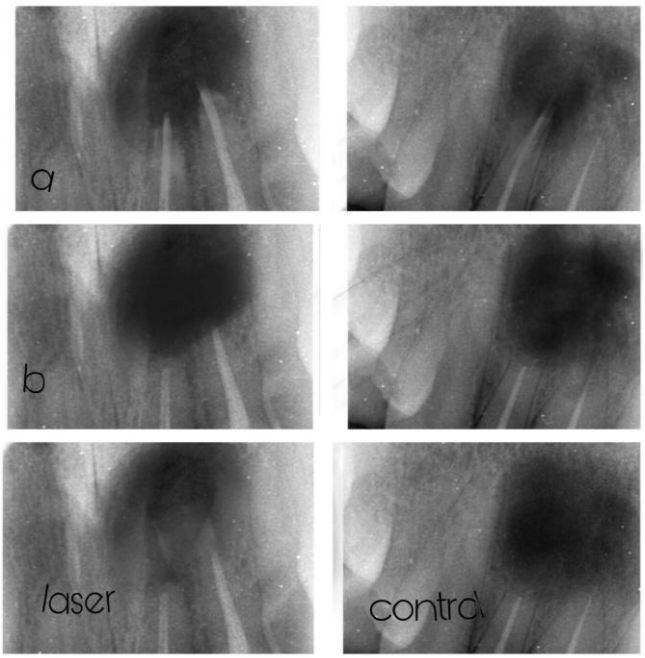
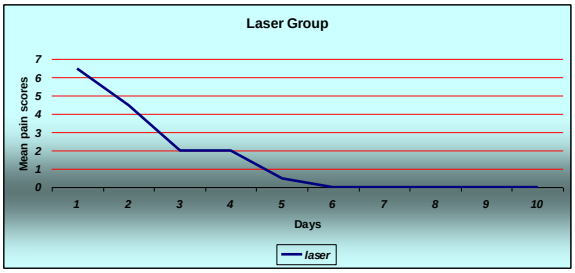


Figure 4: Photo radiographs showing changes in bone density in the two groups where a) preoperative, b) day 1 postoperative and day 90 postoperative the last one

Pain Scores Results

For Laser (A) groups the results showed that there is a significant decrease in pain scores from day 1 (6.5 ± 1) till day 3 (2 ± 0) and day 5 (0.5 ± 0.5) with no pain at all in day 10 (Fig. 5A).

A)



B)

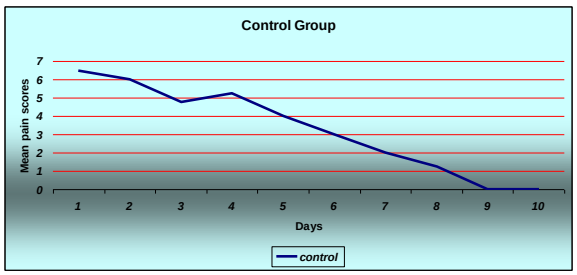


Figure 5: A) Line chart representing pain scores in Laser group; B) Line chart representing pain scores in Control group

For Control (C) groups the results showed that there is a decrease in pain scores from day 1(6.5 ± 1) but there was an increase again in pain on day 4 (5.25 ± 1.25) then a gradual decrease in pain till day 8 (1.25 ± 0.75) (Fig. 5B).

The results showed that there was a significant decrease in pain scores in Laser group as compared to the control group.

Discussion

After enucleation of jaw cysts, the residual cavity is a common bone defect. Osteogenesis of bone in the cystic cavity begins with the formation of a blood clot, which is later replaced by osteogenic granulation tissue [9]. So, the objective of the current study was to evaluate the stimulatory effect of low-level laser therapy on osteogenesis following enucleation of maxillary cystic lesions. The results showed more superior effects of laser on bone density.

For the preservation of morphological contour, which is obligatory for prohibition the surrounding soft tissue incorporation into the bony defect, restoration of mechanical strength & function, and prevention of infection is very important. The applying new modalities for promoting bone repair are very important [10-12].

LLLT and its biostimulation effect were studied in different fields. Most of these studies were directed towards the effect of LLLT on wound healing. But its effect on bone remodelling and repair is deficient in the literature [13-15].

Results of many studies seem to depend on delivery of appropriate energy levels, the type of laser (wavelength) used, the frequency of session and duration of exposure. Several of these studies did not describe the levels of laser energy used to stimulate stimulatory effects or the exposure parameters. This caused controversy when determining whether or not these lasers influence healing effect [16, 17].

In this study, sixteen patients were presented with intrabody maxillary cystic lesions were divided into two equal groups, eight patients each; Laser group, where patients were subjected to low intensity diode laser with 870 nm wavelength the spot diameter 2mm with the intensity 1.592 mW/cm^2 and the dosage 24 J/cm^2, that corresponds to action by continuously modulated radiation (CW mode) of power 50 mW and exposure time 60 sec. Application after surgery to the area of the enucleated lesion and a Control group where patients were not subjected to low-level laser therapy.

In this study, enucleation was chosen over marsupialization following the universal agreement to the treatment goals and basic therapeutic principles of surgical enucleation of the cyst and obturation of the root canal of the affected teeth to eliminate the necrotic tissues (bacteria and toxins) and avoid the recurrence [18].

In laser group patients were subjected to low-intensity diode laser immediately after surgery for three times per week for two weeks. This was in accordance with a study which discussed the effect of low-intensity laser on bony cavities after removal of cysts of the jaws and applied low-level laser therapy starting from the second day of the surgery for two weeks, three times per week.

But there was another study [18] which discussed the effect of low-level laser therapy (LLLT), using a GaAlAs diode laser device, on bone healing and growth in rat calvarial bone. Diode laser was applied immediately after surgery and then daily for 6 sequential days. The tissue samples from the experimental animals contained significantly more calcium, phosphorus, and protein with additionally maintained angiogenesis than the control group. Furthermore, connective tissue formation and more advanced bone formation were found in the experimental group than in the controls.

The bone density was measured at day 1 and day 90 postoperatively. This time was chosen in the current study close to the time of a previous study in which the cone beam digital radiographs were performed to measure the bone density within the

bony cavity in groups of study at time intervals of day 1 and 4 months after the surgical procedure.

Comparing both groups regarding the change of bone density throughout the study period showed there was no significant difference between both groups at day 1 post surgically, while the bone density in laser group was statistically highly significant than the control group at four months post-surgically.

Radiography is the major non-surgical method for detecting bone formation in a healing osseous defect. Thus it is useful in clinical situations because of its continuity of measurements, and non-invasive nature. Bone healing is radiographically expressed as an increase in radiopacity, resulting in a higher optical density of the bone image. Computed tomography is a more precise method to evaluate the bone healing process after cyst enucleation, but it produces a relatively high cost for routine follow-up examination [19].

Evaluation of bone density was carried out at the apical area to assess the changes in density within three months from the day of cyst enucleation. The results of the current study revealed an increase in the bone density in laser group throughout the follow-up period. This was the same results revealed in many other studies [13, 14, 20] which found that the area of greatest bone destruction is usually centred around the apex of the tooth with sclerotic pattern located at the periphery. This is an area which has the highest healing activity.

The bone density of the patients in laser group was higher than that the control group three months post-surgically. These results were found to be significant. This could be explained according to the results of several studies [2, 9, 23-25] which detected that, low-intensity laser was found to stimulate microcirculation, enhance fibroblast proliferation, increase osteoblastic activity and stimulate the production of ATP which plays an important role in accelerating mitosis, as well as improving the host immune response. Moreover, the use of low-level laser therapy (LLLT) could enhance callus development in the early stage of the healing process [26], with doubtful improvement in biomechanical properties of the healing bone.

The rate of increase of bone density was significantly higher in the laser group. This enhancement of osteogenesis was thought to be due to the increase of osteoblastic activity induced by laser application [23]. LLLT induces the expression of bone morphogenic protein-2 (BMP-2), transforming growth factor-β (TGF-β) in 1% hypoxic cultured human osteoblasts, osteocalcin, type I collagen and so enhance osteogenesis. That also matched with anther study [25] which reported a significance increase in the recorded levels of alkaline phosphatase enzyme, which is a common parameter that measures the differentiation of osteoblasts and identifies osteogenic activity. It is known to be associated with bone metabolism and differentiation of osteoblasts. This also was in agreement with another study which concluded that soft diode laser has a biostimulation effect in the process of bone formation after surgical bone removal and that it increase bone repair at early bone healing.

These results also were in agreement with several studies [8, 14, 26], which the authors recorded an increase in the percentage of bone density values in the laser groups when compared with the non-laser groups. The low-intensity laser was proven to have a stimulatory effect on bone regeneration and apposition.

Furthermore, these findings have a great support with histological observations, which explained by further studies [8, 18, 27]. By using atomic absorption spectroscopy, colorimetry, and photometry used to determine the levels of calcium, phosphorus, and protein. They found that in the experimental group [GaAlAs (980 nm) diode laser was applied], the tissue samples contained significantly more calcium, phosphorus, and protein than the controls. Similarly, histological analyses disclosed more pronounced angiogenesis and connective tissue formation, and more advanced bone formation in the experimental group than in the controls.

The dose of the laser is still a complicated subject. And any mistake in parameters may give an illusory picture. We have chosen our laser parameters by getting specifications from manufacturers of the entire laser used in the reported trials and we have recalculated all power densities, dose per treatment sessions and time of treatment sessions. Most of the literature on LLLT is full of conflicting reports and this due to lack of dosage unanimity [28]. In one study the authors assessed whether LLLT enhances bone regeneration and osseointegration of dental implants following sinus augmentation, this experimental study in a sheep model using cancellous bone graft. The authors didn't find a positive impact of LLLT on bone regeneration. This is opposite to our findings but in our opinion, this may be due to the authors applied LLLT for only three times during the first postoperative week with a diode laser (75mW-680 nm) and this is a lower wavelength and laser duration of application of LLLT from a current study.

In conclusion: (1) low-level laser therapy has a significant effect on bone healing after enucleation of cystic lesion regarding bone density measured by direct digital radiography; (2) the results from this study will possibly aid in the prevention of pathological bone fracture following enucleation of large cysts and tumours. However, this needs large sample size with larger defect sizes to confirm help in avoiding pathological bone fracture after large cysts or tumour removal; (3) this also will be helpful for more researchers that will help in early loading in case of dental implants as it can accelerate osseointegration.

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Stomatology – Review Article

Implant Supported Fixed Restorations versus Implant Supported Removable Overdentures: A Systematic Review

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Keywords: fixed restoration; removable over denture; all on four; systematic review; implant supported restorations.

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Abstract

AIM: The aim of this study is to systematically evaluate and compare implant retained fixed restoration versus implant retained over denture.

MATERIAL AND METHODS: Search was made in 2 databases including PubMed and PubMed Central. Title and abstract were screened to select studies comparing implant retained fixed restorations versus implant retained removable overdentures. Articles which did not follow the inclusion criteria were excluded. Included papers were then read carefully for a second stage filter, this was followed by manual searching of bibliography of selected articles.

RESULTS: The search resulted in 5 included papers. One study evaluated the masticatory function, while the other 4 evaluated the patient satisfaction. Two of them used Visual Analogue Scale (VAS) as a measurement tool, while the other two used VAS and Categorical Scales (CAT). Stability, ability to chew, ability to clean, ability to speak and esthetics were the main outcomes of the 4 included papers.

CONCLUSION: Conflicting results was observed between the fixed and removable restorations.

Introduction

Being without teeth is a disability, and the main target of implant placement is to add support of fixed prostheses or to maintain complete dentures in the edentulous arch [1]. Difficulties are faced by edentulous patients using their traditional complete dentures due to lack of fit, support and security, adding to that the related compromise in mastication function [2].

Implant supported restorative restorations show high success rates [3, 4]. Reconstruction by implant-supported single-unit crowns or fixed bridges represents a valid tool to rehabilitate partially edentulous patients [5-7]. Furthermore, long-term data of implant-supported fixed prostheses or overdentures in edentulous jaws are available and presents a reliable treatment [8].

Overdenture is a removable dental prosthesis that covers and rests on one or more remaining natural teeth, the roots of natural teeth, and/or dental implants [9]. Overdentures resting on implants have been shown to provide a successful long-term end result, particularly when used to rehabilitate edentulous jaws [10, 11]. High Implant survival rates and patient satisfaction has been reached with this treatment option [12].

Fixed-implant prostheses in the edentulous jaw are also a scientifically justified treatment option [13]. Two fixation methods are used for fixed implant-supported restorations. They can be attached to implants with screws, or they can be cemented to abutments which are attached to implants [14]. Despite their high survival rates, patients concerns have been reported periodically for implant supported fixed bridges, resulting in low patient comfort [15].

When selecting between a fixed or a removable restoration: the available bone quantity and quality, the number, location and implant distribution,

the available inter-arch distance and maxilla-mandibular relationship, the nature of the opposing occlusion, the expenses as well as the time frame required to assemble and maintain the prosthesis, are all considered crucial factors prior to treatment planning [2, 16].

The aim of this study was to systemically evaluate & compare the implant retained fixed prosthesis and implant retained removable overdentures, for the completely edentulous patients.

Materials and Methods

Search Strategy

An Electronic Search of the literature was performed on PubMed and CENTRAL, using the following search terms: 1- Edentulous Jaw; 2- Edentulous Mouth; 3- Edentulous Jaws; 4- Edentulous Mouths; 5- Completely Edentulous; 6- Complete Edentulism; 7- Edentul*; 8- 1 OR 2 OR 3 OR 4 OR 5 OR 6 OR 7; 9- Implant Supported Dental Prosthesis; 10- Overlay Denture; 11- Overdent*; 12- Screw Retained Prosthesis; 13- Hybrid Prosthesis; 14- Removable Implant Overdenture; 15- Implant Overdenture; 16- All On Four; 17- 9 OR 10 OR 11 OR 12 OR 13 OR 14 OR 15 OR 16; 18- 8 AND 17.

A hand search was done on the bibliography of the included papers. Last hand search was performed on the 25th of April – 2016.

Selection criteria

Clinical trials comparing the screw retained prostheses and the removable overdentures , for the completely edentulous patients were selected according to the following inclusion criteria: human studies; studies comparing fixed prostheses with removable prostheses with no surgical intervention prior to implant placement; minimum of four implants placed per arch; complete edentulism.

Study Selection

Retrieved titles were all screened, and all papers that met the inclusion criteria were selected. Abstracts of all headings chosen were screened and obtained for inclusion criteria. After abstracts were screened, full-text studies were retrieved for the selected papers. In case both the heading and the abstract of an article wasn't enough to obtain data needed to make a decision regarding inclusion criteria, full texts were retrieved. Full-text papers meeting inclusion criteria were screened upon their methodology and results. Two reviewers performed the screening procedure.

Critical appraisal

The risk of bias was assessed according to Cochrane handbook of systematic reviews. Six criteria were evaluated which included: study design, selection randomization, allocation concealment, addressing inclusion/exclusion criteria, patient's attrition (reporting of lost follow-up) and objective/numerical evaluation of the results (Table 1).

Table 1: Risk of Bias

Study	Type of Study	Selection Randomization	Allocation Concealment	Inclusion/ Exclusion criteria	Patient's Attrition	Objective/ Numerical Evaluation	Risk of Bias
Feine et al, 1994	Prospective	NO	NO	YES	NO	YES	HIGH
Grandmont et al, 1994	Prospective	NO	NO	YES	NO	YES	HIGH
Feine, et al,1994	Prospective	NO	NO	YES	YES	YES	HIGH
Heydecke et al, 2002	Prospective	NO	NO	YES	YES	YES	HIGH
Heydecke et al, 2004	Prospective	NO	NO	YES	YES	YES	HIGH

Results

A total of 504 titles were identified by the electronic search. After initial titles and abstracts screening, 495 irrelevant articles were excluded and a total of 9 articles were selected for full-text screening. No additional articles were found through hand searching. After a full-text screening, 5 articles [17-21] were included in the present analysis. Jacobs et al, the study [22], was excluded as the overdenture group was supported by only two implants which didn't match the inclusion criteria. Ferrigno et al, the study [23] were excluded as the study had surgical procedures before implant placement. Katsoulis et, al study [24], was excluded as patients had natural teeth, while Feine et al, study full text was not available [25] (Fig. 1).

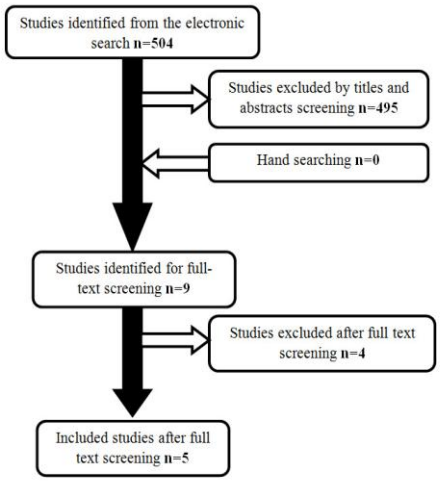

Figure 1: Flow diagram of study selection process

Table 2: Included studies

Study	Arch	Number of patients			Gender		Age (years)	Number of implants/Arch		Outcome
		Total	Fixed	Overdenture	Male	Female		Fixed	Overdenture	
Feine et al, 1994 [17]	Mandible	15	8	7	N	N	30-62	4-5	4-5	- Masticatory Time - Cycle duration - Vertical Amplitude
Grandmont et al, 1994 [18]	Mandible	15	8	7	N	N	30-62	4-5	4-5	- G. Satisfaction - Esthetics - Ability to speak - Fit/Retention - Function
Feine et al,1994 [19]	Mandible	15	8	7	N	N	30-62	4-5	4-5	- Stability - Ability to clean - Ability to chew - Ability to speak - Esthetics
Heydecke et al, 2002 [20]	Maxilla	13	8	5	6	7	45.1	4-6	4-6	- G. Satisfaction - Choice of Prosthesis
Heydecke et al, 2004 [21]	Maxilla	30	15	15	N	N	30-60	6	4	- Speech Analysis - Ability to speak

The 5 articles included in this study were published in a period ranging from 1994 to 2004. They differed widely with respect to methodology, study designs and outcomes. So the possibility of attempting a meta-analysis was eliminated. In all studies, patients were divided into a fixed restoration group and another removable group except Grandmont et al, study [18], which had an extra group of conventional complete denture under investigation. Three studies [17-19] used the same settings while reporting three different outcomes in each, where all implants were placed in the mandible. The other two studies [20, 21] used the maxilla as the arch of interest (Table 2).

Speech analysis

Four studies [18-21] evaluated the ability to speak within the comparison of two groups of patients. A 100 scale VAS (mm) was used to assess the ability to speak for all the four studies. No significant difference between both groups was reported regarding the ability to speak in Grandmont, Feine and Heydecke studies [18, 19, 21]. In those studies, VAS records were higher for the fixed group in studies [18, 19], while in Heydecke study [21], VAS records were higher for the removable group. While a significant difference in favour of the removable group was reported in a study [20].

A single study [21] has reported into depth the speech quality & errors by using a fixed prosthesis in one trial and a removable prosthesis in another trial. This study tested stops, fricatives & vowels between both the removable and fixed groups. The study revealed a statistically significant difference in the favour of the removable group in case of correctly produced sounds, especially for stops & fricatives. While non-significant difference was observed for vowels, with higher means for the removable prosthesis.

Patient satisfaction

Four studies [18-21] reported the patient satisfaction with different reporting methods and outcomes. Two studies [18, 20] out of four used a 100 mm VAS (Visual Analogue Scale) and CAT (Categorical Scales), to assess the patients' own words in describing their satisfaction about the prosthesis in different aspects. While the other two [19, 21] only used VAS (Table 3).

In studies [18-20] using the VAS, the significant difference was obvious between the treatment groups regarding ease of cleaning in favour of the removable denture restoration. While non-significant difference between both groups was reported regarding the esthetics, where Grandmont and Feine studies [18, 19] recorded higher means for the fixed group, opposed by Heydecke's study [20] where the removable group had higher means for esthetics. Chewing ability was significantly better for the fixed group in studies [18, 19]. On the contrary, it was significantly better in favour of the removable prosthesis in a study [17]. While no significant difference was noticed in study [20] between both groups.

A contradicting outcome evaluating the general satisfaction between the two groups, gave a significant difference for the removable group in the study [20] while reporting no significant difference between studied groups in a study [18], with higher VAS means for the fixed group. Grandmont et al study [18] which used CAT scale has mentioned that regarding fit, retention, function & quality of life, there was no difference between both types of implant-supported groups. While Heydecke et al study [20] have reported a significant difference regarding embarrassment at work and avoiding conversations, both for the favour of the removable group.

Choice of prosthesis

Feine and Heydecke Studies [19, 20] reported the number of patients who chose either a fixed or a removable prosthesis at the end of the trial. Eight patients chose the fixed solution in a study [19], while only four patients in a study [20]. For the removable prosthesis, seven patients chose that type in a study [19], while only four patients in a study [20].

Table 3 - Patient satisfaction

Study	Measurement Tool	Stability		Ability to chew		Ability to clean		Ability to speak		Esthetics		Occlusion		Comfort		General Satisfaction		General Satisfaction vs. natural teeth	
		F	R	F	R	F	R	F	R	F	R	F	R	F	R	F	R	F	R
Grandmont et al 1994 [18]	VAS (mm)	N	N	N	N	N	N	90.1	86.0	85.3	85.2	N	N	N	N	N	N	N	N
Feine et al,1994 [19]	VAS (mm)	94.4	79.0	92.7	79.3	71.4	85.6	89.3	83.4	82.7	76.5	N	N	N	N	N	N	N	N
Heydecke et al, 2002 [20]	VAS (mm)	84.3	96.4	86.7	95.8	36.5	86.0	61.7	94.0	76.8	94.6	86.7	95.8	76.7	96.5	48.5	89.2	49.1	94.2
Heydecke et al, 2004 [21]	VAS (mm)	N	N	N	N	N	N	79.2	88.6	N	N	N	N	N	N	N	N	N	N

VAS = Visual Analog Scale , F= Fixed Restoration , R=Removable Overdenture , N=Not reported.

Masticatory function

A single study [17] has evaluated the efficiency of mastication between both the fixed and removable prostheses. Jaw mastication muscles and mandibular movements were recorded using an electromyographic activity. Three measurements were considered: Mastication time, vertical amplitude & cycle duration. The study showed higher cycle duration with the removable denture group while for the vertical amplitude, results were less. Mastication time was found to be faster for certain types of food when the patient used the overdenture.

Discussion

Edentulism is often correlated with functional and esthetic concerns for the patient and is related to psychological problems possibly affecting routine activities [26]. Tooth absence can severely affect a patient's psychosocial status, even for patients who seem to adapt well to a conventional complete denture [27].

Restorations supported by implants offer multiple advantages over ordinary removable prostheses. Efficient retention and stability are simply achieved by creating a fixed restoration, or at least by using overdenture attachment instead of counting on the weak physical ways used with regular dentures [28].

Commonly used abutment types connecting over dentures include magnets, balls and bars [29]. The anatomic configuration of the jaws must be taken into consideration when selecting the convenient type [30]. In our included studies, the bar type was the attachment type used for the removable dentures, due to its superiority in better force distribution amongst the implants. That is mainly due to its primary splinting effect, load splitting, better fit and the least after insertion maintenance [31].

When evaluating the end result of implant therapy, it is important to consider both the clinicians' and the patients' opinions [32]. For the clinician: implant success, prosthesis long-term durability, and the rate of complications are the most important measures. On the contrary, the patient's degree of satisfaction depends on aspects such as function, comfort, aesthetics, taste sensation, speech difficulties, and personal confidence [33]. Patient's preference may be the chief controlling factor for selecting the prosthesis design [34]. Dental implants studies generally target the success and failure from a biological point of view, whereas fewer investigations have been carried out on patient satisfaction [35].

More than one type of scale is used to measure patient's satisfaction. Out of those is the OHIP (Oral Health Impact Profile) which is meant to provide information about perceptions of oral health. However, the complete 49 item version is not always applicable in a clinical study because its time consuming .This led to the development of a simplified version, the OHIP-14 [36]. This questionnaire includes 14 items, two from each domain, selected because they have been shown to be the most frequently reported. The OHIP questionnaire includes seven main scopes [37]. Another type is the Visual analogue scales (VAS) and categorical scales (CAT) which are known to be predictable assessment tools [38]. VAS is frequently used to measure subjective perceptions, while CAT questions are used to collect information about the patients' physical, psychological function and general health. Patients are asked to choose a word from a four-point scale that best described their response. Our included studies used a VAS scale from 0 to 100, other authors have used VAS with scores from 0 to 10 or from 1 to 5 [39]. The need for multidimensional evaluation of implant therapy, using consistent instruments and valid tools when available, has been displayed in literature for many years [40].

According to Jacobs et al, complete edentulism can affect speech quality. That is interpreted by the absence of the periodontal ligaments which is responsible for speech sensation [41]. In our study, the ability to speak was for the favour of the removable group in two studies [20, 21]. That could be explained that the gap between soft

tissue and fixed prostheses is thought to be a dominant cause of speech flaws [15]. Removable overdentures are similar to the pattern of the complete dentures previously used by patients, whereas the bases of fixed prostheses are generally narrower, which could explain the cause of speech adaptation problems encountered by patients [20].

Significant results were observed for an ability to speak especially in the studies using the maxilla as the arch of interest [20, 21]. One must be aware that although rehabilitation for soft and hard tissue deficits can be adequately provided with fixed implant prostheses in the mandible, but in case of a resorbed maxilla, a prosthesis design may cause detrimental effects on phonetics [42] and with the fact that more sounds are produced with the tongue approximating the maxilla, than the mandible [43]. In the other two studies [18, 19], fixed restorations showed higher VAS scores for the ability to speak than that of the removable prosthesis. While it is well known that the slot between the soft tissue and prosthesis could cause speech problems, it's also documented that when the palate of a patient is covered, the pronunciation of consonants is often atypical, even after long periods of adaptation [44].

For the cleaning efficiency, higher VAS scores were favouring the removable group in two studies [19, 20]. Patients receiving oral implant therapy are usually well motivated to practice enough oral hygiene. But also they found that overdentures are to be more hygiene friendly than the fixed prostheses, which were described as very complicated to clean [45]. Moreover, it has been noted that an increase in the number of implants involves a greater hygiene difficulty, in comparison with the natural teeth and traditional dentures [46]. That was presented by Heydecke at al study [20] with the highest number of implants placed per arch compared to the rest of the studies.

Since the ability to clean the prosthesis had the most influence on choice for the removable group [19], it may aid in treatment planning if the clinician can determine which patients consider cleanliness as a crucial factor. After having worn conventional dentures for many years, candidates may be unconscious of the problems related t**o** preserving oral hygiene around implants [19].

A conflict in results was for the esthetics. In Heydecke et al study [20], where patients chose the removable solution that was mainly because of the lip support sustained by this type [47]. While for patients choosing the fixed solution as for better esthetics, possible reasons may include their dissatisfaction of flanges appearance of the overdenture, or excessive tooth display as a result of the additional bulk of components required to fabricate a long bar overdenture [47].

Stability and chewing ability had higher scores for the fixed prosthesis than the removable in the lower jaw. Patients often need a fixed prosthesis to feel teeth integrity as a part of their mouth which cannot be provided through a removable prosthesis. Such enhancement has a positive dramatic effect on improving masticatory efficiency [34]. Adding to that, its documented that full lower-jaw prostheses seem to provide lower satisfaction, probably due to the centrifugal resorption pattern of the mandible that affects the osteomucosal support of the residual ridge, which frequently results in flat ridges [48].

In our review, stability and chewing ability were linked together in their scores, where higher scores were observed for the fixed group in the mandible, while better scores for the removable group in the maxilla. A homogenous VAS scores for the ease of cleaning regardless the jaw under investigation were observed in favour of the removable overdenture. A controversy was noted for the esthetics & ability to speak results. For esthetics, higher scores were for the removable restoration in the maxilla, while for speaking ability, patients chose the fixed solution when mandible was tested, while a removable one when maxilla was the arch of interest [18-21].

Conflicting results in our systematic review is mainly due to lack of randomization in all of the included studies [18-21], outcomes vary obviously when true randomization takes place, rather than a patient-centred protocol, in the matter of directing patients to a specific treatment group. Follow up period wasn't enough to judge satisfaction scores, only two months were given for patients to test their perception. Only five studies were included in our review, which isn't a valid number to synthesise a definitive conclusion about the treatment options. In two studies [19, 21] only a single scale measurement (VAS) was used, while its recommended to use more than one assessment tool for more reliable outcome results. The degree of patient satisfaction is the result of a complicated interaction between psychological and physiological factors [49]. Even though, up till now, there is no any accurate scale or a questionnaire with items related to personal behavioural habits, which might be relevant for motivating the patient to shift their choices towards a specific prosthesis design, keeping in mind conservation of the oral tissues functions [50].

In conclusion, this study clarified that fixed prostheses showed higher scores in the mandible regarding stability, ability to chew, aesthetics and ability to speak. While removable prostheses showed higher scores in the maxilla. On the other hand, an ability to clean showed higher scores for the removable group in both mandible and maxilla. Unfortunately, this conclusion is based on a limited number of articles, indicating the need for more clinically controlled randomised studies.

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Medical History

Macedonian Medical Association – Seventy Years from Its Establishment, 1945-2015

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Abstract

AIM: To present the phases of development and activities over the 70-year period of existence and work of the Macedonian Medical Association, from its establishment in 1945 to 2015.

METHODS: A retrospective study based on available archive materials, encyclopaedias and other sources of information and reviews of the relevant literature, and personal experiences and observations of the author.

RESULTS: Macedonian Medical Association was established on August 12, 1945, with science and health educational mission and program. Dr Boris Spirov was elected as the first president of the Association, one of the main initiators and facilitators of activities in health care sector, including the establishment of the Faculty of Medicine in Skopje in March 1947. Over the past 70 years, the Association is the main carrier and has a key role and contribution in continuing medical education, vocational and scientific advancement of medical staff and improvement the dignity and reputation of the medical profession. The journal of the Association Macedonian Medical Review has contributed to spreading and advancement of knowledge and skills of modern medicine, as well as presenting professional and scientific achievements of physicians in the past 70 years. Macedonian Medical Association is a member of the World Medical Association and many other international associations and organisations contributing to international collaboration in education and science and promoting the Republic of Macedonia in Europe and worldwide.

CONCLUSION: Macedonian Medical Association over the 70-year period of its existence has been one of the pillars and lighthouse in the healthcare system in the Republic of Macedonia with great contribution to the advancement of medical and related sciences and continuing medical education, strengthening of health services and health care for the population and overall socio-economic development of the Republic of Macedonia during the past 70 years.

Introduction

Macedonian Medical Association (MMA) was established on August 12, 1945. In 2015 MMA celebrated its jubilee - 70 years of existence with a successful and fruitful work and versatile huge contribution to the development of the health system and health care of the population and overall social development in the Republic of Macedonia [1, 2]. Immediately after the World War II, the then People's Republic (PR) of Macedonia faced a very difficult economic, cultural and health status of the population. Besides devastated and ruined country, poverty and hunger in the exhausted predominantly agricultural population with cultural regression and poor economic conditions, the health system was underdeveloped with very weak human resources. At that time there were only 120 doctors, a few stomatologists and less than 100 high-school dentists and dental technicians, 96 pharmacists, for about 1,200,000 inhabitants in PR Macedonia. On average, there were 1 doctor and 1 nurse per 10,000 residents. The health condition of the population was very poor with neglected pathology, the unfavourable hygienic-epidemiological situation with many endemic spots and high morbidity and mortality from respiratory, diarrheal and other communicable diseases, especially in infants and young children [3-5]. Basic demographic and health

indicators of the population and network of health institutions and health workers in PR Macedonia, immediately after the World War II, are presented in Table 1.

Table 1: Basic demographic and health indicators of the population and network of health institutions and health workers in Macedonia in the period 1945-48 [6-10]

Demographic and vital statistics indicators:
• Total population in Macedonia (census 1948): 1,152,986; agricultural population 70.6%;
• Life expectancy (1948): males 45.9; females 48.2 years;
• Birth rate : 35 per 1,000 population;
• Mortality rate: 13.7 per 1,000 population (95% of the dead were not examined and treated by a doctor before the death);
• Population growth rate: 21.3 per 1,000 population; the average number of members per family 5.3.
Indicators of the health status of the population:
• Infant mortality rate (1948): 136.1 per 1,000 live births;
• 300.000 infected with malaria each year;
• 30.000 disease with active tuberculosis;
• High morbidity from typhus and para typhus, cholera and other diarrheal diseases;
• High morbidity from diphtheria, pertussis and other acute infectious diseases in children;
• High morbidity / mortality from anthrax, tetanus, rabies;
• High morbidity from skin and sexually transmissible diseases;
• High morbidity from goitre, nephropathy and other endemic diseases.
Structure of the network of health care facilities:
• Dispensaries: 3 (2 for children, one for TBC);
• Doctor's offices: 56 (50 general and specialist medicine, 6 for school-children);
• Dental offices: 7;
• Antimalarial stations: 18;
• Hospitals: 9; hospital beds: 868 / hospital beds per 1,000 population: 0.72;
• Public health institutes: 1.
Number of doctors and other health professionals:
• Doctors: 120/ 1 doctor per 10,000 inhabitants;
• Pharmacists: 96/ 1 pharmacist per 12,000 inhabitants;
• Nurses: 120/ 1 nurse per 10,000 inhabitants.

In such extremely unfavourable conditions, in 1945, the newly-formed government, with official authorities but an insufficient number of health personnel, entered the gigantic battle against diseases, poverty and centuries-long regression, as well as an affirmation of national identity and autonomy of PR Macedonia. Adverse conditions and problems had to be overcome, centuries to be bridged and PR Macedonia to become not only *de jure* but also *de facto* an equal member of the fraternal community of the Federal People's Republic of Yugoslavia. It was necessary to take urgent measures to establish organised health services for to improve the health status of the population and in-depth examinations of national pathology, particularly widespread infectious diseases as a health problem of the highest priority. One example of creating a foundation for organised work is the activity of uniting highly educated health personnel (doctors, dentists and pharmacists) in a professional association through the formation of Macedonian Medical Association [1, 3, 5].

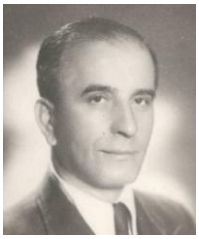
Establishment of the Macedonian Medical Association

In the postwar period with adverse health and social circumstances, but in the setting of emphasised enthusiasm and sincere desire of each person to help his people and country, the Macedonian Medical Association (MMA) was established as an association of doctors, dentists and pharmacists in PR Macedonia. The mission of MMA was to promote the medical and related sciences, to protect interests of doctors and to contribute to the growth and dignity of the medical profession, as well as development of the educational system for health professionals toward improvement the quality and accessibility of healthcare services and health status of the population. Pharmacists separated from MMA in July 1947 and dentists in June 2004 and thus formed their own special associations. Dr Boris Spirov was playing the key pioneering role as one of the leading initiators and founders of MMA [2, 3, 11, 12]. The general medical conference was held on August 12, 1945, and MMA was founded with science and health educational mission and program. Dr Boris Spirov was elected as the President of MMA, and Vice-president was Dr Done Miovski. In the first composition of the Board, there were elected 9 members, including doctors who later on became professors at the Faculty of Medicine in Skopje, i.e. Haralampie Mančev, Sterio Bozdov and Anton Čakmakov. In the years after the liberation, Dr Boris Spirov as a member and secretary of the ASNOM Presidium had great authority and served on many high state offices in the Government of PR Macedonia. As president of MMA, Dr Spirov was one of the main initiators and facilitators of activities in health care sector, including the establishment of the Faculty of Medicine in Skopje in March 1947. At that time he was elected professor of internal medicine and the history of medicine and was one of 19 teachers in the first group of the newly established faculty, most of them members of MMA.

Prof. Spirov was a man of vision, doctor humanist and tireless fighter, educator, enthusiast and organiser, who, along with members of the Board, coordinated the overall activities of MMA in the first years after its establishment. He led several important epidemiological studies of infectious and endemic diseases, especially malaria, typhus and goitre in Macedonia. Dr Boris Spirov completed his three-year term as the first president of MMA by organising the First Scientific Congress of Doctors of Macedonia in Skopje on 31.05.1948, with main topics on infectious diseases and child mortality in PR Macedonia.

The new president was elected, Dr Georgi Gavrilski, and Prof. Boris Spirov was elected as Editor-in-Chief of the MMA journal *Macedonian Medical Review*, which editorial function he performed in the period 1948-1950 [11-13]. All 25 presidents of the Macedonian Medical Association in the period 1945-2015, served entirely on a voluntary basis, are presented in Fig. 1.

The current president of MMA is Assist. Prof. Dr Goran Dimitrov from the Clinic of Gynecology and Obstetrics at the Faculty of Medicine in Skopje (Fig. 2).



1945-1948
Prof. Dr Boris Spirov
Internist

1948-1950
Prof. Dr Gjorgi Gavrilski
Epidemiologist

1951-1952
Prof. Dr Sterjo Bozdov
Hygiene Specialist

1952-1955
Prof. Dr Haralampie Manchev
Pediatrician

1955-1956
Prof. Dr Bozhidar Niketic
Neuropsychiatrist

1956-1957
Prim. Dr Ljubomir Savev
Pthysiologist

1958-1959
Prof. Dr Gligor Muratovski
Pthysiologist

1960-1961
Acad. Prof. Dr Pencho Davchev
Gastroenterologist

1962-1963
Prof. Dr Jonche Nedelkovski
Haemathologist

1963-1966
Colonel Dr Blagoja Petkovski
Internist

1967-1970
Prof. Dr Dushan Teodosievski
Paediatrician

1971-1972
Prof. Dr Borislav Karanfilski
Patophysiologist

1973-1977
Prof. Dr Stefan Stefanovski
Neuropsychiatrist

1978-1980
Colonel Dr Dushan Karanovski
Internist

1981-1982
Prof. Dr Predrag Ugrinski
Pathologist

1982-1983
Prof. Dr Kiril Velkov
Radiologist-onkologist

1983-1984
Colonel Prim. Dr Stevan Grdanoski
Epidemiologist

1984-1986
Acad. Prof. Dr Momir Polenakovic
Nephrologist

1986-1987
Prof. Dr Milan Jovanovski
Pathologist

1988
Dr Zhivko Velkovski
Neuropsychiatrist

1988-1991
Colonel Prim. Dr Kosta Sekulovski
Surgeon

1993-1998
Prim. Dr Petko Chadikovski
Dermatovenerologist

1998-2002
Prof. Dr Vitomir Micev
Neuropsychiatrist

2002-2003
Prof. Dr Ante Popovski
Neuropsychiatrist

2004-2015
Prof. Dr Jovan Tofoski
Gyncologist

Figure 1: Presidents of the Macedonian Medical Association in the period 1945-2015 [3, 5, 14]

Figure 2: President of the Macedonian Medical Association, Assist. Prof. Dr Goran Dimitrov (from the left side), with the president of the Doctors' Chamber of Macedonia, Prof. Dr Kocho Chakalaroski, at working meeting

Activities and contribution of MMA shortly after the World War II

Activities of MMA in the period of postwar years were directed to investigation of health behaviour and to the eradication of harmful vices of the people, the study of the causes of acute and chronic infectious and endemic diseases and medical-social problems. Special attention was devoted to building the Macedonian medical terminology and popularisation of medical science, an organisation of medical courses and lectures, scientific excursions and medical congresses, the formation of medical libraries and exchange of printed papers, books, magazines, pamphlets and other propaganda materials.

Thus MMA became the core factor and carrier of modern ideas about the organisation of health services in Macedonia, with an orientation towards professional and scientific work and continuing education of health personnel, who at that time were of enormous shortage and badly need for society [5, 15, 16]. In the period from March 1947 to April 1948, the medical units of MMA in Macedonia delivered 170 lectures for doctors and other health personnel and 10,039 popular lectures on health education of the population. Popular medical lectures about the priority health problems were delivered by practitioners in the framework of organised weekly shares, mostly to members of the Antifascist Front of Women (AFZH) and of the Red Cross of Macedonia, such as malaria and tuberculosis prevention and control, kids' week etc. Some of these lectures were broadcast via Radio Skopje [1, 5, 8, 9, 15, 16].

Educational activities and continuing medical education of doctors

The main mission of MMA is to improve the medical and related sciences, preserving and promoting the dignity and reputation of the medical profession and protect the interests of doctors. There is a wide spectrum with a continuous upward trend of the medical, educational and health promotional activities of MMA from the beginning of the newly created medical units of MMA (in Skopje, Bitola, Stip, Veles, Kumanovo, Ohrid, Strumica and Tetovo). Later on, with the increase in the total number of physicians and specialists throughout the 1950s and beyond, specialist sections and associations in MMA were established. Then a new period of intensifying the professional activities of MMA began. Over the past 70 years, MMA is the main carrier and has a key role and contribution in continuing medical education (CME), vocational and scientific advancement of medical staff and improvement of the medical profession. There were 18 congresses of all physicians and more than 70 congresses of specialist associations held, often with international participation, especially after the independence of Republic of Macedonia in 1991. The number of presented papers, printed in special collections of papers (proceedings) or supplements of *Macedonian Medical Review* exceeds the number of 13,000 articles. Specialist sections and associations have organised regular meetings with presentations and discussion about at least 3 professional themes, on average 4 times a year. In addition, most of the specialist associations practiced to organize additional professional and educational activities, such as specific schools (ultrasound, anesthesiology and resuscitation, allergology, health management, etc.) to improve the level of knowledge and skills of their members and other interested, first of all doctors who work at the level of primary health care [1, 4, 5].

Within the organisational activities related to CME, MMA has realised numerous activities. The most notable were the establishment of the Educational Centre for CME, production, translation and publication of several important manuals, tutorials, posters and other educational materials. MMA stressed the need to reactivate the CME centres in other cities besides already renovated centres in Gostivar, Prilep and Veles. Besides the wide range of activities of MMA in the health sector and health care facilities, important activities are taking part in numerous debates and discussions in the Ministry of Health and the Parliament about Health Strategy 2006-2015, an active participation in the adoption of the Law on Patients' Rights, the Law on Doctors, Law on additional work of the doctors, participating in the public debate on amendments to the Law on health care in 2013 and other legislative and policy documents. Special attention MMA devoted to the

preparation of professional guidelines for medical doctors for ensuring higher quality of medical services and more rational and efficient treatment of patients in accordance with the worldwide recognised approaches and evidence-based medicine [17]. MMA and the Section of Doctors of General Medicine had started an initiative and gained support for returning the name of specialisation of general medicine/family medicine [1, 5].

Publishing activities of MMA

Journal of MMA *Macedonian Medical Review* is the first professional scientific journal in the Macedonian language in the field of medicine, which starts to be published in July 1946. The journal is the foundation of the Macedonian medical professional and scientific publishing and debate on current issues in the field of prevention and epidemiology, diagnostics and therapy and a mirror of scientific research in the field of medicine in the Republic of Macedonia. The first issue of *Macedonian Medical Review* was designated as a "journal of medicine, veterinary medicine and pharmacy". Later on, it was transformed into a journal for medicine only, following the establishment of the Faculty of Dentistry and the strengthening of the Section of Dentistry in MMA in 1977 when they started with publishing the journal *Macedonian Dental Review*. About 5,000 peer-reviewed papers were published in *Macedonian Medical Review* by the year 2015. Papers were published in Macedonian with abstracts in English until 2010 when bilingual publishing was introduced. Thus, *Macedonian Medical Review* has contributed to spreading and advancement of knowledge and skills of modern medicine, as well as presenting professional and scientific achievements of physicians in the past 70 years. Today the journal is aimed at the general practitioners, specialists in different medical disciplines and researchers in the field of basic medical and related sciences. It is an open access journal publishing original papers, short communications on clinical and laboratory experiences, case reports, and experience from practice to practice, educational papers/reviews etc. [4, 18].

Since 2009 MMA started to publish a professional informative and educational newsletter entitled *The Doctors newsletter*. It is a monthly magazine for free distribution to 6,000 doctors in Macedonia. Within the period of six years, more than 70 issues were published with circulation over 400,000 copies presenting activities of the units and bodies of MMA, specialist sections and associations, as well as activities related to international cooperation [1, 2].

Social and professional activities and initiatives

After the World War II, a huge shortage of qualified and trained medical personnel in PR Macedonia was recognised. The need to start with an intensive education of medical professionals was perceived the earliest by the MMA. For the rapid development of health care and health care facilities, it was necessary to initiate the establishment of medical faculty as educational, medical and research institution in PR Macedonia. In 1946, the Government of PR Macedonia, besides numerous operational decisions, passed an important strategic decision with long-term significance for improving the health status of the population and development of the health service and health system in Macedonia - to make immediate preparations for the establishment of the Faculty of Medicine in Skopje. The first meeting of the Teachers Council was held on 17.03.1947 and that date marked the formation and the beginning of the real existence and academic activities of the Faculty of Medicine in Skopje. More than 17,000 students were enrolled and about 12,000 doctors graduated at the Faculty of Medicine till 2015 [3, 19-23].

MMA actively monitor the situation and changes in the health sector and participates as initiator and advisor in the creation and improvement of legislation in the field of healthcare and social protection, but also broader in the sphere of education and science, and other organised forms of professional and civic action in the overall social life. Members of MMA have been engaged in the state bodies for creating health policy and social development, in the political, cultural and sports life, in political parties, other professional and scientific institutions, civil society and free associations, as well as in the highest scientific institution - Macedonian Academy of Sciences and Arts [5, 24].

After the independence of the Republic of Macedonia in 1991, MMA has launched an initiative for the establishment of the Doctors' Chamber of Macedonia (DCM) as a professional institution with powers to perform control and assessment of knowledge and skills, to deal with accreditation and licensing of doctors for medical practice in the prevention and treatment under modern advances in medical science and evidence-based medicine. DCM was founded in June 1992, when the constituent assembly was held at the Military Hospital in Skopje. At the beginning, the members of DCM were medical doctors and doctors of dentistry. In 1995 dentists separated and formed a new special Dental Chamber of Macedonia. DCM is the successor of the Doctors' Chamber had existed in this region in the third decade of the last century in the then Kingdom of Yugoslavia. The seat of the Chamber, which covered the former "Vardar's Banovina", was in Skopje. All doctors and dentists were obliged for membership in the Chamber

in order to gain liability for public and private practice [25].

Among other things, under the operation of MMA as a contemporary medical association, several key documents were prepared i.e. the Statute of MMA, Rules for the operation of specialised and regional associations, Criteria for evaluation of the activities of associations, Rules for conducting elections etc. Recently, a decision for the establishment of the Academy of Medical Sciences (AMS) in MMA was adopted, which is to be realised in the near future.

Cooperation with other institutions and associations

The today the Republic of Macedonia has a highly developed medicine with a broad network of health institutions at all levels of the health system and healthcare delivery. According to the official data from the Institute of Public Health, the total number of doctors in 2015 was 5,975, employed in the public (3,803) and private health sector (2,182). All of them are members of the MMA. About 70% of doctors in the public sector and 47.7% of doctors in the private sector are specialists in various fields of medicine. They cover a wide range of activities involving over 100 specialities and subspecialties, with advanced equipment and superior expertise in their professions. Most of them have international experience and recognition. MMA successfully cooperates with the Ministry of Health, Faculty of Medicine and the Doctors' Chamber of Macedonia [1, 4, 5].

MMA membership is organised into 22 regional or local associations in larger cities in the Republic of Macedonia and more than 90 specialist and subspecialist sections and associations (58 specialist associations are members of the relevant international organisations, and some of them are founders of Balkan regional and South East Europe associations). MMA is a member of the World Medical Association (WMA), the European Forum of Medical Associations (EFMA), the World Health Organization (WHO), and Forum of the Medical Association of Southeast Europe (SEEFMA) etc. Through their specialist associations, MMA is a member of more than 70 international specialist associations. Through international collaboration and active participation of members to professional and scientific meetings abroad MMA contributes to the promotion of the Republic of Macedonia in Europe and worldwide and to advance its process of Euro-Atlantic integration [5, 18].

In conclusion, the establishment of MMA in 1945 as the core factor and transmitter of advanced pursuits in the organisation of health care services and improving the health status of the population was an exceptional event of crucial importance for the further development of educational, scientific and highly specialised health care activity in the Republic of Macedonia.

In the past 70 years, MMA asserts itself as a lighthouse and one of the pillars of the Macedonian health care system. MMA is a factor that gave and continues to give great contribution to the advancement of medical and related sciences and continuing medical education, safeguarding the dignity and reputation of the medical profession and protect the interests of doctors, as well as the development of health services and health care for the population in the Republic of Macedonia. Especially significant is contribution to the advancement of quality standards of health services and the application of good medical practice and evidence-based medicine. In a broader sense, MMA has had a continuous contribution to the overall socio-economic development of the Republic of Macedonia during the past 70 years.

Contribution and merits of MMA members are recognised and valued by granting numerous certificates, awards and honours, including the highest award of the medical profession - Charter "Dr Trifun Panovski," which is traditionally given to the most deserving practitioners every year during the celebration of World Health Day – 7th of April. In 1976 MMA received high federal recognition - the Order of Merit of the people with silver rays. On the occasion of 60th anniversary of existence, MMA received a prestigious national award in 2005 - the award "St. Kliment Ohridski". Also, MMA has won the state award "October 11" and the Prize of the City of Skopje "November 13" for outstanding contribution to the advancement of the organisation of health services and health education of the population, as well as successes in the training of professional staff in the Republic of Macedonia.

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Book Review

Jordan Minov. COPD and the Workplace. New York: Nova Science Publishers, Inc.; 2016. 83 pages; ISBN 978-1-63484-249-5

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Keywords: chronic obstructive pulmonary disease (COPD); workplace exposures; tobacco smoke; occupational exposures.

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Abstract

PURPOSE: The aim of this monograph is to present a role of the workplace exposures on the development and progression of chronic obstructive pulmonary disease (COPD), the joint effect of the workplace exposures and tobacco smoke in its development and progression, the diagnostics of the COPD related to occupational exposures, as well as its management and prevention.

CONTENTS: The publication consists of seven chapters supplemented by a list of abbreviations and index of terms. The cited literature at the end of the monograph obtains scientific support to the elaborated professional knowledge.

CONCLUSION: The monograph COPD and the Workplace presents a comprehensive literature dedicated to this problem and a serious effort for improvement of detection and management of COPD related to workplace exposures by medical professionals and its prevention.

Field of medicine: Respiratory medicine.

Audience: Occupational medicine specialists, pneumologists, internists, general practitioners.

Purpose: The aim of this monograph is to present a role of the workplace exposures on the development and progression of chronic obstructive pulmonary disease (COPD), the joint effect of the workplace exposures and tobacco smoke in its development and progression, the diagnostics of the COPD related to occupational exposures, as well as its management and prevention.

Content: The publication consists of seven chapters supplemented by a list of abbreviations and index of terms. The cited literature at the end of the monograph obtains scientific support to the elaborated professional knowledge.

Chronic obstructive pulmonary disease (COPD) is a major cause of disability and is the fourth leading cause of death throughout the world. Although the cigarette smoking is the major and the best-studied causative factor of COPD, there is consistent evidence that a substantial proportion of COPD cases can not be explained by smoking. Other noxious particles and gases, such as workplace dust, gases, vapours or fumes, indoor air pollution from burning biomass fuels from cooking and heating and urban outdoor air pollution are important risk factors of COPD. According to the actual knowledge, 15-20% of COPD cases are like to be caused or made worse by work, around 4,000 COPD deaths every year are related to workplace exposures and 40% of COPD patients are below retirement age and a quarter of those below retirement age are unable to work at all.

In the first chapter the key points related to COPD, including definition, epidemiology, burden, risk factors, pathogenesis, pathology, pathophysiology, clinical presentation, diagnosis and assessment, and

management, are presented.

The several ways by which the workplace exposures may influence the course of COPD, like causing COPD, modifying the effect of tobacco smoke in causing COPD, and accelerating the progression and severity of the disease in the subjects with established COPD, are explained in the second chapter.

In the third chapter, the workplace exposures as a causal factor of COPD are presented. There is sufficient epidemiological evidence from both population-based and workplace-based studies that workplace exposures to coal dust, silica dust, welding fumes, cadmium fume, cotton dust, agricultural dust and wood dust may be associated with the development of COPD in previously healthy subjects. Also, further evidence that majority of the workplace agents mentioned above are capable of inducing COPD (i.e. obstructive bronchiolitis and/or alveolar destruction) comes from experimental studies, particularly in those carried out in an animal model.

Across the world, cigarette smoking is the most encountered risk factor for COPD with a clear dose-response relationship. The combined effect of tobacco smoke and workplace exposures is explained in the fourth chapter. Results from many studies (both population-based and workplace-based) indicated that the joint effect of tobacco smoke and workplace exposures to dust, gases, vapours and/or fumes in the COPD development is greater than their additive effect.

Despite as a causing factor, workplace exposures may influence the course of COPD also as a factor that accelerates the rate of ventilatory function decline in the subjects with established COPD (Chapter 4). The existing evidence suggests that the workplace exposures in many professions (construction, mining, metallurgy, welding, textile manufacture, agriculture, etc.) may worsen the symptoms and lung function loss in the subjects suffering from COPD that is independent of smoking and ageing. As in the case when the workplace agents are causative factors of COPD, the mechanism underlying this effect still are not fully understood.

In the fifth chapter, the diagnostic workup for COPD-related to workplace exposures is presented. The diagnostics are based on standard diagnostic procedure and confirmation of the work-relatedness of the disease. The standard diagnostic procedure of COPD includes diagnostic tests recommended by the actual guidelines. As till today there is no diagnostic tool able to confirm the workplace exposure as a causative factor of the disease (in contrast to occupational asthma), the workplace reasons for persistent and progressive airflow limitation should be assessed by detailed occupational history, as well as by assessment of the patient's workplace (Chapter 6).

In the seventh chapter the actual principles of management and prevention of COPD related to workplace exposures are presented. The management of occupationally-related COPD includes pharmacological and non-pharmacological treatment of the disease, workability assessment and compensation due to disability caused by the disease. As occupationally-related COPD may be severely disabling disease, its prevention must be the primary tool for decreasing the incidence of morbidity, disability and mortality. Primary prevention (i.e. engineering control, personal protective equipment, education, etc.) should be designed to abate workplace hazards before any damage in the exposed workers has occurred. Secondary prevention addresses early detection of the disease and early intervention to minimise its severity and complications, whereas tertiary prevention aimed at treatment and rehabilitation of clinically manifested COPD.

Highlights: As the first publication of this kind in our country, the monograph gives a clear scientific impulse to the development of the professional knowledge related to COPD. Due to the connection of the actual scientific knowledge and specified practical experience, this book will certainly provoke attention to medical practitioners from different disciplines who encounter COPD in their everyday practice, thus becoming a real help in detection, management and prevention of occupationally-related COPD.

Conclusion: COPD represents one of the principal demands of the public health at global level due to high morbidity, early mortality and huge costs to health systems. Workplace exposures play a significant role in the development of the disease and its progression. On the other side, COPD related to workplace exposures, like other occupationally-related diseases, is potentially preventable. The monograph *COPD and the Workplace* presents a comprehensive literature dedicated to this problem and a serious effort for improvement of detection and management of COPD related to workplace exposures by medical professionals and its prevention.

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Letter to the Editor

Aortic Root Enlargement or Sutureless Valve Implantation?

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Abstract

Aortic valve replacement (AVR) in patients with a small aortic annulus is a challenging issue. The importance of prosthesis–patient mismatch (PPM) post aortic valve replacement (AVR) is controversial but has to be avoided. Many studies support the fact that PPM has a negative impact on short and long term survival. In order to avoid PPM, aortic root enlargement may be performed. Alternatively and keeping in mind that often some comorbidities are present in old patients with small aortic root, the Perceval S suturelles valve implantation could be a perfect solution. The Perceval sutureless bioprosthesis provides reasonable hemodynamic performance avoiding the PPM and providing the maximum of aortic orifice area. We would like to see in the near future the role of the aortic root enlargement techniques in the era of surgical implantation of the sutureless valve (SAVR) and the transcatheter valve implantation (TAVI).

We read with great interest the case report written by doctor Selman Dumani et al and we would like to congratulate them for their successful fourth redo procedure and for their publication in this valuable journal [1]. It is well known that in order to avoid the patient-prosthesis mishmash (PPM) there are some techniques like Manugian [2] and other surgical procedures. As referred in the international bibliography [3], stentless valve is another option. Recently the sutureless valves seem to be a good tool to avoid PPM [4]. The sutureless valves, like Perceval S aortic valve, are bioprosthetic valves indicated in old patients with comorbidities and small aortic root. We have used also the Perceval S valve in some ''difficult'' cases like in patient with achondroplastic Dwarf [5] and in porcelain aorta with great results.

Taken all these information about small aortic root, we would like to ask the authors about the use of a Perceval aortic valve in their patient. Their patient was young (52 years old) but high risk and without very long life expectancy. They did not refer at all the sutureless valves; is there any issue using this tool? Then, there is the valve-in-valve procedure in case of sutureless valve degeneration. Wat's the author's opinion about this dilemma? In our opinion, in the era of sutureless aortic valve and the trans-catheter techniques, in order to avoid a severe PPM we may take under consideration these options. Especially in high risk patients with comorbidities and small aortic root the Perceval S valve is the ideal solution. It minimizes the cross clump time, it is not necessary to work with the annulus because it is self-expanding without any suture in the aortic annulus. With the Perceval S valve implantation we achieve the maximum possible of the effective orifice area avoiding the PPM while we offer a brief intervention in patients with small aortic root [4].

We really would like to know the future of the surgical techniques of aortic root enlargement in the ''modern'' cardiac surgery and interventional cardiology era.

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