ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2019 Jan 15; 7(1):1-5.
https://doi.org/10.3889/oamjms.2019.006
eISSN: 1857-9655
Basic Science

Applying the Taguchi Method to the Optimization of Anticancer Activity of Bacterial Alginate-CuO Bionanocomposite

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Citation: Golshah A, Taran M, Safaei M, Mozaffari HR, Imani MM, Sharifi R, Moradpoor H, Upadhyay P. Applying the Taguchi Method to the Optimization of Anticancer Activity of Bacterial Alginate-CuO Bionanocomposite. Open Access Maced J Med Sci. 2019 Jan 15; 7(1):1-5. https://doi.org/10.3889/oamjms.2019.006

Keywords: Anticancer; CuO nanoparticles; Alginate; Nanocomposite; Taguchi method

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Received: 02-Oct-2018; **Revised:** 14-Nov-2018; **Accepted:** 15-Nov-2018; **Online first:** 20-Dec-2018

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

Abstract

AIM: In recent decades, despite various types of cancer inflicting many people worldwide, the existing therapies are not satisfactory and have many side effects. The present study was conducted to optimise the synthesis of novel alginate-CuO nanocomposite with utmost anticancer activity.

METHODS: In this study, 9 nanocomposites were designed using Taguchi method and three factors including copper oxide nanoparticles, alginate biopolymer and stirring times were assessed at three different levels. The anticancer activity of the synthesised nanocomposites was evaluated on the MCF-7 cell line using the MTT method. Using the Qulitek-4 software, we determined the optimum conditions for the synthesis of alginate-CuO nanocomposite with the highest anticancer activity.

RESULTS: The results indicated that all three factors (copper oxide, alginate and stirring time) were effective on the anticancer activity of the alginate-CuO nanocomposite. Also, the nanocomposite produced under the conditions of experiment 9 (8 mg/ml of copper oxide, 2 mg/ml of alginate and 60 min of stirring time) provided the highest growth inhibition rate as 75.63% against cancer cells.

CONCLUSION: The synthesised alginate-copper oxide nanocomposites in this study showed a significant anticancer effect. Therefore, the synthesised nanocomposite under optimal conditions can be used in the design of new anticancer drugs.

Introduction

Despite the synthesis of numerous new drug compounds in recent decades, no effective and definitive treatment has been still provided for some diseases such as autoimmune diseases [1], [2], microbial resistance [3], [4], AIDS [5], chronic pains [6], [7], [8] and cancer [9], [10]. Cancer is one of today's common diseases, which is caused by the uninhibited proliferation of cells. The number of people affected by different types of cancer and the resulting deaths is increasing every year [11], [12]. Some of the factors involved in increasing the risk of cancer include new lifestyles, increased consumption of unhealthy foods, increased elderly population, and tobacco and alcohol consumption [13]. Despite the prevalence of various types of cancer, the current anticancer compounds are not responsive and have some disadvantages and problems. Increased drug resistance, lack of distinction between cancer cells and normal cells, severe side effects and high costs

seem to be some of the current common anticancer drug problems. Hence, much effort is needed to find new anticancer compounds in the view of the increasing growth of this disease. The researchers have recently focused on the use of nanotechnology for the synthesis of various anticancer compounds and reported an optimal anticancer activity for a variety of nanoparticles.

Copper oxide nanoparticles have recently received much attention because of their biocompatibility and anticancer properties. Despite few studies reported in this regard, these studies have shown that copper oxide nanoparticles have a potential ability as an anticancer agent [14], [15]. In recent years, the use of metal nanoparticles in the structure of nanocomposites has expanded rapidly. Some of the advantages of employing nanoparticles in the form of nanocomposites include the easy control of the metals concentrations, low costs of the production process and most importantly the prevention of the agglomeration of nanoparticles. Different compounds are used as the substrate for the synthesis of nanocomposites. In this regard, polymers are one of the most important ones [16]. Due to biocompatibility and biodegradability, alginate biopolymer has come to the focus of attention for various biological applications. This biopolymer is a polysaccharide composed of two subunits of mannuronic acid and glucuronic acid [17]. Polymer alginate is usually synthesised in two forms; chemical and microbial. Microbial alginate can be synthesized by fungi and bacteria. Bacterial alginate is produced using *Azotobacter* and *Pseudomonas*. Also, bacterial biosynthesis of alginate can provide compounds with defined chemical structures and physical properties. Recent developments in the relative ease of modifying bacteria and regulating alginate biosynthesis in bacteria may empower the production of alginate with customised features and extensive biomedical applications [18].

Although much research has been done on the use of nanocomposites as antimicrobial agents, their anticancer properties have been less noticeable. The present study was conducted to use copper oxide nanoparticles in the form of alginate – copper oxides nanocomposite for the synthesis of novel nanocomposite with the most favourable anticancer activity using the Taguchi method.

Material and Methods

The copper oxide nanoparticles were synthesised using the coprecipitation method by an approach introduced in the previous study. Alginate biopolymer was synthesized using *Azotobacter vinelandii* IBRC 10786 prepared from Iranian Biological Resource Center [19].

Aimed at determining the optimal conditions for the synthesis of nanocomposites with the highest anticancer activity, 9 experiments were designed by Taguchi method with different amounts of biopolymer and nanoparticles at different stirring times using the Qulitek-4 software (Table 1). Thus, solutions of copper oxides nanoparticles (2, 4, and 8 mg/ml) and alginate biopolymer (0.5, 1, and 2 mg/ml) were prepared at 3 levels separately. The solution containing the nanoparticles was then added to the biopolymer solution drop by drop and combined with the in situ synthesis method at stirring times of 30, 60 and 90 min. After stirring, the solutions containing the nanocomposite were placed in a 40 °C oven as long as the white powder of the nanocomposite is formed.

The MTT method was used to determine the anticancer activity of 9 nanocomposites synthesised in this study. To do so, the cells of the Michigan Cancer Foundation-7 (MCF-7) line were cultured in the Dulbecco's Modified Eagle's Medium (DMEM) medium in the presence of 5% CO_2 and 95% moisture at 37°C. Then, 180 µl of cell suspension containing 5 × 10^4 cells were added to each well plate to be cultured for 24 h. About 20 µl of the synthesised nanocomposites in 9 experiments was added to each well, and the cells were treated for 72 h. After adding 20 µl of the MTT solution to each well and performing MTT test steps, the absorption rate of each plate was determined by ELISA reader. All experiments of this study included three tests carried out in triplicate. Finally, the inhibition rate of MCF-7 cancer cells growth by the studied nanocomposites was determined using the proposed relation [20].

$$\text{Cell growth inhibition } (\%) = \frac{\text{ODc} - \text{ODt}}{\text{ODc}} \times 100$$

ODt: optical density of treated cells

ODc: optical density of control cells

Results

Based on Taguchi method, nine nanocomposites were synthesised using factors including the content of copper oxide nanoparticles, alginate biopolymer and different stirring times. Synthesised nanocomposites were added to the MCF-7 cancer cells to identify the most potent anticancer compound (Table 1). The nanocomposite produced using 8 mg/ml of copper oxide, 2 mg/ml of alginate and the stirring time of 60 min (Test 9) showed the highest inhibitory growth rate of 75.63% against the cancer cells. The lowest inhibition of cell growth was related to the nanocomposite synthesized under the conditions of experiment 1 (2 mg/ml of copper oxide, 0.5 mg/ml of alginate, and the stirring time of 30 min), which was 42.62%.

Table 1: Taguchi design of experiments and results of anticancer activity of alginate-copper oxide bionanocomposites

Experiment	CuO (mg/ml)			Alginate (mg/ml)			Stirring time (min)			Cell growth inhibition (%)
	2	4	8	0.5	1	2	30	60	90	
1	2			0.5			30			42.62
2	2			1			60			55.39
3	2			2			90			46.11
4	4			0.5			60			66.38
5	4			1			90			72.30
6	4			2			30			68.36
7	8			0.5			90			55.14
8	8			1			30			70.42
9	8			2			60			75.63

The effects of the factors of copper oxide nanoparticles, alginate biopolymer and different stirring times at different levels on the growth inhibition rate of the MCF-7 cells by alginate-copper oxide nanocomposites are presented in Table 2. The second level in all three examined factors showed the highest effect in inhibiting the growth of cancer cells. The effects of factors, including copper oxide nanoparticles, alginate biopolymer and the stirring time in the structure of alginate-copper oxide nanocomposite on the inhibitory effect on the cancer cell growth were 69.01%, 66.04% and 65.80%, respectively.

Table 2: Effect of different levels of factors on the anticancer activity of alginate-copper oxide bionanocomposite

Factors	Level 1	Level 2	Level 3
CuO	48.04	69.01	67.06
Alginate	54.71	66.04	63.37
Stirring time	60.47	65.80	57.85

Table 3 shows the interactions among the studied factors (copper oxide nanoparticles, alginate biopolymer and the stirring time) at different levels. The third level of alginate biopolymer and the second level of the stirring time had the highest interaction effect on inhibiting the growth of cancer cells by a value of 58.57%. The copper oxide nanoparticles at the third level and the stirring time at the second level showed a significant interaction in inhibiting the cells growth by 22.34%. The interaction between the third levels of copper oxide nanoparticles and alginate biopolymer by 10.37% revealed the lowest severity of the effect on reducing the growth of MCF-7 cells.

Table 3: The interactions effects of studied factors on anticancer activity of alginate-copper oxide bionanocomposite

Interacting factor pairs	Column	Severity Index (%)	Optimum conditions
Alginate × Stirring time	2 × 3	58.75	[3, 2]
CuO × Stirring time	1 × 3	22.34	[3, 2]
CuO × Alginate	1 × 2	10.37	[3, 3]

Analysis of the variance of copper oxide nanoparticles, alginate biopolymer and the stirring time on the inhibition of growth of the MCF-7 cancer cells is shown in Table 4. Copper oxide nanoparticles (68.75%) and the stirring time (6.60%) showed the highest and lowest effect on inhibiting the cells growth, respectively. Alginate biopolymer was also effective in reducing the growth of cancer cells by 16.42%.

Table 4: Analysis of variance for anticancer activity of alginate-copper oxide bionanocomposite

Factors	DOF	Sum of Squares	Variance	F-Ratio (F)	Pure Sum	Per cent (%)
CuO	2	805.57	402.78	34.44	782.18	68.75
Alginate	2	210.23	105.11	8.99	186.84	16.42
Stirring time	2	98.49	49.25	4.21	75.10	6.60

The optimal conditions for the synthesis of alginate-copper oxide nanocomposites with the highest anticancer activity were predicted using the Taguchi method (Table 5). Based on the obtained results, the second levels of the factors of copper oxide nanoparticles, alginate biopolymer and the stirring time had the highest role in improving the inhibition of cancer cell growth by 7.64%, 4.66% and 4.43% contribution, respectively. Due to the mean growth inhibition rate of MCF-7 cells under different conditions (61.37%) and the reduced growth rate at optimal conditions (16.73%), it is expected that the growth of cancer cells will be inhibited as 78.10% under the optimal conditions recommended for the synthesis of nanocomposites.

Table 5: Prediction of optimal conditions for the synthesis of alginate-copper oxide bionanocomposite with maximum anticancer activity

Factors	Level	Contribution
CuO	2	7.64
Alginate	2	4.66
Stirring time	2	4.43
The total contribution from all factors		16.73
Current grand average of performance		61.37
Cell growth inhibition at optimum condition		78.10

Discussion

The results indicated that all synthesised nanocomposites had a desirable anticancer activity. Also, all the studied factors (copper oxide nanoparticles, alginate biopolymer and stirring time) were effective on the anticancer activity of alginate-copper oxide nanocomposite. In agreement with the results obtained, previous studies have also reported the anticancer activity of copper oxide nanoparticles and the nanocomposites containing those [21], [22], [23]. Jeronsia et al., [22] synthesised the copper oxide nanoparticles using the coprecipitation method and evaluated their anticancer activity at different levels against the MCF-7 cell line. According to their reports, with increasing dose, the anticancer activity of nanoparticles would increase.

The placement of nanoparticles in the nanocomposite structure improves their structural properties, prevents their agglomeration and increases their contact surface [19]. The sum of the factors mentioned above can be effective in improving the anticancer activity of nanocomposites. The mechanism of the effect of nanoparticles and the nanocomposites containing them on cancer cells and

how to differentiate them from other cells is not exactly known. In this regard, Azizi et al., [24] synthesised a biocompatible nanocomposite using albumin and copper oxide nanoparticles. The anticancer properties of synthesised nanocomposites against breast cancer cells were studied by MTT method. They indicated that anticancer activity of albumin-copper oxide nanocomposite is significantly improved compared to the copper oxide nanoparticles. Also, ROS production was significantly higher in nanocomposite treated cells.

ROS may provide an appropriate explanation for the selective toxicity of nanoparticles against cancer cells [25]. ROS and various signalling molecules are mainly found more in cells with a high proliferation such as cancerous cells because of their high metabolic rate compared to the normal cells. In the treatment of cancer cells, the nanoparticles react with chemicals and the signalling molecules surrounding them with a high rate, leading to the increased production of the ROS. By increasing the amount of ROS, the intensity of oxidative stress increases in the cell, which ultimately leads to apoptosis. However, ROS is also produced in the treatment of normal cells using the nanoparticles, but their production rate is relatively low in the normal cells compared to cancer cells since the normal cells have lower amounts of ROS and less signalling molecules. Therefore, the oxidative stress may not be sufficient to kill the cell, and as a result, they show a relatively low response to toxicity. Hence, the mentioned mechanism may be the main contributor to the selective toxicity of nanoparticles for cancer cells [26].

In this study, the Taguchi method was used to determine the optimum conditions for the synthesis of alginate-copper oxide nanocomposite with the highest anticancer ability considering its applied advantages in reducing the cost, time and the optimal predictive power. The findings of this study suggested that the alginate-copper oxide nanocomposite has an anticancer effect on the MCF-7 cancer cells at low concentrations. Moreover, these findings provided a new perspective on the use of nanocomposites in cancer treatment. Thus, they can be used to combat all types of cancers by performing more thorough studies. Also, animal and clinical studies of alginate-copper oxide nanocomposite and its mechanisms of action are recommended for further studies.

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ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2019 Jan 15; 7(1):6-11.
https://doi.org/10.3889/oamjms.2019.008
eISSN: 1857-9655
Basic Science

Association of Her-2 Expression and Clinicopathological Parameters in Colorectal Carcinoma in Indian Population

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Citation: Hasan R, Bhatt D, Khan S, Khan V, Verma AK, Anees A, Dev K.. Association of Her-2 Expression and Clinicopathological Parameters in Colorectal Carcinoma in Indian Population. Open Access Maced J Med Sci. 2019 Jan 15; 7(1):6-11. https://doi.org/10.3889/oamjms.2019.008

Keywords: HER-2/neu; IHC; Colorectal Cancer (CRC); Prognostic; Biomarker

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Received: 15-Sep-2018; **Revised:** 28-Oct-2018; **Accepted:** 30-Oct-2018; **Online first:** 22-Dec-2018

Funding: This work was funded by the University Grants Commission (U.G.C.), Govt. of India through MRP grant awarded to Kapil Dev (MRP-MAJOR-BIOT-2013-34536) vide F. No. 43- 84/2014(SR) dated 23rd 241 Sept. 2015

Competing Interests: The authors have declared that no competing interests exist

Abstract

BACKGROUND: Human epidermal growth factor receptor 2 (HER-2) is an oncogenic gene and a well-established therapeutic target in several cancers including breast and ovary.

AIM: The present study aimed to compare HER-2 expression status with histological grades as well as Clinicopathological parameters including age, bleeding per rectum, pain/burning sensation in defecation and exercise.

METHODS: Her-2 status was assessed by immunohistochemistry (IHC).

RESULTS: Results of the study shows that 40.96% patients were Her-2 positive for expression and a statistically significant difference (p-value = 0.004) was observed in histological grades where most of the cases were of grade II. We also observed a significant difference in histological grades with gender (p-value = 0.04), as well as in both the age groups ≤ 55 years and > 55 years (p-value = < 0.0001). Patients with the bleeding rectum and pain/burning sensation in defecation had grade II/III tumours (93.4%, 88.7%) respectively. A significant association was observed between bleeding per rectum and pain/burning sensation in defecation. About 95% of patients with pain/burning sensation in defecation had bleeding per rectum.

CONCLUSION: To conclude, Her-2 can be a potential prognostic marker in CRC. The role of age, tumour grade and bleeding per rectum/burning sensation in defecation are of significant worth. Thus, CRC cases of high grades can be screened for HER-2/neu positivity so that they can be subjected to mAb-based individualised therapy.

Introduction

Colorectal cancer is the third most widespread malignancy globally affecting both the genders, with an estimated 1.4 million new cases annually. It accounts for about 9.7% of all cancer [1]. In the earlier studies, it was observed that HER-2 is associated with tumorigenesis, metastases of disease and poor clinical prognosis. The ligand binding causes receptor dimerisation and passes signal by auto-phosphorylation of HER-2 tyrosine kinase domain and activates the target proteins, such as mTOR, Src, STAT, MAPK [2]. In tumour cells, the Her-2/neu gene is amplified in approximately 20% to 25% of all cases instead of having twin copies per cell; there may be as many as 50 or 100 c-erb-B2 gene copies per cell [3]. This gene amplification even results in overexpression of Her-2/neu at both the mRNA and protein levels; amounting approximately 2,000,000 Her-2/neu molecules in a single tumour cell, instead of the 20,000 to 50,000 molecules per cell found in normal cells. When Her-2/neu is overexpressed at these abnormally high levels, the kinase activity becomes constitutively activated possibly due to auto-activation of accumulating Her-2/neu molecules [4].

The overexpression of Her-2/neu protein on the cell membrane leads to hypersensitivity of the cancer cell to the growth factors. Thus, it acts as a proto-oncogene. The role of EGFR (HER-2) is well established in breast cancer pathogenesis and is also one of the most important prognostic marker and target for the gene related therapy in breast cancer management. HER-2 overexpression in malignant cells intensifies metastatic potency and invasiveness [5]. The role of HER-2 in carcinogenesis has been

established in a rodent model by both *In vitro* as well as *In vivo* ways by the formation of tumours upon HER-2 amplification. Some recent studies highlighted novel mechanisms of HER-2 regulation that can be targeted for the treatment of cancer [6], [7].

Thus, the present study aimed to evaluate the immunohistochemical expression of HER-2/neu and its correlation with histological grade as well as clinicopathological parameters in colorectal cancer. The outcome of the study will help in establishing Her-2/neu as a potent prognostic marker for CRC and further helps in the management of Her-2 positive cases of CRC with any single treatment regime solely based on Her-2 expression, which again directs us towards the need of individualised therapy. Secondly, the association of Her-2 expression with clinicopathological parameters will help in establishing a better diagnosis and understanding aetiology of the disease.

Material and Methods

The present study was conducted at the Medical Biotechnology Laboratory, Department of Biotechnology, Jamia Millia Islamia, New Delhi, India after ethical approval from the Institutional Ethics Committee (17/9/10/JMI/IEC/2015). In the present study 83 (Eighty-three) unrelated subjects of Indian origin were included which were histologically diagnosed with colorectal cancer.

A tissue biopsy/colectomy sample was collected from each patient after taking their consent, and their tissues were stored in 10% Buffered Formaldehyde (Merck) at room temperature for immunohistochemical analysis. The adjacent non-cancerous tissue of the biopsies was taken as control for the IHC analysis. For the study purpose, the relevant clinical history of each case was collected in the pretext perform for correlation with clinicopathological parameters.

The tissue biopsy was processed for making paraffin embedded blocks. Further, the thin sections of size (4 µm) from all cases were cut by using ultrathin microtomy. All the cases were routinely processed, and Haematoxylin & Eosin staining was done for diagnosis and histological grading. All these cases were graded into one of three histological grades using WHO criteria as Grade I/II/III (well/moderate/poorly differentiated) respectively. Following the routine histological examination, these cases were further studied for expression of proto-oncogene Her-2/neu using Immuno-histochemical stains.

The Immuno-histochemical stains were performed using Avidin-Biotin technique [8]. Thin sections of paraffin block were cut using microtome and taken on poly-L-lysine coated slides. The sections were deparaffinized in three changes of xylene for 5 minutes each. Three changes of acetone were given for 5 minutes each. The slides were then washed in running tap water for 2-5 minutes. The slides were then given three changes of P.B.S. buffer. 0.03% H_2O_2 was prepared in methanol, and then the slides were kept in this solution for 30 minutes on a shaker. The peroxide block was discarded, and then three changes of P.B.S. buffer was given. Antigen retrieval was done by the microwave oven method [9]. After antigen retrieval, blocking was done using BSA (Merck). Immunohistochemistry is the technique utilised for the study of localisation of antigens in tissue sections using labelled antibodies as specific reagents through antigen-antibody interactions. The primary antibody for Her-2/neu was, Purified mouse anti-human Monoclonal antibody from (Novacastra, USA); the secondary antibody, tertiary antibody and DAB used was from (Novacastra, USA)

Stained slides were evaluated independently. Whole tumour area was observed, and overall percentage positivity of tumour cells for Her-2/neu has counted under x 400 magnification. For Her-2/neu, membranous staining was taken as real positivity. Based on the percentage of positive cells, Her-2/neu cases were graded into one of three grades as follows: Grade I: < 30% tumour cells positive, Grade II: ≥ 30% tumour cells positive (showing membranous as well as cytoplasmic staining), Grade III: ≥ 30% tumour cells positive (showing only membranous staining).

The Image J program and it's IHC profiler plugin (based on colour deconvolution) was used for analysis of captured images. The results are in the form of a histogram depicting count and intensity peaks as well as a log file characterising the negativity/positivity, its magnitude and % categorical values accordingly.

Considering a large number of similar values in the categorical data analysis was done. The expression of HER-2 was divided into positive and negative cases. The differences between the groups were studied by Fischer's exact test and other appropriate algorithms. All the statistical analysis was done by using SPSS 16 software.

Results

In the present study, a total number of 83 cases of colorectal carcinoma were studied. The age range (43) of all the cases in the study was between 30 to 73 years. The mean age of cases in the study group was 55.9 years, while the median age was 56 years and mode age came out to be 54 years. So, we

divided patients into two groups ≤ 55 years and > 55 years of age (Table 1).

Table 1: Clinicopathological characteristics of patients

Gender	Age	Bleeding per Rectum	Pain/Burning Sensation in Defecation	Exercise
Variable: n (%) {Total samples-83}				
Male: 63 (75.9 %)	≤ 55 years: 38 (45.8 %)	Yes: 76 (91.6 %)	Yes: 80 (96.4%)	High: 10 (12.0 %)
Female: 20 (24.1 %)	> 55 years: 45 (54.2 %)	No: 7 (8.4 %)	No: 3 (3.6 %)	Low/Moderate: 73 (88.0 %)

A significant difference was observed in the distribution of histopathological tumour grades among genders (p = 0.04). In both genders, grade II a tumour was most common. CRC patients in ≤ 55 years of age group, were mostly in grade I (31.6%) and grade II (63.1%), only 5.3% patients were in grade III.

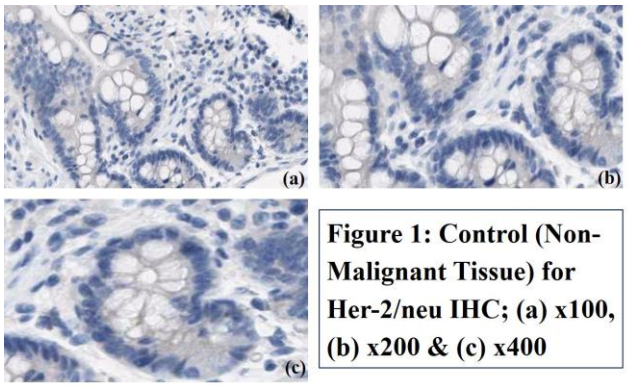

Figure 1: Control (Non-Malignant Tissue) for Her-2/neu IHC; (a) x100, (b) x200 & (c) x400

Patients in > 55 years of age group showed that patients were in grade II (64.4%) and III (35.5%) and the difference among age groups were found to be significant (p < 0.0001) (Table 2).

Table 2: Association of gender and age with histological grades in CRC patients

N = 83	Grade I	Grade I	Grade III	p-value
Male (63)	7 (11.1%)	39 (61.9%)	17 (27%)	0.04*
Female (20)	5 (25%)	14 (70%)	1 (5%)	
≤ 55 years (38)	12 (31.6%)	24 (63.1%)	2 (5.3%)	< 0.0001*
> 55 years (45)	0 (0%)	29 (64.4%)	16 (35.5%)	

It has been observed that cases which reported bleeding with rectum were either grade II/III tumours. However, patients who do not report bleeding were of grade I a tumour, and the difference among them was found to be significant (p < 0.0001).

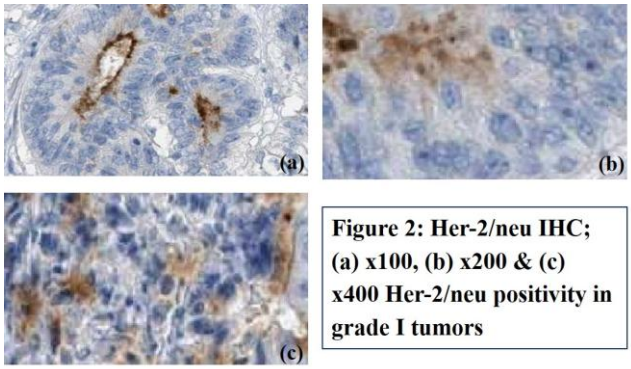

Figure 2: Her-2/neu IHC; (a) x100, (b) x200 & (c) x400 Her-2/neu positivity in grade I tumors

Patients who had burning sensation in defecation were either grade II/III (88.7%) however, patients who do not report burning sensation in defecation were of grade I a tumour (100%), and the difference among them was found to be significant (p = 0.002) (Table 3).

Table 3: Association of bleeding per rectum and pain/burning sensation in defecation with histopathological grades in CRC patients

Clinical status	Grade I	Grade II + Grade III	p-value
Bleeding per rectum			
Yes (76)	5 (6.6%)	71 (93.4%)	< 0.0001*
No (7)	7 (100%)	0 (0%)	
Pain/Burning Sensation in Defecation			
Yes (80)	9 (11.3%)	71 (88.7%)	0.002*
No (3)	3 (100%)	0 (0%)	

A significant association (p = 0.0004) was observed between burning sensation in defecation with bleeding per rectum.

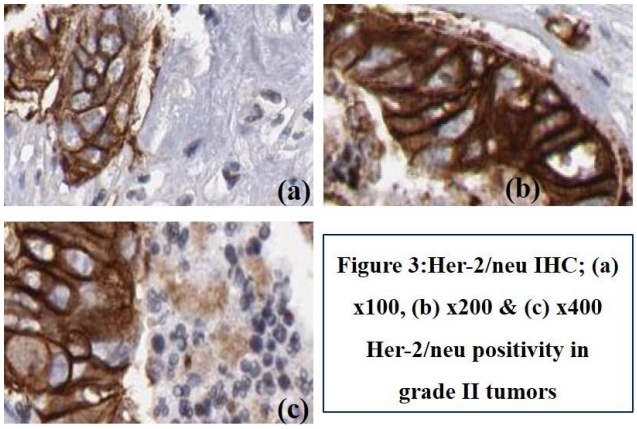

Figure 3:Her-2/neu IHC; (a) x100, (b) x200 & (c) x400 Her-2/neu positivity in grade II tumors

In the study group, it has been observed that most of the patients had bleeding per rectum (95%) with pain/burning sensation in defecation, only 5 % did not show any bleeding per rectum, however bleeding per rectum was also not observed in patients who did not show any pain/burning sensation in defecation (Table 4).

Table 4: Association of pain/burning sensation in defecation with bleeding per rectum in CRC patients

Clinical status	Pain/Burning Sensation in Defecation		p-value
Bleeding per rectum	Yes (n = 80)	No (n = 3)	
Yes (76)	76 (95%)	0 (0%)	0.0004*
No (7)	4 (5%)	3 (100%)	

CRC patients with a high level of exercise/physical activity showed no significant difference in the distribution of tumour grade in comparison to patients with low/moderate levels of exercise/physical activity but, it can also be seen that the number of patients with high level of activity (10/83, 12%) is very less in comparison to the patients with low/moderate level of physical activity (73/83, 88%). Though physical activity level didn't show any significant difference in the distribution of tumour grade, in the present study group, it was observed

that physical activity is helpful in preventing the onset of cancer as cited in previous research studies (Table 5).

Table 5: Association of exercise with histopathological grades in CRC patients

Exercise	Grade I	Grade II	Grade III	p-value
High (10)	1 (10%)	8 (80%)	1 (10%)	0.51
Low/Moderate (73)	11 (15.0%)	45 (61.6%)	17 (23.4%)	

Histological sections of 83 colorectal tumour cases were incubated with anti-Her-2/neu purified mouse anti-human monoclonal antibody. It was found that 49 (59.04%) cases failed to demonstrate any staining while the remaining 34 (40.96%) cases stained positive for Her-2/neu.

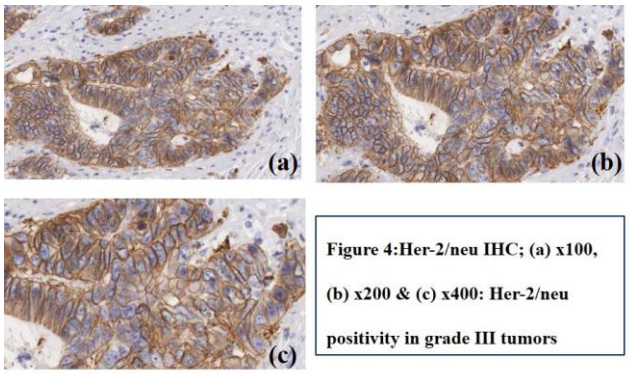

Figure 4:Her-2/neu IHC; (a) x100, (b) x200 & (c) x400: Her-2/neu positivity in grade III tumors

It was found that Her- 2/neu expression was positive in 2 (16.7%), 19 (35.9%) and 13 (72.2%) cases among grade I, grade II, and grade III tumours respectively and the differences were found to be significant (p = 0.004) (Table 6).

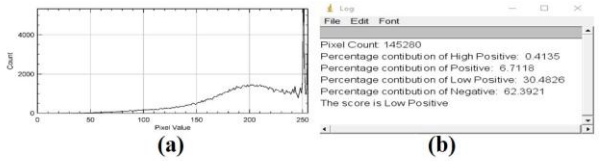

Figure 5.1: Grade I (IHC+) analysis from Image J program; (a) Histogram, (b) Log File

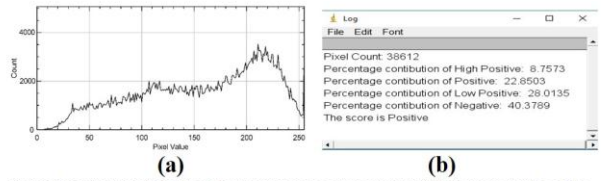

Figure 5.2: Grade II (IHC+) analysis from Image J program; (a) Histogram, (b) Log File

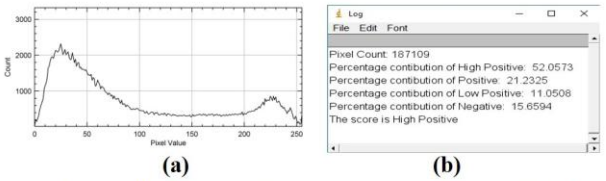

Figure 5.3: Grade III (IHC+) analysis from Image J program; (a) Histogram, (b) Log File

The Image J program and it's IHC profiler plugin (based on colour deconvolution) was used for analysis of captured images. The results are in the form of a histogram depicting count and intensity peaks as well as a log file characterising the negativity/positivity, its magnitude and % categorical values accordingly. Immunohistochemical expression observed was validated with Image J program and the results were found to be in concordance.

Table 6: Distribution of Her-2 immunohistochemical expression status among different tumour grades of CRC patients

Variables	Her-2 +ve (n = 34)	Her-2 -ve (n = 49)	P value
Histopathological grade			
Grade I (12)	2 (5.9%)	10 (20.4%)	
Grade II (53)	19 (55.9%)	34 (69.4%)	0.004*
Grade III (18)	13 (38.2%)	5 (10.2%)	

Discussion

Colorectal cancer is the third most common malignancy globally affecting both the sexes, with an estimated 1.4 million new cases annually. With global urbanisation and economic transition, adoption of western dietary habits and lifestyles and increasing life expectancy in developing nations, the incidence of colorectal cancer is rising steeply. An increasing trend in the incidence rates of Colorectal cancer in India has been reported from the various population-based studies.

Colorectal cancer is a multifactorial disease with various etiological and medical physiognomies. Age, personal history, dietary habits and lifestyle are the major risk factors for colorectal cancer. The chances of CRC diagnosis raises after 40 years of age, gradually increasing from age 40, rise steeply after 50 years of age [10].

In the present study, all 83 cases of colorectal carcinoma were distributed in the range of 30 to 73 years. In our study group, there were 38 cases in ≤ 55 years of age, and 45 were in > 55 years of age. Hence the prevalence rate showed a positive trend with an increase in the age of patients.

The mean, median, and mode age in our study come out to be 55.9, 56 and 54 years respectively. According to National cancer registry programme, hospital-based cancer registry, 2012 the mean age at diagnosis is 45 years in Indian population which is similar to mean age observed in developed nations of the west as stated by National Cancer Institute, USA. Over-expression of Her-2/neu has been notably associated with increased cellular survival, increased proliferation and decreased the apoptotic potential of cells leading to malignant transformation and maintenance of the associated malignancy [11]. A very scarce data is available particularly from Asian and south-east Asian region to indicate expression of Her-2/neu in patients with colorectal adenocarcinomas.

Overexpression of Her-2/neu in colorectal cancer shows a wide range of variability between 0-84% in different studies [12]. In neoplastic cells, dysregulation of the mentioned pathways and also increased expression of HER2/neu promotes tumour cell growth, progression and migration [13]. Overexpression of the HER2/neu receptor is detected in 25%-35% of breast cancer patients and has been known as an important prognostic and predictive factor [14]. In the present study, the overall expression rate of Her-2/neu was 40.96% which was confined mainly in the membrane with intermittent cytoplasmic localisation. This was in accordance to few studies which reported both membranous as well as cytoplasmic overexpression with rates streaking up to the tune of 60% [19]. On the contrary, most of the studies reported the rate of membranous overexpression between zero and 15% [17], [19]. According to histological grade, its expression varied from 16.66% (2/12) in grade I tumors to 72.22 % (13/18) in grade III tumors. Thus, Her-2/neu expression was positively related to histological grade, and the difference in expression rate across different grades was statistically significant p-value = 0.0045 (*, $p < 0.05$). Our results are in concordance with previous studies of [17]; these studies also showed very similar positive correlation between Her-2/neu expression and histological grades in their respective studies.

This positive correlation between Her-2/neu expression and histological grade accounts for the poor prognostic significance of Her-2/neu in case of colorectal carcinoma. Based on these findings only this gene has become a target for the successful management of CRC. Further, in the present study group, most of the cases reported bleeding per rectum and pain/burning sensation in defecation showed to have grade II/III a tumour and those who do not report was found to be in grade I a tumour. Hence, pain/burning sensation in defecation and bleeding per rectum are important indicators for high-grade CRC.

It has been reported that the (grade II) moderately differentiated carcinoma was the most common type and explained that the variation between different studies be related to the randomised selection and also small sample size [18], [20]. In clinicopathological parameters role of age, tumour grade and bleeding per rectum/burning sensation in defecation are significant in the diagnosis of CRC. Further, the Her-2 positive cases especially with high grades of CRC can be subjected to mAb-based individualised therapy targeting Her-2/neu. Therefore, we hypothesise that Her-2 may be a potent prognostic marker in CRC and can play a pivotal role in the better management of the disease.

Acknowledgement

The authors are indebted to all the study participants. Rameez Hasan would like to thank and acknowledge University Grants Commission (U.G.C.), Govt. of India and Ministry of Minority Affairs, Govt. of India for providing Maulana Azad National Fellowship (F1- 17.1/2013-14/MANF-2013-14-MUS-RAJ-20039 dated 6th Feb. 2014) to him.

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ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2019 Jan 15; 7(1):12-18.
https://doi.org/10.3889/oamjms.2019.009
eISSN: 1857-9655
Basic Science

Vancomycin MIC Distribution among Methicillin-Resistant *Staphylococcus Aureus*. Is Reduced Vancomycin Susceptibility Related To MIC Creep?

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Citation: Othman HB, Abdel Halim RM, Gomaa FAM, Amer MZ. Vancomycin MIC Distribution among Methicillin-Resistant Staphylococcus Aureus. Is Reduced Vancomycin Susceptibility Related To MIC Creep? Open Access Maced J Med Sci. 2019 Jan 15; 7(1):12-18. https://doi.org/10.3889/oamjms.2019.009

Keywords: VISA; h-VISA; Vancomycin screening agar; BMD; Vancomycin MIC creep

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Received: 07-Sep-2018; **Revised:** 29-Oct-2018; **Accepted:** 30-Oct-2018; **Online first:** 22-Dec-2018

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

Abstract

AIM: To determine the distribution of vancomycin MIC and the frequency of *S. aureus* strains with reduced vancomycin susceptibility among Methicillin-Resistant *Staphylococcus aureus* (MRSA) isolates.

METHODS: MRSA isolates (n = 100) were tested for reduced susceptibility to vancomycin using MIC broth microdilution method (BMD), vancomycin screening agar with different vancomycin concentrations with and without casein, and Vitek 2 system.

RESULTS: BMD detected (22%) vancomycin-intermediate *S. aureus* (VISA) and (78%) vancomycin-susceptible *S. aureus* (VSSA) but couldn't detect nine (Heterogeneous VISA) (hVISA) isolates (9%) with MIC ≤ 2 µg/ml that grew on screening agar 4 µg/ml or 6 µg/ml. Adding casein to vancomycin screening agar increased detection rate of VISA by 4.5%. Screening agar with 6 µg/ml vancomycin overall detection rate for VISA was 95.45%. Probable 'pre-hVISA'isolates (17%) showed growth on vancomycin screening agar 2 µg/ml with casein. Vitek 2 system failed to detect any VISA isolates.

CONCLUSION: Vancomycin screening agar; 2 µg/ml and (4 and 6 µg/ml) were able to detect; probable "pre hVISA and (hVISA and VISA) isolates respectively based on their BMD MIC values. Decreased vancomycin susceptibility in MRSA isolates might be related to MIC creep. Analysis of vancomycin MIC values over longer periods is recommended to further study this phenomenon and its impact on vancomycin treatment failure.

Introduction

Staphylococcus aureus is a virulent microorganism responsible for many serious infections among the general population. The emergence of vancomycin resistance in *S. aureus* has been anticipated since vancomycin-resistant enterococci (VRE) has been recognised. Hiramatsu et al. in 1997 described the first documented case of infection caused by *S. aureus* with reduced susceptibility to vancomycin [1].

Reduced susceptibility could be either due to strains that showed intermediate resistance to vancomycin with MIC 4-8 µg/mL [vancomycin intermediate *S. aureus* (VISA)] or heteroresistant strains (Heterogeneous VISA) (h-VISA) that are defined as strains with minimal inhibitory concentrations (MICs) within the susceptible range (MIC ≤ 2 µg/ml), but containing subpopulations of cells in the vancomycin-intermediate range (VISA, MIC 4-8 µg/ml) These strains have been described for both MRSA and methicillin-susceptible *S. aureus* (MSSA) respectively [2].

There has been special interest in vancomycin MIC creep phenomenon that was associated with greater rates of complications, and vancomycin therapeutic failures with vancomycin MICs within the susceptible range (MICs of 1-2 mg/L) [3], [4], [5]. Unfortunately, there has been uncertainty regarding optimal laboratory detection of *S. aureus* with reduced susceptibility to vancomycin [6].

Center for Disease Control and Prevention (CDC) in 2012 recommended that screening of VISA should be done by MIC method plus vancomycin screening agar method with 6ug of vancomycin per ml, but this is not reliable to detect all VISA; some strains for which the vancomycin MICs are 4 ug/ml will fail to grow [7], [8].

Screening for h-VISA by the population analysis profile-area under the curve (PAP-AUC) method has been the most reliable and reproducible approach but is labour-intensive, costly, and unsuitable for routine use in clinical laboratories [9], [10].

Moreover, standardized reference methods for susceptibility testing, such as CLSI broth microdilution, agar dilution, and standard Etest methods, can detect VISA but fail to detect h-VISA due to several factors as small inoculum size, the relatively poor support of growth on Mueller-Hinton agar plates, the slow growth of h-VISA strains and its unique pleomorphic features, such as small-colony variant [9], [11].

Satola et al. showed that the use of BHI screen agar with 4 µg/ml vancomycin with increase incubation time to 48 hours and the addition of casein could increase the sensitivity and specificity for detection of VISA and may be useful for clinical detection of h-VISA [12].

The study aimed to determine the distribution of vancomycin MIC, and the frequency of *S. aureus* strains with reduced vancomycin susceptibility among Methicillin-Resistant *Staphylococcus aureus* (MRSA) isolates.

Materials and Methods

The study included 100 MRSA isolates analysed by Kirby Bauer disc diffusion method [13] recovered from different specimens referred to Central Microbiology Laboratory of Ain Shams University Hospitals for routine culture and sensitivity. Isolates were collected throughout four months from January till April 2017 and were preserved on tryptone soy broth with 15% glycerol at -80°C until use.

Detection of MRSA with reduced susceptibility to vancomycin was performed using vancomycin screening agar with different vancomycin concentrations 2, 4, and 6 µg/ml with and without casein and compared to MIC broth microdilution method for vancomycin (BMD) [13]. Finally, MIC susceptibility testing for MRSA isolates was performed by Vitek 2 automated system (Biomerieux, France).

Brain heart infusion (BHI) agar without casein (Oxoid, UK) was prepared according to the manufacturer's instructions and BHI agar with casein was similarly prepared but with the addition of eight gram pancreatic digest of casein (Sigma Aldrich, USA) to every 500 ml of media [12]. A stock solution of vancomycin was prepared by dissolving 500 mg of vancomycin powder in 10 ml of sterile distilled water (final concentration was 50 mg/ml). At the time of media preparation, further dilution of 1:10 was done twice to produce a working solution of 0.5 mg/ml vancomycin. For the final preparation of vancomycin screening agar with and without casein; six ml, four ml and two ml of 500 ml prepared media were removed under complete aseptic precautions and replaced by 6 ml, 4 ml and 2 ml of working solution of vancomycin to prepare *Vancomycin screening agar* with 6 µg/ml, 4 µg/ml and 2 µg/ml respectively.

All isolates were subcultured by taking a small piece of a frozen organism with a sterile loop and plated twice onto blood agar plates. The used vial was returned immediately to the deep freezer to be used if needed as repeated thawing and re-freezing can reduce the viability of the organism. The cultivated plates were incubated aerobically at 35°C for 24 hours. Two to three colonies were picked up by the sterile loop and adjusted to 0.5 McFarland standards in 5 ml sterile tubes. Quadruplicate technique was performed (i.e. four droplets, 10ul each, from 0.5 McFarland MRSA suspension, was dropped by a pipette onto the 2, 4, and 6 vancomycin screening agars) and the plates were incubation for a full 48 hours at 35°C to enhance the sensitivity of detection of MRSA with vancomycin reduced susceptibility [12]. Plates were examined at 24 and 48 hours. Vancomycin-Resistant *Enterococcus Faecalis* ATCC 51299 and *Staphylococcus aureus* ATCC 25923 (NAMRU-3) were used as positive and negative controls respectively.

No growth in any of the four droplets was denoted as sensitive to vancomycin. Growth in any of the four droplets was considered as MRSA with vancomycin reduced susceptibility.

The broth microdilution method was used for determination of the MICs of vancomycin [14]. Vancomycin suspension used was prepared by dissolving 500 mg of vancomycin powder in 10 ml of sterile distilled water (50 mg/ml), then further dilution 1:10 was done twice (0.5 mg/ml). From the prepared dilution, 640 µl was added to 10 ml of D.W to reach a final concentration of 32 µg/ml vancomycin.

Serial two-fold dilution of the prepared vancomycin concentration was carried in a 96 well plate. Fifty microliters of double-strength Muller Hinton Broth (MHB), 50 µl of the antibiotic dilutions, and (5 µl of the organism suspension adjusted to 0.5 McFarland standards and then diluted 1:20) were mixed and incubated at 35°C for 24 hours. MICs; ≤ 2 µg/ml is considered as sensitive, 4-8 µg/ml as VISA and ≥ 16 µg/ml as vancomycin resistant *S. aureus* (VRSA) (Figure 1).

Susceptibility testing on Vitek 2 system was performed with AST PG 76 cards according to the manufacturer's instructions, and susceptibility breakpoints of *Staphylococcus aureus* were interpreted per CLSI 2015 [13].

Results

Broth microdilution (BMD) method (Figure 1) revealed that, out of 100 MRSA isolates, 22/100 (22%) were VISA (14/22 VISA with MIC = 8 µg/ml and 8/22 VISA with MIC = 4 µg/ml) and 78/100 (78%) were VSSA (VSSA MIC ≤ 2 µg/ml).

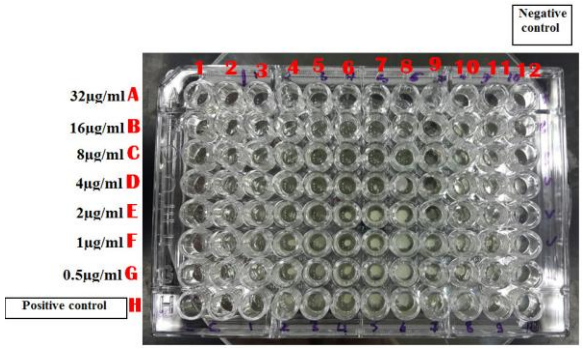

Figure 1: Broth microdilution plate for detection of vancomycin susceptibility in MRSA isolates; A12 to G12 are negative growth control wells. H1 to H11 wells are positive growth control wells. MIC of vancomycin for isolate; 4 (well E4) is 2 µg/ml, 5 (well E5) is 2 µg/ml (VSSA), 6 (well D6) is 4 µg/ml and for isolate 8 (well C8) is 8 µg/ml (VISA)

In vancomycin screening agar method, h-VISA was reported if one or two colonies on at least one droplet showed growth on screening agar with 4 µg/ml or 6 µg/ml [12]. Among MRSA isolates that showed MIC ≤ 2 µg/ml by BMD; 9 isolates (9%) grew on screening agar 4 µg/ml or 6 µg/ml and were designated as h-VISA (Figure 2).

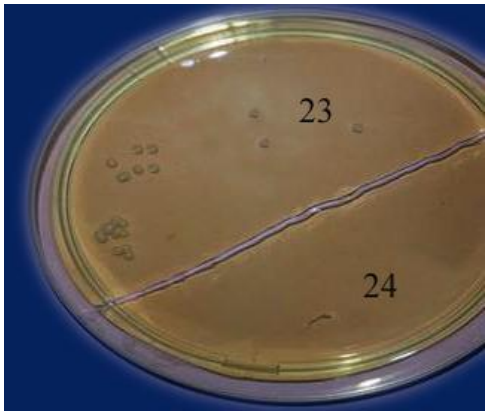

Figure 2: Vancomycin screening agar 6 µg/ml with casein; isolate [23] shows the growth of hVISA. Isolate [24] shows no growth (VSSA)

Seventeen isolates with susceptible MIC by BMD (17%) showed growth on vancomycin screening agar 2 µg/ml, six out of them with MIC of 2 µg/ml by BMD. These isolates were considered as probable 'pre-hVISA', which represent small subpopulations of cells capable of growth in the presence of 2-4 mg/L vancomycin [14] (Table 1 and Figure 3).

Table 1: Detection rate of MRSA with reduced susceptibility to vancomycin among 100 tested isolates

Vancomycin screening Agar	BMD MIC	
	VSSA (MIC ≤ 2 µg/ml)	VISA (MIC 4-8)
No growth	52 (52%)	0
2 µg/ml (probable pre-hVISA)	17 (17%)	0
4 µg/ml or 6 µg/ml	9 (9%) hVISA	22 (22%)
Total	78 (78%)	22 (22%)

All of the results of screening agar with and without casein were similar except for two isolates; one isolate showed growth on screening agar with 4 µg/ml, with casein but not in that without casein, and one more isolate grew on screening agar 2 µg/ml without casein only after 48 hours. So, adding casein to vancomycin screening agar increased detection rate of VISA by 4.5% (only one VISA out of 22). Screening agar with 6 µg/ml vancomycin (with and without casein showed similar results) detected 7 out of 8 VISA with BMD MIC equal to 4 µg/ml (87.5%) and 14 out of 14 with BMD MIC equal to 8 µg/ml (100%), with overall detection rate of VISA 95.45% (Table 2).

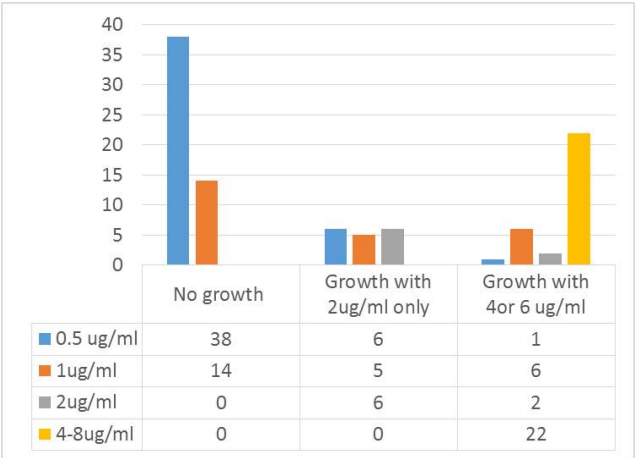

Figure 3: Results of Broth microdilution and vancomycin screening agar among 100 tested isolates

Increasing incubation time did not increase the detection rate for vancomycin with reduced susceptibility among screening agar with casein and only affected one isolate grown on 2 µg/ml screening agar without casein (Table 2).

Table 2 shows vancomycin MIC results using Broth microdilution and vancomycin screening agar. It is noted that Broth microdilution method was not able to detect nine (9) h-VISA isolates.

Table 2: Vancomycin MIC results using Broth microdilution and vancomycin screening agar for detection of MRSA with reduced susceptibility to vancomycin

	BMD MIC	Vancomycin screening agar MIC						
		No growth (< 2 µg/ml) with and without casein	> 2 µg/ml (< 4 µg/ml)		> 4 µg/ml-< 6 µg/ml		> 6 µg/ml	
			With casein*	Without casein	With* casein	Without* casein	With* casein	Without* casein
Sensitive	MIC ≤ 1	52	11	10 + 1●	0	0	7	7
	MIC 2	0	6	6*	0	0	2	2
Interme-diate	MIC 4	0	0	0	1	0	7	7
	MIC 8	0	0	0	0	0	14	14
Total		52	17	16 + 1●	1	0	30	30

*No difference between 24 and 48 hours; ●the Only one showed no growth at 24 hours but detected at 48h.

All of MRSA isolates (100%) were susceptible for both vancomycin and linezolid by VITEK 2 system. (Figure 4) shows the result of susceptibility testing for MRSA isolates on the Vitek 2 system.

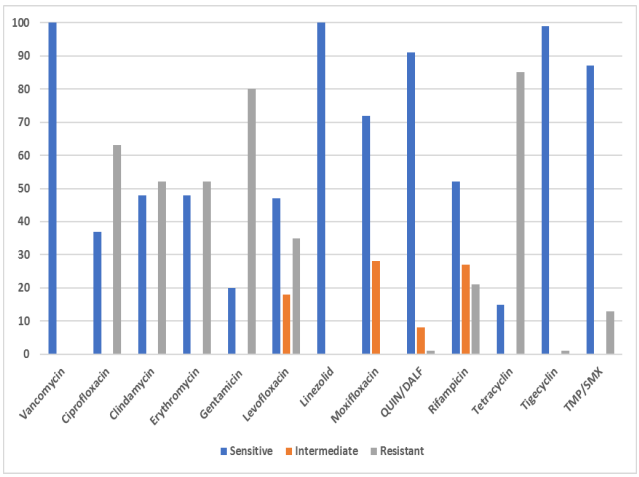

Figure 4: Result of susceptibility testing for 100 MRSA isolates using AST GP 76 cards on Vitek 2 system [14]

Discussion

Overuse of vancomycin has led to the development of a selective pressure over time with the result of the emergence of *S. aureus* with reduced vancomycin susceptibility. The emergence of MRSA with reduced susceptibility to vancomycin is worrisome as the available drugs for MRSA treatment are limited [6].

We report 22% VISA isolates by broth microdilution method. Vaudaux et al. reported that Broth microdilution assay led to under detection of the vancomycin-intermediate *S. aureus* VISA) phenotype, yielding only three VISA isolates, for which vancomycin MICs were 4 µg/ml compared to 8 and 19 VISA isolates detected by microdilution and agar testing, respectively [14].

In the present study, among the MRSA isolates that showed MIC less than or equal to 2 µg/ml by BMD; (9%) of h-VISA isolates showed growth on screening agar 4 µg/ml or 6 µg/ml. Whereas, (17%) of isolates with susceptible MIC by BMD showed growth on vancomycin screening agar 2 µg/ml (probable 'pre-hVISA') [15]. The pre-hVISA strains may be correlated with the 'MIC creep' phenomenon observed in hospitals where anti-MRSA chemotherapy is frequently implemented [15].

Lodise et al. observed that patients with MRSA bloodstream infections with elevated vancomycin MICs but within the susceptible range (≥ 1.5 mg/mL) had higher probabilities of recurrent bacteremia and longer hospital stays [16]. Sakoulas et al. reported that the likelihood of treatment success is significantly lower in patients with MRSA isolate with a vancomycin MIC of 1-2 mg/mL compared with patients infected by isolates with a vancomycin MIC ≤ 1.5 mg/Ml [17]. Edwards et al. suggested lowering vancomycin breakpoints further, to avoid clinical failure and the increased risk of mortality [4].

Satola et al., tested 140 MRSA blood isolates with vancomycin MICs 2 µg/ml by reference broth microdilution and screened for reduced susceptibility to vancomycin using PAP-AUC as the reference method, where they detected 15% h-VISA. They evaluated brain heart infusion (BHI) screen agar containing 16 g/liter casein and 4 mg/liter vancomycin for the detection of h-VISA, revealing 90% and 95% sensitivity and specificity with a 0.5 McFarland inoculum and 100% and 68% sensitivity and specificity with a 2.0 McFarland inoculum respectively [12].

In the present work, adding casein to vancomycin screening agar increased detection rate of VISA by 4.5% (only one VISA out of 22). The base medium of the screening agar might be as important as the vancomycin concentration. Enhancement of detection of h-VISA by screen agar methods could be obtained by the addition of supplements to the agar. Willey et al. reported that the addition of pancreatic digest of casein to BHI agar and 4 g/ml vancomycin improved the detection of VISA on screen agars, as 97.7% of VISA strains in their study were successfully detected with high specificity within 24 h [18]. Other supplement suggested differentiating between h-VISA and VSSA was the addition of 20% horse serum to BHI [19].

Riederer et al., tested 485 MRSA blood isolates with vancomycin MICs 0.5 to 4 µg/ml using BHI-V3, BHI-V4 and other methods. The modified PAP/AUC was measured for all isolates revealing seven VISA and 33 h-VISA phenotypes. The sensitivity and specificity for detecting VISA were 100% and 94.6% for BHI-V3, 100% and 99.2%, for BHI-V4 respectively [20]. These observations differ from those of Burnham et al., who reported 100%

sensitivity and 65% specificity for detecting VISA with BHI-V3 [21]. The reason for the difference is unclear but might be related to isolates selection as Burnham et al., selected their isolates based on MIC results and did not perform PAP/AUC [21].

In the present study, screening agar with 6 µg/ml vancomycin detected 7 out of 8 VISA with BMD MIC equal to 4 µg/ml (87.5%) with overall detection rate of VISA 95.45%. CDC 2015 stated that growth of more than one colony on screening agar with 6 µg/ml vancomycin is considered a positive result for VISA [13]. All *S. aureus* isolates for which the vancomycin MIC ≥ 8 µg/ml grow on these plates and some isolates for which the vancomycin MIC = 4 µg/ml will also grow [22].

As a vancomycin MIC of 4 to 8 µg/ml is considered an intermediate susceptibility, the use of an agar medium such as BHI-V6 as a means to screen for vancomycin-intermediate strains of *S. aureus* (VISA) is not adequate for this purpose, as those strains having a vancomycin MIC greater than 2 but less than 6 µg/ml could not be detected by this method [23].

Swenson et al. reported that BHI-V6 agar failed to detect 33% (12 of 36) of VISA isolates with MIC 4 µg/ml [24]. Similarly, Walsh et al. reported low sensitivity (22%) for the agar screening method using brain heart infusion agar (6 mg of vancomycin per litre), and 97% specificity [23].

In the present study, the VITEK 2 system failed to detect any isolates with reduced susceptibility to vancomycin. Swenson et al. reported that the Vitek 2 system tended to categorise VISA isolates as susceptible [24]. This was justified by Edwards et al., who demonstrated that MICs from automated systems and the E-test were significantly lower after cryopreservation if compared with those from the E-test analysis, at the time of isolation [4]. Also, Mason et al. pointed out that the prevalence of vancomycin MIC creeps may be underestimated because of the cryopreservation effect [25].

On the other hand, the study performed by Burnham et al., showed that Vitek2 using card GP67 had the worst sensitivity (7.7%), detecting only one out of the 13 VISA isolates compared to Microscan system which had the highest sensitivity (92%), followed by Etest (85% sensitive) and then Sensititre (54% sensitive). Thereby, they suggested that laboratories using the GP67 AST card for vancomycin susceptibility testing of *S. aureus* should consider additional testing to rule out VISA when MIC 2 µg/ml is generated and/or the concomitant use of a screening medium such as BHI-V3 to ensure detection of VISA isolates [21]. Also, Kruzel et al. stated that it became evident that the automated susceptibility testing methods are inappropriate for the detection of VISA [26].

All of our MRSA isolates were susceptible to vancomycin using VITEK 2 system. They were also sensitive to linezolid (100%) followed by tigecycline (99%) then Quinupristin-dalfopristin (91%). A study by Cook et al. described the successful treatment of a ventriculoperitoneal shunt infection caused by h-VISA with linezolid due to its tolerability and excellent blood-brain barrier penetration [27]. High-dose of Quinupristin-dalfopristin (Synercid) significantly reduced the number of bacteria detected in the VISA hematogenous infection in murine models [28].

Since the first reports of hVISA/VISA, their prevalence differed among geographic regions: the incidence of h-VISA was 6.81% in Asia and 5.60% in Europe/America, and that of VISA was 3.42% and 2.75%, respectively. Several factors may be responsible for such condition; i) high public hygiene standards and meticulous antimicrobial treatments in most European and American countries [29], [30], [31], ii) the control of nosocomial infections is more successful in European and American countries [32, 33], iii) Asia is the most populous region of the world, susceptible to microbial transmission, and iv) more MRSA infections occur in Asian countries [34].

In the present work, Vancomycin screening agar; 2 µg/ml and (4 and 6 µg/ml) were able to detect; probable “pre hVISA and (hVISA and VISA) isolates respectively based on their broth microdilution MIC values. We believe that decreased vancomycin susceptibility in MRSA isolates might be related to MIC creep, but we could not indicate this phenomenon since an earlier data from our lab was not available for comparison. Similar factors as that found in Asia could be responsible for the occurrence of MRSA with reduced susceptibility to vancomycin in our country. Further studies on a large scale are needed to determine the prevalence of VISA and h-VISA and also to study the phenomenon of vancomycin MIC creep.

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ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2019 Jan 15; 7(1):19-23.
https://doi.org/10.3889/oamjms.2019.007
eISSN: 1857-9655
Basic Science

Assessment of Density of Neovascularization in Lower Lip Squamous Cell Carcinoma in Relation To Neoplasm Differentiation Grade in Patients with and without Neck Lymph Nodes Metastasis

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Citation: Kakasheva-Mazhenkovska L, Kostovski M, Gjokik G, Janevska V. Assessment of Density of Neovascularization in Lower Lip Squamous Cell Carcinoma in Relation To Neoplasm Differentiation Grade in Patients with and without Neck Lymph Nodes Metastasis. Open Access Maced J Med Sci. 2019 Jan 15; 7(1):19-23. https://doi.org/10.3889/oamjms.2019.007

Keywords: Squamous cell carcinoma; Lower lip; Neoangiogenesis; Grade of differentiation; Neck metastasis

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Received: 09-Nov-2018; **Revised:** 17-Dec-2018; **Accepted:** 18-Dec-2018; **Online first:** 03-Jan-2019

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

Abstract

BACKGROUND: Squamous cell carcinoma (SCC) of the lower lip is a relatively rare carcinoma, with the incidence of 1 to 2%, but it is the most common carcinoma in the oral cavity accounting for 25-30% of all malignant oral tumours.

AIM: This study aimed to determine the role of neovascularisation in the process of tumour progression.

METHODS: We analysed the surgical specimens obtained from 60 patients with squamous cell carcinoma (SCC) of the lower lip. The examined group consisted of 45 specimens of patients without metastasis and 15 specimens of patients with metastasis in the regional lymph nodes. Histopathological slides were immunohistochemically stained with an antibody against CD34 and by hematoxylin & eosin staining for routine histopathological examination.

RESULTS: The results obtained showed a statistically significant difference in the density of neovascularisation between groups of the SCC with different grade of differentiation (Kruskal-Wallis test: $H(2, N = 60) = 30.0943$, $p = 0.00001$). Statistical analysis also showed a significant difference in the density of vascularisation of lower lip SCC between patients without metastasis and patients with neck metastasis (Mann-Whitney U test, $p = 0.000198$). Applying Pearson's chi-square test, we found a highly significant statistical difference in grade of SCC differentiation in patients with and without neck metastasis ($p = 0.0000$).

CONCLUSION: In conclusion, the density of neoangiogenesis is increased in tumours with poorer differentiation and in patients with neck metastasis. So, the density of neovascularisation of the primary lip SCC may predict the tumour progression.

Introduction

Squamous cell carcinoma (SCC) of the lower lip is a relatively rare carcinoma, which incidence ranges between 1% and 2%, but it is the most common carcinoma in the oral cavity accounting for approximately 25-30% of all malignant oral tumours. Over the last years, an increasing tendency of this carcinoma has been observed [1], [2], [3].

Metastases in the neck lymph nodes from SCC of the lower lip are found in less than 20% of patients, and they are more frequent in patients with worse neoplasm differentiation. In patients with smaller tumours (pT1 and pT2) at the moment of diagnosis, metastases are present in about 8% [4], [5], [6], [7].

The density of the newly formed blood vessels from the preexisting capillaries (neoangiogenesis), has been examined in squamous cell carcinoma in different locations of the body by many authors. Neoangiogenesis as a parameter can be a good indicator of tumour progression or its aggressiveness, and consequently of disease prognosis [8], [9], [10], [11].

This study aimed to determine the density of neovascularization at the invasive front in lower lip squamous cell carcinomas in patients with and without

neck metastasis about the grade of neoplasm histological differentiation. We also aimed to determine the role of neovascularization in the process of tumour progression, that is, in the onset of neck metastasis.

Material and Methods

The study included surgical specimens obtained from 60 patients with squamous cell carcinoma of the lower lip, who underwent surgical treatment at the University Clinic of Maxillofacial Surgery in Skopje. This was a retrospective study, for which purposes the archival material from the Institute of Pathology was retrieved, paraffin blocks, histopathological slides and histopathological reports were used. Specimens were histopathologically analysed at the Institute of Pathology, Medical Faculty in Skopje.

The examined group consisted of 60 specimens of patients with squamous cell carcinoma of the lower lip, of which 45 specimens of patients without regional lymph nodes metastasis and 15 specimens of patients with regional lymph nodes metastasis (Figure 1 and 2).

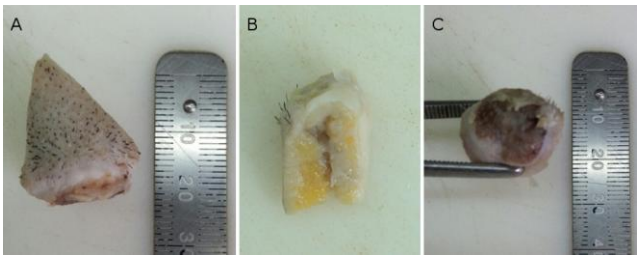

Figure 1: a) Lower lip squamous cell carcinoma. Triangle-shaped excision with central ulceration and crust over it; b) Side view of the specimen; c) another specimen of SCC with central ulceration

The density of the blood vessels (neoangiogenesis) in the stroma of the invasive front of the squamous cell carcinoma was determined on histological sections from both, the examined and the control group of specimens. From the resection margins of every triangle surgical excisions (60 cases) with low lip carcinoma, a specimen of non-tumour tissue (normal healthy tissue) was taken for the control group.

Immunohistochemical staining was performed with the standard procedure used at the Institute of Pathology, Medical Faculty in Skopje, by using Immunoperoxidase LSAB + system and specific primary monoclonal antibodies against CD 34 DAKO, Monoclonal Mouse Anti-Human CD 34 Classe II Clon QBEnd 10, code M716501-2.

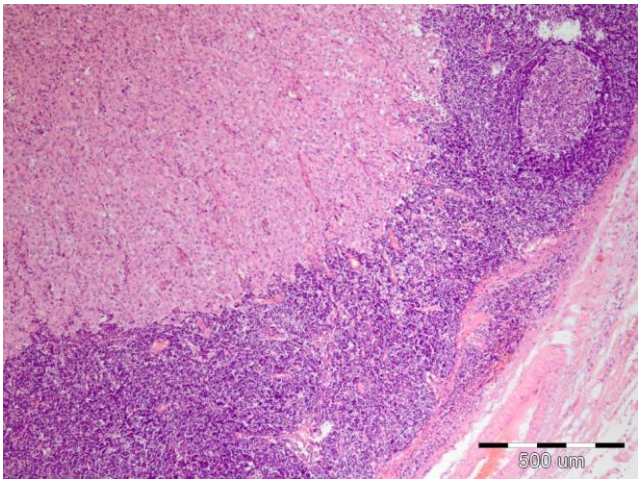

Figure 2: Neck lymph node with a metastatic deposit from a patient with moderately differentiated lower lip squamous cell carcinoma (HE 10 x 10)

Negative controls were carcinoma tissues in which primary antibody was omitted, and positive controls were considered carcinoma tissues with high expression of relevant proteins.

Staining with CD34 antibody was used for visualisation of tumour neoangiogenesis in the stroma at the invasive neoplasm front by endothelial brown staining cells in the blood vessels. At a magnification of 10 x 4, areas with the highest vascular density (hot spots) were identified, and at a magnification of 10 x 40, blood vessels were counted including groups of proliferative endothelial cells with clearly formed lumen in a set of 10 visual fields (Figure 3 and 4).

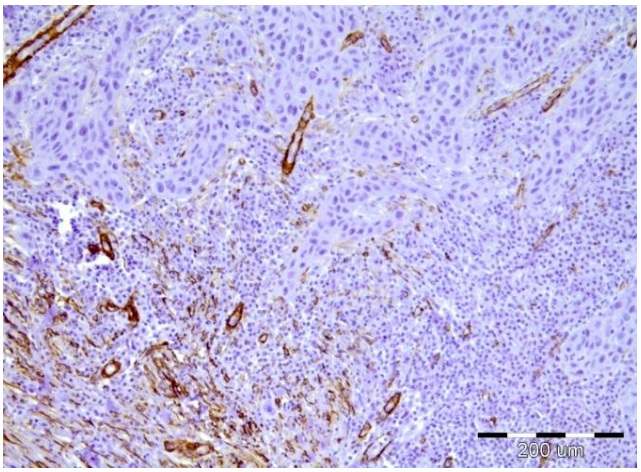

Figure 3: Vascular density with immunohistochemical staining for CD34 (10 x 4)

The analysis of histopathological sections was made with a light microscope. Also, a standard histopathological analysis was made of the surgical material from histopathological sections stained with hematoxylin-eosin. The histological results were analysed and presented in attributive and numerical statistical series. The results were evaluated with modern computer methods and software package and

tests for statistical processing: Kruskal Wallis test, Mann Whitney *U* test and Pearson's chi-square test.

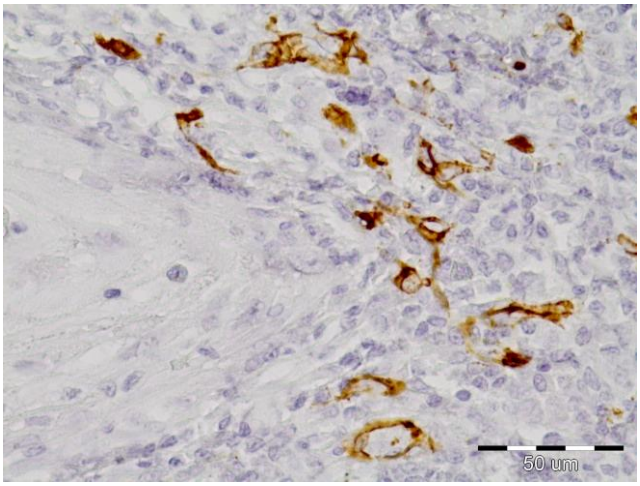

Figure 4: Visualization of vascular areas with immunohistochemical staining for CD34 at invasive front (10 x 100) with clearly formed lumina

Results

In this study, there were 13 out of 60 (21.7%) female patients, with age from 65 to 75 years, mean 70.2 ± 2.9 years and 47 (78.3%) male patients with age from 41 to 78 years, mean 66.4 ± 8.5 years.

All 15 patients with a metastatic deposit in the neck lymph nodes were men, aged 47 to 77 years, mean age 67 ± 8.2 years.

Regarding the grade of histological differentiation of SCC in patients with and without metastasis, the statistical analysis of data by applying the Pearson's chi-square test showed a highly significant statistical difference (p = 0.0000).

Figure 5 illustrates that the largest number of SCC in patients with metastasis were with poor histological differentiation. In patients without metastasis, SCCs with well histological differentiation were prevalent.

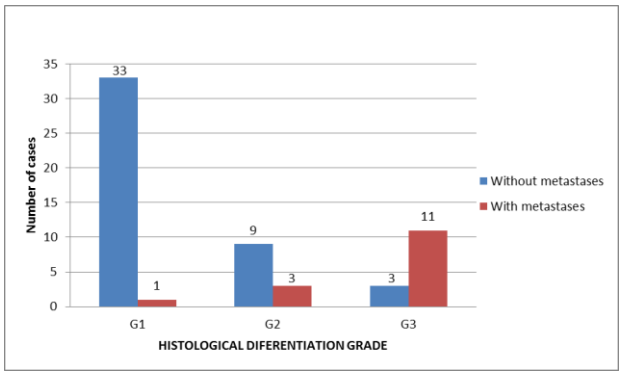

Figure 5: Comparison of the number of tumours with and without metastasis in the three groups with different grade of neoplasm differentiation (G1, G2, G3)

The lowest vascular density (smallest mean value) at the invasive front of the neoplasm was found in the well-differentiated SCC of the lower lip, and the highest in the poorly differentiated SCC of the lower lip.

Table 1: Vascular density at the invasive front of lower lip SCC in 60 patients about the grade of histological neoplasm differentiation (G)

Density of vascularization	Number of cases	Number of blood vessels			
		Mean value	Minimum value	Maximum value	Standard deviation
SCC of the lower lip					
G1	34	20.37	10.60	32.20	4.36
G2	12	24.90	18.60	37.30	6.27
G3	14	37.17	20.10	46.30	6.90

The mean values of vascularisation ranged from 20.4 to 37.2 (Table 1) and showed a statistically significant difference in the density of neovascularisation between groups of carcinoma with different grade of differentiation (Kruskal-Wallis test: H (2, N = 60) = 30.0943, p = 0.00001) (Figure 6).

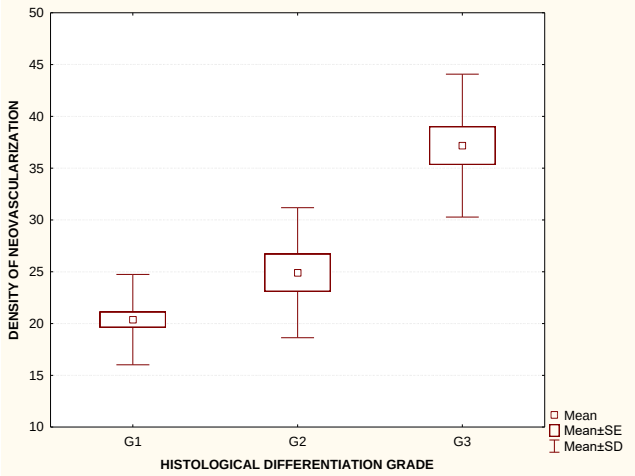

Figure 6: Statistically significant difference in the density of neovascularisation in lower lip SCC in 60 patients according to histological differentiation grade (G1, G2, G3). Legend: (G1) – well-differentiated carcinoma, (G2) – moderately differentiated carcinoma, (G3) – poorly differentiated carcinoma

Table 2 present the values of neovascularisation density in SCC and the surrounding non- tumour tissue.

Table 2: Vascular density in the invasive front of low lip SCC and surrounding non-tumour tissue

Density of vascularisation	No of cases	Mean value	Minimum value	Maximum value	Standard deviation
Non tumor tissue	60	6.4	4.70	8.70	0.97
SCC	60	25.20	10.60	46.30	8.71

The difference between the mean values of the vascular density in a tumour and non-tumour tissue was statistically significant (Mann-Whitney U тест-z = 9.447550, p = 0.0000001).

Table 3: Density of neovascularization in patients with neck metastasis from primary SCC of lower lip according to the grade of differentiation

Parameter	Number of cases	Number of blood vessels			
		Mean value	Standard deviation	Minimum value	Maximum value
G1	1	17.40	/	17.40	17.40
G2	3	27.13	9.08	19.80	37.30
G3	11	37.26	7.64	20.10	46.30

Table 3 and 4 present the values of neovascularisation (number of counted vascular spaces) at the invasive neoplasm front in patients with and without neck metastasis.

Table 4: Density of neovascularisation in patients without neck metastasis from primary SCC of lower lip according to the grade of differentiation

Parameter	Number of cases	Number of blood vessels			
		Mean value	Standard deviation	Minimum value	Maximum value
G1	33	20.46	4.39	10.60	32.20
G2	9	24.16	5.57	18.60	36.70
G3	3	36.86	4.17	32.10	39.90

The statistical analysis showed a significant difference in the density of vascularisation of lower lip SCC between neoplasms of patients without metastasis and neoplasms of patients with neck metastasis (Mann-Whitney U test, p = 0.000198) (Figure 7).

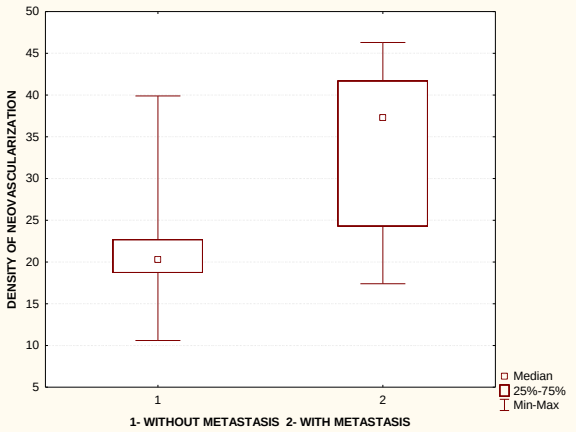

Figure 7: Statistically significant difference between vascular density in lower lip SCC in neoplasms without metastasis and neoplasms with metastasis

Discussion

Oral squamous cell carcinoma accounts for 95% of all forms of carcinomas that appear in the region of the head and neck. Squamous cell carcinoma is the most common carcinoma of the oral cavity, and the lower lip carcinoma is the most common carcinoma in the oral cavity [12]. Metastatic deposits in the regional lymph nodes are a predictive and prognostic parameter in patients with squamous cell carcinoma of the lower lip [13].

Metastases are less frequent in patients with a well-differentiated tumour (about 5%) than in patients with undifferentiated carcinomas (20%). Early detection of this carcinoma is important for obtaining esthetic and functional postoperative results and for favourable prognosis in patients [7].

Prognosis of squamous cell carcinoma of the lower lip depends on several factors, which include exposure to various risk factors and their combinations [4], stage of the disease [14], size of the tumor [15], presence of metastasis in the regional lymph nodes, different histological variables such as tumor grade, maximal thickness, perineural invasion and proteins expression [16], as well as other factors including surgical criteria [4]. The microvascular density of neoplasms is one of the histological elements that have been investigated in squamous cell carcinoma, but also other malignant neoplasms, aimed at early prevention of metastasis development and poor prognosis in patients with malignancies [17].

It has been determined that microvascular density (MVD) in tumours from the oral cavity does not differ significantly between oral mucosa and epithelial dysplasia, but is significantly increased in the tumour tissue. Also, it has been demonstrated that there is no expression of angiogenetic factors in the oral mucosa, but it is expressed in the tumour tissue and is in correlation with microvascular density, T status, tumour differentiation and grade of invasion [18]. Several studies that have examined MVD in a tumour have shown that it is higher in the tumour tissue than in the controls, it is a risk factor for the onset of metastasis [19], is in correlation with the grade of differentiation (G) and the onset of nodal metastasis [10]. Some authors consider that neoangiogenesis happens in the early phase of the development and its density is parallel to tumour progression [8].

Li C at al., reported that MVD was related to T status, stage of invasion and tumour differentiation as we also have found considering tumour differentiation [18]. Sedivy R at a.,l analyzing expression of vascular endothelial growth factor-C in correlation with MVD and the nodal status in oral SCC cell cancer reported that MVD (lymphatic and blood) results in a higher risk for cervical lymph node metastasis and that the angiogenetic effect of VEGF-C may also favour the onset of late lymphatic and haematogenous metastases [19]. These findings correspond to our findings that MDV predict the onset of neck nodal metastasis.

In this study, the density of neovascularisation was statistically significantly higher in carcinomas with a higher grade G3 of differentiation (poorly differentiated neoplasms) compared to those with a lower grade G1 (well-differentiated neoplasms) as well

as in patients with neck metastasis compared to those without neck metastasis.

In conclusion, the density of neoangiogenesis is increased in tumours with poorer differentiation and in patients with neck metastasis. So, the density of neovascularisation of the primary lip SCC may predict the tumour progression.

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ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2019 Jan 15; 7(1):24-28.
https://doi.org/10.3889/oamjms.2019.017
eISSN: 1857-9655
Basic Science

The Immunomodulatory Activities of *Picria Fel-Terrae* Lour Herbs towards RAW 264.7 Cells

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Citation: Auliafendri N, Rosidah, Yuandani, Suryani S, Satria D. The Immunomodulatory Activities of *Picria Fel-Terrae* Lour Herbs towards RAW 264.7 Cells. Open Access Maced J Med Sci. 2019 Jan 15; 7(1):24-28. https://doi.org/10.3889/oamjms.2019.017

Keywords: Picria fel-terrae Lour; inflammatory biomarkers; Cell viability; MTT assay; Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

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Received: 15-Oct-2018; **Revised:** 28-Nov-2018; **Accepted:** 29-Nov-2018; **Online first:** 05-Jan-2019

Funding: This research was funding by PDUPT 2018 from the ministry of research technology and higher education

Competing Interests: The authors have declared that no competing interests exist

Abstract

AIM: To investigated immunomodulatory activities of *Picria fel-terrae* Lour herbs extract against inflammatory biomarkers by conducting cell culture experiments in vitro.

MATERIAL AND METHODS: The herbs of *Picria fel-terrae* Lour were dried and extracted with n-hexane, ethyl acetate, 96% ethanol, followed by evaporation and freeze-drying. Phytochemicals screening were analysed with thin layer chromatography method. Cell viability was assessed with MTT assay. The genes of Tumor Necrosis Factor (TNF)-α, Interleukin (IL)-6, interleukin (IL)-1β and inducible Nitric Oxide Synthase (iNOS), Cyclooxygenase (COX-2) in lipopolysaccharide (LPS)-induced macrophages were analysed by Reverse Transcription-Polymerase Chain Reaction (RT-PCR) method.

RESULTS: Phytochemicals screening were showed steroids from the extract of n-hexane (ENPFH) and flavonoids, glycosides, saponins, tannins from extracts of ethyl acetate (EEAPFH) and ethanol (EEPFH). The Viability of RAW 264.7 cell toward ENPFH, EEAPFH, and EEPFH (1-200 μgmL^{-1}) was showed no toxicity effects. At the gene level, ENPFH; EEAPFH; EEPFH have decreased the gene expression of TNF-α, IL-6, IL-1β, iNOS, and COX-2 which induced with LPS (1 μgmL^{-1}).

CONCLUSIONS: Our results suggest that extracts of *Picria fel-terrae* Lour Herbs had immunomodulatory activities with inhibits selected inflammatory biomarkers at the gene levels in LPS-induced macrophages.

Introduction

Among various immune system-related cells, macrophages are versatile cells that exist in almost all tissues and play necessary roles in immune responses. In particular, macrophages were recruited in infection sites where they are activated to perform many functions through increasing phagocytosis. This process is the first line of defence against microbial and parasitic infections and in removing senescent and dead cells, immune mediator secretion and antigen presentation. Activated macrophages also prevent the invasion of pathogens by secreting inflammatory mediators, including cytokines, such as TNF-α and interleukins (ILs) and protein inflammatories such as inducible nitric oxide synthase (iNOS) and COX-2. Recent data showed that natural compounds had been widely and safely consumed over centuries, and many studies have indicated that most natural compounds have a wide range of diverse biological activities but few side effects. These natural products could affect the development and progression of cancer in various ways, such as inhibiting tumour cell growth, angiogenesis, and metastasis, immunomodulating, and enhancing effects of chemotherapeutic drugs. Therefore, traditional herbal medicinal resources have been investigated extensively for their immunomodulatory potential for therapeutic agents in immune-related functions [1].

Poguntano (*Picria fel-terrae* Lour.) have been used a drug of colic, malaria, diuretic, fever, and skin

disease [2]. Modern pharmacological investigations indicated that the extract of *P. fel-terrae* Lour exerts diuretic, antipyretic, hepatoprotective, cardioprotective, antidiabetic, antioxidant, anti-inflammatory, anthelmintic, and analgesic activities [3], [4], [5], [6], [7], [8], [9], [10], [11]. Moreover, *P. fel-terrae* inhibits hepatitis B (HB) e-antigen excreted by HepG2 2215 cell lines, suggesting having anti-HB virus activity [12]. It can be developed a co-chemotherapeutic regimen for breast cancer by inducing apoptosis, and cell cycle arrest and suppressing cyclin D1 and Bcl-2 expression based on the recent studies and it has antioxidant and antiproliferative activities of ethyl acetate fraction [13], [14], [15], and *n*-hexane, ethyl acetate, and ethanol fractions of *P. fel-terrae* Lour herbs have cytotoxic activity toward 4T1 and MCF-7 cell line [16].

Therefore in this study, we have examined the immunomodulatory activity of *P. fel-terrae* Lour herbs in RAW 264.7 mouse monocyte-macrophages.

Material and Methods

Fresh *P. fel-terrae* Lour herbs were collected from Tiga Lingga village, Dairi regency, Sumatera Utara province, Indonesia. RAW 264.7 cells were obtained from Parasitology Laboratory, Faculty of Medicine, Gadjah Mada University. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal bovine serum and kept at 37°C with a CO_2 supply of 5%. Lipopolysaccharides are from Escherichia coli O111.B4 (Sigma), Dexamethasone (Harsen), *n*-hexane, ethyl acetate, ethanol 96%. TLC Silica gel 60 F_{254} (Merck). All chemicals and reagents used in this work were of analytical grade. Total RNA Mini Kit (Geneaid), ReverTra Ace (Toyobo), GoTaq®Green (Promega), Nuclease-Free Water (Promega), TBE (Vivantis), agarose gel (Promega), Flurosafe (Smobio), DNA ladder 100 bp (Smobio).

The powder of *P. fel-terrae* Lour herbs (1 kg) was repeatedly extracted by maceration with *n*-hexane (3 x 3 day, 10 L) (ENPFH). The powder was dried in the air and extracted with ethyl acetate (3 x 3 day, 10 L) (EEAPFH), and then the powder was dried in the air and extracted with ethanol (3 x 3 day, 10 L) (EEPFH) at 25-30°C with periodical stirring. The filtrate was collected, and then evaporated to obtain a viscous fraction and then freeze-dried to dry [13], [15].

The Phytochemicals: Alkaloids, Flavonoids, Glycosides, Saponins, Tannins, Steroids were determined using thin layer chromatography standard procedures [17], [18], [19].

RAW 264.7 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, 100 units/mL of penicillin and 100 µg mL^{-1} of streptomycin as previously described by Chi et al., 2016. Cells were incubated in the presence of 5% CO_2 at 37°C. The cells (passage 7-12) were seeded at a concentration of 3 x 10^3 cells mL^{-1} in 96-well plates and incubation 24 h. The effects of *P. fel-terrae* Lour herbs extracts on cell viability were evaluated with the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-Tetrazolium bromide (MTT) colourimetric assay (Sigma-Aldrich). Extracts of *P. fel-terrae* Lour herbs were dissolved in 100% DMSO, and the stock solution of the extract at a concentration of 50.000 µgmL^{-1} was prepared in 10% DMSO. The final concentrations of the extract ranged from 1-200 µgmL^{-1} in the culture media. Dexamethasone and lipopolysaccharides were used as positive and negative controls [20], [21].

The gene's expression of TNF-α, IL-6, IL-1β, iNOS, and COX-2 was determined by RT-PCR. Total RNA from the control cell, LPS, positive control, and treatment groups were extracted using the Total RNA Mini Kit (Geneaid) according to the manufacturer's protocol. The oligonucleotide primers for TNF-α, IL-6, IL-1β, iNOS, COX-2, and β-actin were designed according to a PCR primer selection program at the website of the Virtual Genomic Center from the GenBank database (Table 1).

Table 1: Mouse oligonucleotide primers sequences used for RT-PCR (5-3') and Annealing temperature

Gen		Primer Sequences	Size (bp)	Temp (°C)
TNF-α	F	5'-TGTGCCGCCGCTGTCTGCTTCACGCT-3'	374	55
	R	5'-GATGAGGAAAGACACCTGGCTGTAGA-3'		
IL-6	F	5'-GATGCTACCAAACTGGATATAATC-3'	269	55
	R	5'-GGTCCTTAGCCACTCCTTCTGTG-3'		
IL-1β	F	5'-CCCTGCAGCTGGAGAGTGTGGA-3'	447	62.5
	R	5'-TGTGCTCTGCTTGTGAGGTGCTG-3'		
iNOS	F	5'-CGAAACGCTTCACTTCCAA-3'	311	60
	R	5'-TGAGCCTATATTGCTGTGGCT-3'		
COX-2	F	5'-CCTGTGTTCCACCAGGAGT-3'	249	55
	R	5'-GTCCCTGGCTAGTGC TTCAG-3'		
β-actin	F	5'- TGGAATCCTGTGGCATCCATGAAAC-3'	349	55
	R	5'- TAAAACGCAGCTCAGTAACAGTCCG-3'		

PCR consisted of 35 amplification cycles and each cycle carried out for 30 s at 95°C, 1 min at annealing temperature (55°C for TNF-α, IL-6, COX-2 and beta-actin and 60°C for iNOS) and 45 s at 95°C, 1 min at annealing temperature 62.5°C for IL-1β.) and 1 min at 72°C in a thermal cycler (ProFlex™ 3 x 32-well PCR System, Applied Biosystems). The β-actin was used as an internal control to standardise the relative expression levels for all biomarkers. PCR products were separated electrophoretically on a 2% agarose and fluorosafe (Smobio) with Tris-Borate-EDTA (Vivantis) 0,5x. The stained gel was visualised by using Gel-Doc Quantity One software (Syngene) [22].

Triplicate experiments were performed throughout this study. All data were presented as the mean ± Standard Error Minimum (SEM), which were analysed using the SPSS 22 software. The significant difference between Lipopolysaccharide and treated groups were analyzed by the paired Turkey HSD ($p < 0.05$).

Results

The results of phytochemicals screening from extract ethyl acetate and ethanol of *Picria fel-terrae* Lour. Contains flavonoids, tannins, saponins, glycosides while extract *n*-hexane only contains steroids which seen in Table 2.

Table 2: Phytochemicals Screening Result

NO	Phytochemicals	ENPFH	EEAPFH	EEPFH
1.	Alkaloids	-	-	-
2.	Flavonoids	-	+	+
3.	Tannins	-	+	+
4.	Saponins	-	+	+
5.	Glycosides	-	+	+
6.	Steroids	+	-	-

The results were showed the cell viability test on extracts of *n*-hexane, ethyl acetate and ethanol of *Picria fel-terrae* Lour Herbs and dexamethasone as the positive control. The best results were shown on an extract of *n*-hexane with the concentration of 12.5 and 25 μgmL^{-1} which the resulted in the highest viability percentage. Percentage of cell viability on RAW 264.7 cells for extract and Dexamethasone was showed in Figure 1 A and B.

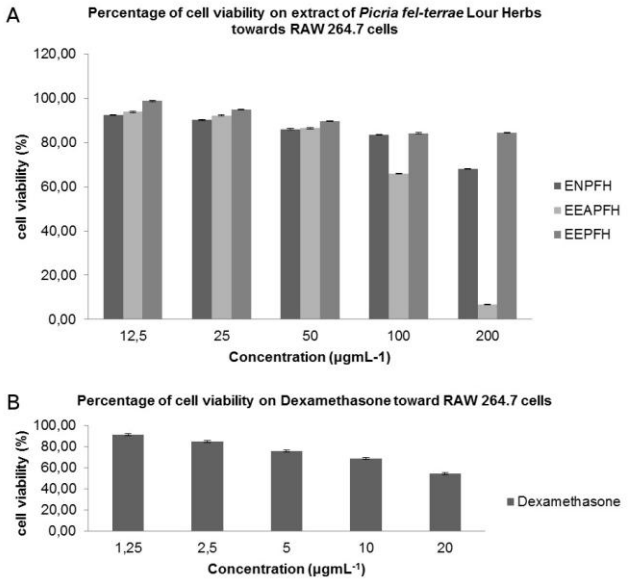

Figure 1: A) Percentage of cell viability on RAW 264.7 cells treated by ENPFH, EEAPFH and EEPFH; B) Percentage of cell viability on RAW 264.7 cells treated by dexamethasone as a positive control

The results expression of genes which treated with ENPFH, EEAPFH, EEPFH and dexamethasone were analysed using RT-PCR methods, and the results were shown in Figure 2.

Furthermore, we were tested whether ENPFH; EEAPFH; EEPFH has potential immunomodulatory in the expression of inflammatory cytokines in LPS-induced macrophages. As shown in Figure 2, resulting treatment with ENPFH; EEAPFH; EEPFH (25 μgmL^{-1}) were inhibited the expression of the gene of cytokines (TNF-α, IL-6, IL-1β), iNOS and COX-2 in macrophages treated with LPS.

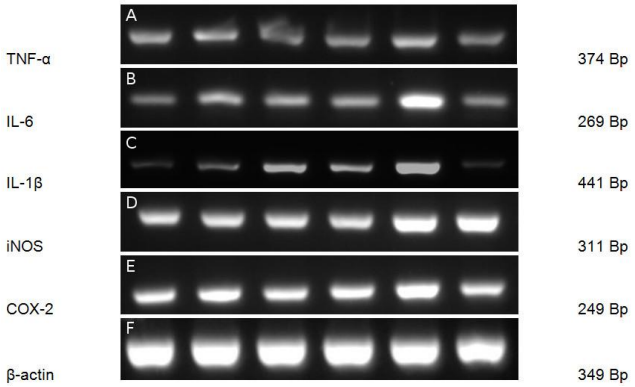

Figure 2: The effect of extracts on the expression of genes in RAW 264.7 cell which induced LPS 1 μgmL^{-1} for 6 hours. The total RNAs were isolated, and RT-PCR was performed using the indicated primers in Materials and Methods. Dexamethasone 2.5 μgmL^{-1}(A); EEPFH 25 μgmL^{-1}(B); EEAPFH 25 μgmL^{-1}(C); ENPFH 25 μgmL^{-1}(D); LPS (E); control cells (F). β-actin was used as the internal control. LPS, Lipopolysaccharide; RT-PCR, reverse transcription-PCR; iNOS, Inducible Nitric Oxide Synthase; IL, interleukin; COX-2, Cyclooxygenase-2; Bp, Base Pair

The results were shown from the value of genes expression from ENPFH, EEAPFH, EEPFH toward LPS showed a significant difference with $P < 0.05$.

Table 3: The value of genes expression in RAW 264.7 cells which induced LPS

Gene	*Mean ± SEM*					
	Dexamethasone	EEPFH	EEAPFH	ENPFH	LPS	Control cell
TNF-α	1,23 ± 0,01	1,26 ± 0,01	1,03 ± 0,01	1,08 ± 0,02	1,46 ± 0,03	1,00 ± 0,00
IL-6	0,78 ± 0,02	1,33 ± 0,01	1,29 ± 0,02	1,27 ± 0,02	2,61 ± 0,02	1,00 ± 0,00
COX-2	1,02 ± 0,01	1.23 ± 0,02	1,05 ± 0,01	1,16 ± 0,01	1,50 ± 0,02	1,00 ± 0,00
IL-1β	1,06 ± 0,02	1,80 ± 0,03	2,78 ± 0,03	2,33 ± 0,04	4,02 ± 0,04	1,00 ± 0,00
iNOS	0,65 ± 0,03	0,69 ± 0,01	0,75 ± 0,01	0,67 ± 0,01	1,03 ± 0,02	1,00 ± 0,00
β-actin	1,02 ± 0,01	1,05 ± 0,01	1,06 ± 0,02	1,09 ± 0,02	1,04 ± 0,01	1,00 ± 0,00

Discussion

The scientific evidence of *P. fel-terrae* Lour has been widely investigated, but the immuno-modulatory effects extract of *P. fel-terrae* Lour Herbs is rarely to be explored. In this present study, previously we were examined of phytochemicals screening from ENPFH, EEAPFH, EEPFH. Cells viability was performed to determine the toxicity of that extracts of *P. fel-terrae* Lour herbs towards RAW 264.7 cells. RAW 264.7 cells in culture media were treated with ENPFH, EEAPFH, EEPFH with various concentrations. After 24 hours of incubation, the culture medium was aspirated, and cell viability was measured using an MTT solution [23]. Cell viability was showed that extracts did not cause toxicity to RAW 264.7 cells. As shown in Figure 1 (cell viability > 85%) were selected for further analysis [21], and we were determined at concentration 25 μgmL^{-1}, which exhibited immunomodulatory activity on decreasing the production of various inflammatory cytokines (i.e.,

TNF-α, IL-6, IL-1β), inducible enzyme iNOS and COX-2 in macrophages treated with LPS.

Meanwhile, LPS is widely recognised as the major inducer for the production of inflammatory cytokines which in turn stimulates iNOS induction during the inflammatory process in macrophages [24], [25]. These cytokines can be produced from macrophages in response to bacterial LPS, infection, and inflammatory stimulation. They also play an important role in the immune system by aiding cytotoxic and cytostatic effects on infected or malignant cells. Among them, TNF-α is one of the earliest factors to be induced or activated in macrophages for eliciting tumour immunity. TNF-α plays as a key mediator of T lymphocyte, and macrophage activation. Similarity, IL-1β and IL-6 are produced by various immune cells, including macrophages [1]. Our results suggest that ENPFH; EEAPFH; EEPFH may reduce the expression of cytokines TNF-α, IL-1β, and IL-6, subsequently leading to the blockade of inflammatory enzyme induction (iNOS) and COX-2, and the value of gene expression significant difference with $P < 0.05$, which indicates that it has an immunomodulatory effect on RAW 264.7 macrophages. It has been recognised that the blockade of inflammatory cytokines by natural anti-inflammatory products is a potent strategy for the management of various inflammatory diseases, immune system upset [22].

Based on the results of phytochemical screening ENPFH contained triterpenoid/steroid compounds. Steroids were a group of compounds which have a basic framework of cyclopentane perhydro phenanthrene, having four integrated rings. These compounds have certain physiological effects. In previous studies, steroids can inhibit TNF-α, IL-6, IL-1β, COX-2 and iNOS gene expression [26], [27]. In phytochemical screening, EEAPFH and EEPFH contained flavonoid, saponin, tannin, glycoside compounds. Flavonoids were a group of natural compounds in the polyphenol group found in plants. Flavonoids are composed of two aromatic rings which can or cannot form a third ring with a C_6-C_3-C_6 arrangement [28]. Saponin is a complex glycoside compound, which is the result of a sugar condensation compound with an organic hydroxyl compound which when hydrolysed will produce sugar (glycone) and non-sugar (aglycone). In previous studies, flavonoids can inhibit TNF-α, IL-6, IL-1β, COX-2, and iNOS gene expression [29], [30], [31], [32], Saponins can also inhibit TNF-α, IL-6, IL-1β, COX-2 and iNOS gene expression [33], [34], [35].

Based on the immunomodulatory profile exposed through various assays, we summarised that *P. fel-terrae* Lour. Herb extracts significantly decreased genes expression of TNF-α, IL-6, IL-1β, iNOS, and COX-2, in LPS-induced macrophages in vitro. The extract of *P. fel-terrae* Lour Herbs could be potentially used as a herbal medicine to immunomodulatory. Further study on molecular mechanisms by which extract of *P. fel-terrae* Lour herbs modulated the expression of inflammatory cytokines and proteins in macrophages in response to LPS is still needed. Also, in vivo study using animal models is needed to determine the exact immunomodulatory potential of that.

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ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2019 Jan 15; 7(1):29-32.
https://doi.org/10.3889/oamjms.2019.026
eISSN: 1857-9655
Basic Science

The Role of Polymorphism Gen *Methylene Tetra Hydrofolate Reductase (MTHFR) C677T* in Ischaemic Stroke Patients with and Without Hypertension

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Citation: Arina CA, Amir D, Siregar Y, Sembiring RJ. The Role of Polymorphism Gen *Methylene Tetra Hydrofolate Reductase (MTHFR) C677T* in Ischaemic Stroke Patients with and Without Hypertension. Open Access Maced J Med Sci. 2019 Jan 15; 7(1):29-32. https://doi.org/10.3889/oamjms.2019.026

Keywords: Polymorphism gen *MTHFR C677T*; Ischaemic stroke; Hypertension

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Received: 20-Sep-2018; **Revised:** 21-Nov-2018; **Accepted:** 22-Nov-2018; **Online first:** 04-Jan-2019

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

Abstract

BACKGROUND: Stroke is a leading cause of disability and remains the second leading cause of death in the world. Some of the pathogenesis of stroke are interactions between genetic and acquired risk factors, the interaction is related with the atherosclerotic which is the main pathogenesis of ischaemic stroke. Previous studies demonstrated an association between methylene tetra hydro folate reductase (MTHFR) genotype and ischaemic stroke; the *MTHFR C677T* genotype is one of the independent risk factor.

AIM: This study aims to know about the role of polymorphism gen *MTHFR C677T* in ischaemic stroke patients with and without hypertension.

METHODS: This study is a cross-sectional study, the sample was taken consecutively, after approval by the Medical Faculty Science's Ethics Committee at University Sumatera Utara. All sample matched with inclusion and exclusion criteria, demography data and blood sample were taken. Demography data were analysed using descriptive statistic.

RESULTS: Of the 106 ischaemic stroke patients were divided into two groups, the first group is patients with hypertension (53 patients), and the second group is without hypertension (53 patients). We have done the PCR-RFLP to all the patients, we got 78 patients with *677CC* of *MTHFR* genotype, 23 patients with *677CT* genotype and 5 patients with *677TT* genotype. We found polymorphism *C677T* is more frequent in ischaemic stroke patients with hypertension (16 patients; 69.5%), and all the patient with *677TT* genotype are an ischaemic stroke with hypertension (5 patients; 100%).

CONCLUSION: We concluded that polymorphism *MTHFR C677T* have an important role in hypertension and ischaemic stroke.

Introduction

Stroke remains a health problem in the world and as the major cause of morbidity, mortality and disability in developing country [1], [2], also in Asia, Russia, East Europe [3] and as multifactorial disease and complex caused by vascular, environment and genetic [4], [5]. The incidence of stroke increased in lower and middle-income countries. WHO predict in the year 2050, 80% of cases of stroke happened in that countries [4].

Aetiology and pathogenesis of ischaemic stroke are complex and involved some risk factor [6], many evidence showed the role of genetic in stroke [4], but identification of the gen that related to stroke is still controversy [7]. *A cohort* study showed that family history with stroke would increase the risk of stroke [8], [9], and case-control study in twins also related to the inherited risk [10].

Over the past decades, the role of a different candidate gene in the pathogenesis of stroke has been examined in numerous association studies [11]. The epidemiologic study shows the existence of basic polygene in stroke incidence and many stroke

pathogenesis, they act as interactions between genetic, and others risk factors [5], [7], the interaction related to atherosclerosis which is major pathogenesis of stroke [12]. Many gene and polymorphism have been studied as the cause of hypertension and act as a major risk factor for stroke [13].

The methylenetetrahydrofolate reductase *C677T* mutation is one of the most common gene polymorphisms. Mapped to chromosomal region 1p36.3, the *MTHFR* gene spans a 2.2-kb length with 11 exons and 10 introns [14] and encodes an enzyme composed of 656 amino acids [15]. The product, known as MTHFR, is a critical enzyme in homocysteine metabolism. The *C677C* to *T* mutation in the catalysing region of the *MTHFR* gene may induce the displacement of alanine by valine [16]. This change may lead to the thermolability of the enzyme and the inhibition of *MTHFR* activity, thus decreasing the transformation from 5,10 methylenetetrahydrofolate to 5 methyltetrahydrofolates [11], which act as a co-substrate for the conversion from homocysteine to methionine [14].

A lot of case-control studies have shown the role of C to T mutation in 677 nucleotides in the *MTHFR* gene; they found a significant relationships between *C677T* with hypertension in Caucasian and Asian population [13]. The *MTHFR C677T* polymorphism has previously been found to associate with various vascular disease including stroke, hypertension and congestive heart failure [11].

Prevalence *MTHFR C677T* relatively high in general population, while the prevalence of T allele is about 0.34 (0.29-0.39) in whites, 0.42 (0.34-0.50) in Japanese and 0.08 (0.06-0.12) in African [17]. Other research shows that T allele frequency is much varied in different ethnic and country, 22.6% in the Guangxi Yao population, 32.2% in Australia, and 63.1% in Shandong [18].

People with *MTHFR 677TT* gene only have 30% of enzyme activity, while the *MTHFR C677T* has 60% of enzyme activity compared with the wild type [6]. T allele from *MTHFR* gene polymorphism is an independent risk factor of idiopathic ischaemic stroke [19], and prognostic factor for cardiovascular disease [20].

Genetic studies have a role in optimized stroke prevention and therapy [9], also can be used to predict the risk of the disease, but the results of the studies did not mention about the type and numerous gene that act as a stroke risk factor [7]. Significant improvement in the last few years about the role of genetic in stroke, giving deep knowledge about the basic molecular disease that can be used for the management patients [21] and to get new therapy target [10]. Identification of stroke risk factor can be modified to get stroke prevention more effective and decrease the mortality [3].

This study aims to know about the role of polymorphism gen *MTHFR C677T* in ischaemic stroke patients with and without hypertension.

Material and Methods

This study is a cross-sectional study; the sample was taken consecutively. The study was conducted with the approval of the ethics committee of the institutions involved, and informed consent was obtained from all subjects or their relatives (for comatose patients). Inclusion criteria were the presence of ischaemic stroke at the present hospital admission, age > 18 years old; the neurological deficits were confirmed in all cases by computerised tomography (CT) scan. We analysed the *MTHFR* genotypes in subjects with ischaemic stroke. The polymorphism was classified into three groups, (1) allele CC, (2) allele CT, (3) allele TT.

A polymerase chain reaction followed by the restriction fragment length polymorphism was used to genotype the *C677T* polymorphism of *MTHFR*. The conditions of amplification and digestion have been well documented previously in our laboratory. The digested PCR products after separation on a 3% agarose gel, stained with ethidium bromide, showed one band of 198bp corresponding to the wild-type homozygous (CC), three bands of 198, 175, and 23bp for the heterozygous (CT), two bands of 175 and 23bp for the mutated homozygous (TT).

Results

This study includes 106 ischaemic stroke patients were divided into two groups, with and without hypertension. A total of 55 men had an ischaemic stroke, while 51 women had an ischaemic stroke, 60 patients at the age below 60 y.o, 46 patients at the age above 60 y.o, Bataknese 33 patients, family history was found in 32 patients.

Table: 1 Distribution of characteristic frequency ischaemic stroke with and without hypertension

Variable	Category	Ischaemic Stroke					
		Hypertension		Non-hypertension		Total	
		N	%	N	%	N	%
Age	< 60 y.o	30	28,3	30	28.3	60	56.6
	> 60 y.o	23	21.7	23	21.7	46	43.4
Gender	Female	25	23.6	26	24.5	51	48.1
	Male	28	26.4	27	25.4	55	51.9
Ethnicity	Bataknese	33	31.1	29	27.3	62	58.4
	Javanese	11	10.4	10	9.43	21	19.8
	Acehnese	4	3.77	3	2.83	7	6.60
	Minangnese	2	1.88	2	1.88	3	2.83
	Melayumese	2	1.88	9	8.49	11	10.3
	Niasnese	1	0.94	-	-	1	0.94
Family history		32	30.1	17	16.0	49	46.2

The differences in the characteristics of patients with and without hypertension are shown in Table 1.

Table: 2 Distribution of frequency polymorphism ischaemic stroke with and without hypertension

Variable	Category	Ischaemic Stroke						p-value*
		Hypertension		Non-Hypertension		Total		
		n	%	n	%	n	%	
Polymorphism	CC	32	60.3	43	81.1	76	71.7	
	CT	16	30.2	10	18.9	26	24.5	0.035
	TT	5	9.5	0	0	5	3.8	

**Fisher Exact Test*

This study found that among 106 patients ischaemic stroke with hypertension and without hypertension, the wild-type homozygous (CC) was found in 76 patients; 71.7% (32 patients with hypertension, 43 patients without hypertension). The heterozygous (CT) was found in 26 patients; 24.5% (16 patients with hypertension, 10 patients without hypertension). The mutated homozygous (TT) was found only in ischaemic stroke patients with hypertension (5 patients).

Fisher exact test was done for this variable category; the result showed a significant correlation (p = 0.035) (Table 2).

Discussion

Stroke is a common complex trait and does not follow Mendelian pattern of inheritance: Gene–gene or gene-environment interactions may be responsible for the complex trait. How the interactions contribute to stroke is still under research [12].

Now we know single polymorphism may have a weak effect on the risk of stroke when analysed individually, but their influence may be more pronounced in the presence of a permissive background. Many cases of stroke are due to a complex interaction between lifestyle factors and genetic susceptibilities. Genetic predisposition for stroke may result from synergistic co effects [11].

Many studies have shown that T allele frequencies in different ethnicities and countries vary widely. The Guangxi Yao population rate was 22/6%, and in Australia, it was 32.2 %. The Yang et al., the study of 15,357 Han patients found that the mutation frequency in Hainan was the lowest with T allele of 245 and in Shandong it was the highest (63.1%) [18].

This study found that polymorphism *C677T* is more frequent in ischaemic stroke patients with hypertension (16 patients), and all the patient with *677TT* genotype are an ischaemic stroke with hypertension (5 patients),

This gene is responsible for producing *Methylenetetrahydrofolate reductase* (MTHFR) enzyme that functional. The function of this enzyme is to transform 5, 10 methylenetetrahydrofolates to 5 methyltetrahydrofolates [22]. The Val form of *MTHFR* encoded by 677 T allele is thermolabile and has reduced enzymatic activity [23]. The mutated homozygous (TT) is the risk of thrombotic disease in young age [24].

The result of this study showed a significant correlation between the polymorphism and ischaemic stroke with and without hypertension (p = 0.035).

There is a strong association between polymorphism gen *MTHFR* with atherosclerosis; even the mechanism of it is indirect. Patients with MTHFR genotype *677TT*, have a vascular occlusion, and infarct, had increased level of blood homocysteine. Those are the reason for the association between polymorphism and hypertension [12].

In conclusion, polymorphism *MTHFR C677T* has an important role in hypertension and ischaemic stroke, because both of the diseases is caused by atherosclerotic vascular disease.

There are several limitations to our study. First, the study enrolled hospital-based stroke patients rather than patients from a community based general population. Secondly, this study did not collect data on other several major risk factors of ischaemic stroke.

Acknowledgements

The authors gratefully acknowledge that the present research was supported by the Ministry of Research and Technology and the Higher Education Republic of Indonesia by Basic Research Award from the TALENTA Universitas Sumatera Utara, Indonesia.

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ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2019 Jan 15; 7(1):33-37.
https://doi.org/10.3889/oamjms.2019.014
eISSN: 1857-9655
Basic Science

CRP Gene Polymorphism and Their Risk Association With Type 2 Diabetes Mellitus

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Citation: Jebur HB, Masroor M, Ahmad H, Khan NA, Akther J, Bharali D, Singh VK, Verma A, Khan S, Khan V, Hasan R, Bhatt D, Goyal Y, Dev K. CRP Gene Polymorphism and Their Risk Association With Type 2 Diabetes Mellitus. Open Access Maced J Med Sci. 2019 Jan 15; 7(1):33-37. https://doi.org/10.3889/oamjms.2019.014

Keywords: Type 2 Diabetes; CRP gene polymorphism; Biochemical parameters

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Received: 10-Aug-2018; **Revised:** 18-Oct-2018; **Accepted:** 20-Oct-2018; **Online first:** 11-Dec-2019

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

&: Equal contribution of authors

Abstract

BACKGROUND: C-reactive protein (CRP) is an inflammatory marker associated with T2DM, obesity, insulin resistance, and cardiovascular disease.

AIM: The present study evaluates the association of CRP +1059 G/C polymorphism of the *CRP* gene in 100 T2D cases and 100 healthy controls.

METHODS: Present study was done by allele specific PCR method to study the CRP gene polymorphism in study subjects.

RESULTS: Study found that *CRP* (+1059 G/C) genotype distribution among case and controls was found to be significant (p=0.001), Higher *CRP* C allele frequency (0.16) was observed compared to controls (0.04). *CRP* +1059 GC and CC had 2.72 (1.12-6.61), 20.56 (1.16-362.1) risk for T2D. It has been observed, HTN, Obesity, Smoking and alcoholism was found to be associated with increased risk of T2D, and a significant difference was observed in biochemical parameters.

CONCLUSION: Study concluded that *CRP* gene polymorphism was found to be associated with risk of Type 2 Diabetes and risk was linked with heterozygosity and mutant homozygosity. Hypertension, Obesity, Smoking and alcoholism increases the risk of occurrence of Type 2 Diabetes.

Introduction

Type 2 diabetes mellitus (T2DM) is one of the major health-related global health problems [1]. Genetic alterations played an important role in determining why some diabetic patients develop these complications while others do not [2]. Several evidence showed the importance of inflammation in the pathogenesis of diabetic complications, especially enhancement of atherosclerosis and complications [3]. According to the International Diabetes Federation revealed that currently, around 415 million people with diabetes all over the world are likely to increase to 642 million by 2040 [4]. T2D accounts for high morbidity and mortality due to complications like renal failure, amputations, cardiovascular disease, and cerebrovascular events [5]. In 2015 there were approximately 5.0 million deaths by diabetes worldwide [4]. C-reactive protein (CRP) is an inflammatory marker associated with T2DM, obesity, insulin resistance, and cardiovascular disease [6].

Furthermore, according to recent data, CRP is not an acute phase protein only, but it is a mediator of atherogenesis, serving as a marker for cardiovascular disease (CVD) [7]. CRP levels display extensive interindividual variability. Although plasma C reactive protein levels are influenced by sociodemographic,

behavioural, and lifestyle factors and obesity and type 2 diabetes, twin and family studies have estimated that genetic factors could contribute up to 35–50% of the variation of CRP [8] [9]. Several population-based association studies have shown that common genetic variants at the *CRP* locus are significantly associated with plasma CRP levels [10]. CRP gene polymorphisms of were found to be in mild to moderate association with the basal CRP levels in healthy men and women [11].

Thus, the current study aimed to evaluate the role of *CRP* (+1059G/C) polymorphism in type 2 diabetic patients.

Material and Methods

The present case-control study enrolled a total of 200 individuals. There were 100 newly diagnosed type 2 diabetes and 100 healthy controls collected. This study was approved by the institutional ethical committee, Jamia Millia University. Written informed consent was obtained from all individual participants included in the study.

All the clinical parameter such as fasting glucose and postprandial glucose were taken care as per standard criteria. Total 3 ml of blood sample were withdrawn, 1 collected in EDTA vials and 2 ml in plain vials from all the study subjects included in the study.

DNA isolation was done by a phenol-chloroform method from blood samples collected in EDTA vials from cases as well as controls. Genomic DNA was analysed on 1% agarose gel to confirm and observed under UV transilluminator.

Isolated DNA was then amplified to determine the genotypes of CRP by allele-specific primers forward: 5′ CATTTGTACAAGCTGGGAGT 3′, 2. Allele C specific reverse: 5′ ATGGTGTTAATCTCATCTGGTGGG 3′, Allele G specific reverse: 5′′TGGTGTTAATCTCATCTGGTGGC 3′ using thermocycler. PCR was performed in 25 µl reaction volume containing 3 µl of 100 ng template DNA, 0.25 µl of 25 pmol each primer, 2.5 µl of 10 mM dNTPs, 1.5 µl of 20mM MgCl2, 0.3 µl of 5 U/µl Taq polymerase with 2.5 µl of 10X Taq Buffer (Fermantas) and 14.7 µl of nuclease-free ddH_2O. The PCR was performed with initial denaturation at 94°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 40 seconds, annealing at 58°C for 40 seconds, extension at 72°C for 40 seconds and the final extension was at 72°C for 10 minutes. The amplified product of 237 bp was visualised under UV transilluminator.

Genotype frequencies between the cases and controls were evaluated using the Chi-square test and values ≤ 5 were analysed by Fisher exact test. Allele frequency was calculated by the Hardy–Weinberg Equilibrium (HWE) equation. The associations between *CRP* genotypes and risk of T2D were estimated by computing the odds ratios (ORs) with 95% confidence intervals (CIs). The parametric and nonparametric test was used to analyse the quantitative data, p-value less than 0.05 considered to be statistically significant.

Results

Demographic characteristic of study subjects investigated in the present study has been summarised in Table 1.

Table 1: Distribution of selected characteristics among T2D patients and healthy controls

Variables	T2D patients n (%)	Healthy controls n (%)
Total no.	100 (100%)	100 (100%)
Gender		
Males	62 (62%)	64 (64%)
Females	38 (38%)	36 (36%)
Age at diagnosis (Years)		
≤ 50	61 (%)	66 (%)
> 50	39 (%)	34 (%)
Mean age (years)	49.82	47.89
HTN		
Yes	63 (63%)	28 (28%)
No	37 (37%)	72 (72%)
Obesity		
Yes	20 (20%)	5 (5%)
No	80 (80%)	95 (95%)
Smoking status		
Yes	58 (58%)	30 (30%)
No	42 (42%)	70 (70%)
Alcoholism		
Yes	49 (49%)	26 (26%)
No	51 (51%)	74 (74%)

The difference observed in genotype among cases and controls was found to be significant (p = 0.001) (Table 2). It was observed that high percentage of heterozygous GC 16 (16%) and mutant homozygous CC 8 (8%) genotype was found in patients compared to controls, heterozygous GC 8 (8%) and CC 0 (0%) while lower GG 76 (76%) genotype in patients compared to control homozygous GG 90 (90%) genotype. The higher allele frequency of C allele (0.16) was observed in T2D patients compared to control (0.04).

Table 2: Genotype distribution and allele frequencies of *CRP* (+1056G/C) gene among T2D patients and controls

Variables	GG n(%)	GC n(%)	CC n(%)	p value	Allele frequency	
					G allele	C allele
Patients (n = 100)	76 (76)	16 (16)	8 (8)	0.001	0.84	0.16
Controls (n = 100)	92 (92)	8 (8)	0 (0)		0.96	0.04

Odds ratio with 95 % confidence intervals was calculated for each group to estimate the degree of association between the *CRP* genotype and risk of T2D in Indian patients depicted in Table 3. Compared to the GG genotype, the OR 2.72 (1.12-6.61) and 20.56 (1.16-362.1) for the heterozygous GC and homozygous CC genotypes were estimated,

suggesting a possible dominant effect of *CRP* polymorphism on T2D risk.

Table3: Risk of T2D associated with *CRP* (+1056G/C) genotype

Genotype	Healthy controls (n = 100)	T2D patients (n = 100)	OR (95% CI)
GG	92 (92%)	76 (76%)	**(ref)**
GC	8 (8%)	16 (16%)	2.42 (0.98-5.96)
CC	0 (0%)	8 (8%)	20.56 (1.16-362.1)
GC+CC	8 (8%)	24 (24%)	3.63 (1.54-8.54)

The risk associated with Hypertension (HTN), Obesity, smoking and alcoholism was calculated in T2D patients and depicted in (Table 4). In HTN, compared to the GG genotype, the OR 1.52 (0.47-4.79), 4.82 (0.56-41.20) for the heterozygous GC and homozygous CC genotypes. In obesity, compared to the GG genotype, the OR 1.61 (0.44-5.80) and 2.90 (0.61-13.72) for the heterozygous GA and homozygous AA genotypes. Patients with smoking, alcoholism status compared to the GG genotype, the OR for GC 1.87 (0.59-5.92), 3.02 (0.95-9.56) and for CC 2.56 (0.48-13.51), 4.12 (0.78-21.79) respectively.

Table 4: Risk of *CRP* genotype in T2D patients associated with different variables

Genotype	HTN No (n = 37)	HTN Yes (63)	OR* (95% CI)
GG	**31**	**45**	**(ref)**
GC	**5**	**11**	1.51 (0.47-4.79)
CC	**1**	**7**	4.82 (0.56-41.20)
Genotype	**Obesity No (n = 80)**	**Obesity Yes (n = 20)**	**OR* (95% CI)**
GG	**63**	**13**	**(ref)**
GC	**12**	**4**	1.61 (0.44-5.80)
CC	**5**	**3**	2.90 (0.61-13.72)
Genotype	**Smoker No (n=42)**	**Smoker Yes (n=58)**	**OR* (95% CI)**
GG	35	41	**(ref)**
GC	5	11	1.87 (0.59-5.92)
CC	2	6	2.56 (0.48-13.51)
Genotype	**Alcohol No (n=49)**	**Alcohol Yes (n=51)**	**OR* (95% CI)**
GG	32	44	**(ref)**
GC	11	5	3.02 (0.95-9.56)
CC	6	2	4.12 (0.78-21.79)

Other serum markers such as cholesterol, TG, LDL, VLDL, HDL, serum uric acid, bilirubin total, fasting blood glucose and postprandial glucose were compared between T2D patients, and healthy controls were shown in table 6. High lipid parameter such as TG, LDL, VLDL and were observed higher in T2D patients compared to controls and the differences was found to be significant ($p < 0.0001$, $p < 0.0001$, $p < 0.0001$), however no significant difference was observed in cholesterol level among T2D cases and controls ($p = 0.73$). Serum uric acid and total bilirubin were observed to be high compared to healthy control group ($p < 0.0001$, $p < 0.0001$). High fasting and postprandial blood glucose were observed in T2D cases compared to healthy controls, and the difference was found to be significant ($p < 0.0001$, $p < 0.0001$).

Table 5: Serum markers level of T2D patients and healthy controls

Serum markers	T2D patients (Mean ± SD)	Healthy controls (Mean ± SD)	p-value
Cholesterol	206.8 ± 43.02	205.2 ± 19.05	0.73
TG	183.3 ± 42.62	139.5 ± 24.92	<0.0001
LDL	147.4 ± 28.77	113.3 ± 14.10	<0.0001
VLDL	34.16 ± 5.62	25.32 ± 4.81	<0.0001
Serum uric acid	5.44 ± 1.85	4.20 ± 0.74	<0.0001
Bilirubin total	0.86 ± 0.44	0.64 ± 0.20	0.0003
Fasting blood sugar	171.1 ± 39.55	92.75 ± 10.88	<0.0001
Postprandial blood sugar	257.7 ± 51.94	126.7 ± 9.58	<0.0001

Patients and controls with different risk factor were analysed to see the effect on biochemical parameters, and it was observed that the patients and controls had hypertension history had a significant impact on biochemical parameters. T2D patients with HTN history had elevated level of TG, LDL, VLDL, Serum uric acid, bilirubin, fasting blood sugar and postprandial blood sugar compared to healthy control with HTN history. In the same way, others risk factors were found to affect most of the biochemical parameters and found to be significant.

Table 6: Effect of different risk factor positive on serum markers level among T2D patients and healthy controls

HTN history yes	In T2D	Healthy control	p-value
Cholesterol	208.3 ± 43.49	210.2 ± 17.95	0.82
TG	182.8 ± 43.10	138 ± 23.67	< 0.0001
LDL	138.9 ± 27.19	118.4 ± 15.54	0.0003
VLDL	34.62 ± 5.74	26.32 ± 3.78	< 0.0001
Serum uric acid	5.51 ± 1.73	4.26 ± 0.59	< 0.0001
Bilirubin total	0.83 ± 0.43	0.67 ± 0.20	0.23
Fasting blood sugar	174.8 ± 42.39	92.54 ± 11.66	< 0.0001
Postprandial blood sugar	256.2 ± 54.99	127.1 ± 1.60	< 0.0001
Obesity history yes	**In T2D**	**Healthy control**	**p-value**
Cholesterol	207.6 ± 45.59	208 ± 27.16	0.98
TG	194.4 ± 47.36	174.8 ± 49.06	0.42
LDL	140 ± 27.69	109.2 ± 12.66	0.02
VLDL	33.80 ± 5.55	23.60 ± 4.27	0.0009
Serum uric acid	5.41 ± 1.51	4.02 ± 0.73	0.009
Bilirubin total	0.68 ± 0.33	0.78 ± 0.16	0.16
Fasting blood sugar	167.1 ± 31.61	93.0 ± 11.90	< 0.0001
Postprandial blood sugar	251.5 ± 56.60	128.8 ± 10.03	< 0.0001
Alcoholism history yes	**In T2D**	**Healthy control**	**p-value**
Cholesterol	210.7 ± 45.08	201.6 ± 18.35	0.33
TG	191.1 ± 44.13	136.0 ± 14.46	< 0.0001
LDL	139.1 ± 28	117.2 ± 14.20	0.0004
VLDL	34.22 ± 5.46	24.81 ± 4.36	< 0.0001
Serum uric acid	5.75 ± 2.05	4.19 ± 0.87	0.0004
Bilirubin total	0.87 ± 0.44	0.68 ± 0.20	0.08
Fasting blood sugar	180.4 ± 46.93	93.42 ± 11.73	< 0.0001
Postprandial blood sugar	262.6 ± 55.92	123.3 ± 9.04	< 0.0001
Smoking history yes	**In T2D**	**Healthy control**	**p-value**
Cholesterol	208.2 ± 42.54	203.5 ± 20.04	0.56
TG	185.8 ± 48.01	136.3 ± 24.74	< 0.0001
LDL	139.4 ± 29.59	113.1 ± 12.85	< 0.0001
VLDL	35.09 ± 6.06	25.0 ± 4.08	< 0.0001
Serum uric acid	5.44 ± 1.73	4.23 ± 0.92	0.0002
Bilirubin total	0.82 ± 0.39	0.66 ± 0.21	0.03
Fasting blood sugar	173.7 ± 42.02	92.0 ± 10.86	< 0.0001
Postprandial blood sugar	261.1 ± 57.51	128.3 ± 8.92	< 0.0001

Discussion

Identification of the genetic determinants could facilitate the risk prediction of disease development and the implementation of individualised treatment for therapy. Up to now, genome-wide association studies (GWAS) for diabetes have identified more than 80 susceptibility loci, but only a small part of the heritability of diabetes can be explained by those findings [12]. In the present study we observed a significant difference in the distribution of *CRP* genotype among T2D case and healthy controls, higher mutant allele distribution was observed among T2D cases compared to healthy controls. An independent association of mutant CC genotype and GC heterozygous genotype of *CRP* gene were found to be associated with increased risk of T2D.

It was observed that the CC and GC genotype in patients showed more than 20 and 2 fold increase risk of T2D compared to healthy controls. Patients with different status like hypertension (HTN), doing alcoholism showed more than 4 fold increased the risk of T2D with mutant CC genotypes of the *CRP* gene. However, obesity, smoking had mutant CC genotypes of *CRP* gene showed more than 2 fold higher risk of T2D. Hypertension, alcoholism, obesity and smoking behaviour are suggestive to a possible risk factor for developing T2D. It has been observed that several lipid parameters such as TG, LDL, and VLDL were found to be elevated in T2D cases compared to healthy controls.

Serum uric acid and total bilirubin were found to elevated in T2D compared to healthy controls as the basal values of *CRP* appear to be significantly heritable [13]. D. Thalmaier et al. found that polymorphisms in the CRP gene and in genes controlling *CRP* expression influence *CRP* levels and *CRP* (+1059G/C) polymorphism involved in the same way [14]. It has been observed that +1059 G/C (rs1800947) nucleotide polymorphism (SNP) in exon 2 of the *CRP* gene. +1059G/C is a synonymous polymorphism, which has been reported to affect the protein levels of *CRP* and contribute to the progression of CAD (coronary artery disease) and T2D [15].

Hypertension is recognised globally as a major risk factor for CVD, stroke, diabetes, and renal diseases [16]. Several studies have shown that most of the hypertensive patients have significant instability of serum lipid parameters in hypertensive patients [17, 18]. Smoking may be linked to impaired glucose and lipid metabolism, and a wealth of evidence indicates that insulin resistance, abnormal glucose and lipid metabolism [19]. Parchwani DN et al. reported that glucose and altered lipid profile are directly found to be correlated with smoking and length of smoking years [20], and Haggard also reported that smoking even a single could be the cause of increased blood sugar [21].

It has been suggested moderate alcohol intake increases the risk of type 2 diabetes, especially in Japanese lean subjects [22]. Considering facts that East Asian subjects with normal BMI level (< 25 kg/m^2) can easily develop type 2 diabetes [23], and large differences in the polymorphic distribution of alcohol-metabolizing enzymes have been reported between East Asian and Caucasian, the effects of alcohol intake on type 2 diabetes might vary according to ethnicity [24].

The study concluded that *CRP* gene polymorphism was found to be associated with risk of Type 2 Diabetes and risk was linked with heterozygosity and mutant homozygosity.

Hypertension, Obesity, Smoking and alcoholism significantly altered biochemical parameters in Type 2 Diabetes patients. These interesting results of the study deserve further validation on a larger population.

Acknowledgements

We gratefully acknowledge the study subjects who were part of this study.

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ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2019 Jan 15; 7(1):38-44.
https://doi.org/10.3889/oamjms.2019.019
eISSN: 1857-9655
Basic Science

Balinese Cultivar of Purple Sweet Potato Improved Neurological Score and BDNF and Reduced Caspase-Independent Apoptosis among Wistar Rats with Ischemic Stroke

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Citation: Adnyana IMO, Sudewi R, Samatra P, Suprapta S. Balinese Cultivar of Purple Sweet Potato Improved Neurological Score and BDNF and Reduced Caspase-Independent Apoptosis among Wistar Rats with Ischemic Stroke. Open Access Maced J Med Sci. 2019 Jan 15; 7(1):38-44. https://doi.org/10.3889/oamjms.2019.019

Keywords: Ischemic stroke; Balinese cultivate of purple potato extract; Neurological score; AIF; BDNF; Apoptosis

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Received: 31-Oct-2018; **Revised:** 28-Nov-2018; **Accepted:** 29-Nov-2018; **Online first:** 14-Jan-2019

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

Abstract

BACKGROUND: Ischemic stroke occurs due to the abrupt occlusion in the brain which leads to neuronal death. Neuronal death in ischemic stroke is due to increase production of reactive oxygen species (ROS). Neuronal death occurs via necrosis and apoptosis mechanisms. Apoptosis can either occur via extrinsic or intrinsic pathway. Meanwhile, the intrinsic pathway can be caspase-dependent or independent. Anthocyanin is a natural pigment with antioxidant, anti-inflammatory, anti-cancer, and neuroprotective properties. Balinese cultivate of purple potato extract contains a high level of anthocyanin and has been proven for its antioxidant activity.

AIM: Antioxidant effect of Balinese cultivates purple potato extract has not been studied on an animal model with ischemic stroke. Accordingly, we would like to study the effect of antioxidant properties from Balinese cultivate of purple potato extract by assessing the neurological score, BNDF concentration, and caspase-independent apoptosis by measuring AIF concentration on Wistar rats with ischemic stroke.

METHODS: This was an experimental study using male Wistar rats age between 12-14 weeks weigh between 200 to 250 g.

RESULTS: This study demonstrated a significant difference of neurological score on day 3 among control versus treatment groups. Balinese cultivate of purple potato extract markedly reduced AIF, increased BDNF, and suppressed apoptosis among treatment group when compared with the control group.

CONCLUSION: We have proven the efficacy of antioxidant activity of anthocyanin derived from Balinese cultivar of purple sweet potato by elevated AIF levels, lower apoptosis rate, improved neurological score on day-3 to day-7 post-stroke, as well as increased BDNF levels.

Introduction

Stroke is a degenerative disease leading to number one morbidity and the second cause of mortality worldwide. Ischemic stroke occurs due to the abruption of blood flow in a specific part of the brain which impairs neuronal homeostasis, leading to neuronal death. Neuronal death in ischemic stroke occurs due to increased production of reactive oxygen species (ROS) [1], [2]. Neuronal death in ischemic stroke occurs via either necrosis or apoptosis mechanism. Neuronal death can occur via extrinsic and intrinsic pathway.

Furthermore, the intrinsic pathway can be either caspase-dependent or independent. Also, mitochondria play an important role in apoptosis via extrinsic and intrinsic mechanisms. Intrinsic mechanism occurs via caspase activation, i.e. the release of cytochrome c from mitochondria, which in turn binds to apoptosis platelet activating factor (APAF) to form apoptosome, which then facilitates activation of procaspase 9 into caspase 9. Afterwards, caspase 9 activates procaspase 3 into caspase 3 which then becomes apoptosis executor. Also, mitochondria also play a role in caspase-independent apoptosis which releases proapoptotic proteins, including AIF, Diablo/Smac that triggers DNA fragmentation [3], [4].

Anthocyanin is a natural pigment that belongs to the flavonoid group. It exerts multiple colours to fruits, vegetables, and yams, while also possess intrinsic benefits as an antioxidant, anti-inflammation, anti-cancer, and neuroprotection [5], [6], [7].

Balinese cultivate of purple potato extract contains 209.8 mg/100 g of anthocyanin [8] and has been proven to possess intrinsic antioxidant activity [9]. It also induces intrinsic antioxidant activity [10]. Given the account of oxidative stress as the underlying cause of neuronal apoptosis in ischemic stroke, we would like to study the effects of anthocyanin derived from Balinese cultivate of purple potato extracts against the neurological score, BDNF, and AIF concentration as markers for caspase-independent apoptosis among Wistar rat with ischemic stroke model.

Material and Methods

The process of purple potato extraction was done in the Technological laboratory of farming products at Udayana University with the following described procedure.

Purple potatoes derived from the farmer were then washed with sterile water, peeled for its skin, and subsequently sliced transversally with a thickness of 2 to 2.5 cm. The sliced purple potato then mixed with sterile water with a ratio of 1 kg of purple potato to 1 L of water, and in turn was mixed and liquefied using a blender before filtered using three-layered bands. The obtained filtered water was heated until it boiled for 30 minutes for sterilisation purpose. The anthocyanin content from this sample was 147.0 mg/mL according to the letter from the Technological Laboratory of Farming Products Udayana University no. 145/AN/Lab/FTP/XII/2015.

Wistar rats age between 3-3.5 months weighing 200 to 250 g were obtained from Biopharma Laboratory, Bandung. The study was conducted at the Bioscience Laboratory of Brawijaya University, Malang after had been formally permitted by the Ethical Committee of Brawijaya University, Malang. Animals were acclimatised for a week before put into the bite-resistant cage: twenty rats, each 10 for positive control and treatment group which underwent cerebral artery ligation without and with the administration of purple potato extracts, respectively. AIF and BDNF levels and the number of cerebral cortex neurons which underwent apoptosis were measured from 33 Wistar rats, each of which consists of 11 rats of the negative and positive control, and treatment group. Negative controls underwent neck skin incision and subsequently stitched, while positive controls underwent cerebral artery ligation without purple potato extracts administration, whereas the treatment group underwent cerebral artery ligation with subsequent purple potato extract administration. All animal models were previously anaesthetised using 10 mg/kg body weight of ketamine given intravenously via caudal vein. Ischemic stroke upon Wistar rats was induced by the previously described method [11], i.e. by ligating the right common carotid artery, right external carotid artery, and internal carotid artery on its root to the middle cerebral artery using silk one med soak for 2 hours. The ligation was in turn released after 7 days for reperfusion. Three mL of anthocyanin per day was administered per orally among the treatment group, as soon as there were any neurologic deficits after reperfusion. This was in accordance to the dose used by Jawi et al., [9] for 7 days, only among treatment group. Neurological score among positive control and treatment group were evaluated on the first day at the first, second, and third hour, the third day, and the seventh day. On the eighth day, serum was obtained from animal models to evaluate BDNF and AIF levels, before decapitated using 10 mg per kg of body weight of ketamine using the caudal vein to evaluate the apoptosis.

Neurological score was assessed by well-trained doctoral students of the Veterinary Faculty, Brawijaya University. Neurological score was assessed by using Bederson criteria [12] as follows: rat was picked up by holding the distal tip of its tail and subsequently observed for the position of both of its feet. If both of their frontal feet were aligned, no neurological deficit was assumed. On the other hand, if the contralateral frontal foot from the ligated cerebral artery was in a flexed position, then a neurological deficit was assumed to occur. Rats were then put into a flat cover and given pressure on its back afterwards. Their reactions were then recorded (Table 1).

Table 1: Neurological score

Parameter	Scale	Interpretation
Normal	Level 0	No neurological deficit
Moderate	Level 1	Frontal foot flexion
Severe	Level 2	Resistance toward lateral pressure was decreased with the absence of any rotation.
	Level 3	Identical to level 2, accompanied by rotation.

Coating microplate with protein derived from blood serum was done undercoating buffer for 2 hours on room temperature, before being washed using washing buffer (PBST) 3 x 3 minutes, then put into 50 µL of 1% BSA for 1.5-hour incubation period.

The sample was subsequently washed using washing buffer thrice for each for 3 minutes and added with 100 µL of biotin-antibody BDNF and AIF (rabbit anti-rat BDNF and AIF) afterwards on each well with subsequent incubation for 1.5-hour period on 37°C. The samples were then washed with washing buffer thrice and added with TMB substrate before incubated for 20 minutes under room temperature and added NaOH. Samples were, in turn, read with ELISA reader on 450 nm wavelength.

Rat brain of those had been necropsied was soaked in 4% paraformaldehyde solution for 1-7 days before being dehydrated. The tissue was mixed with 70% alcohol thrice, and each process was left for 10 minutes while being agitated, with subsequent mixing with 80%, 90%, and 95% of alcohol, and absolute alcohol for 10 minutes each while being agitated.

Samples were then added to xylol for clearing process which lasted overnight. Samples were then infiltrated by xylol paraffin and paraffin with a ratio of 1:1 for 30 minutes. Samples were then covered with paraffin to form paraffin block. Liquid paraffin was subsequently added to the paraffin block, which then followed by tissue addition to the block, and waited until the paraffin solidified. The next step was sectioning with 5 µm thickness. The ribbon-like tissue sections were then attached to the object glass which had been wiped with Mayer Albumin.

The next step was deparaffination, in which the paraffin was eliminated using bathing the slide in the xylol for 10 minutes. Samples were soaked into absolute ethanol twice before being put into 96-90-80-70% ethanol, respectively, each for 5 minutes, before rinsed with PBS pH 7.4, thrice, each for 5 minutes. Samples were then soaked in 3% hydrogen peroxide (inside DI water) for 5-10 minutes and subsequently rinsed with PBS pH 7.4, thrice, each for 5 minutes, then soaked into 5% BSA in PBS for 30 minutes on room temperature before finally rinsed again with PBS solution pH 7.4, thrice, each for 5 minutes.

Samples were then mixed with rabbit anti-rat apoptosis and were incubated overnight at 4°C temperature. It was then rinsed again with PBS thrice, each for 3 minutes, and were in turn mixed with the secondary antibody of anti-goat IgG Biotin conjugated. Incubation was done for an hour under ambient temperature and was then rinsed using PBS, thrice, each for 3 minutes, and subsequently, SA-HRP (Strep Avidin-Horseradish Peroxidase) was added to the mixture for 10-20 minutes before rinsed again using PBS, thrice, each for 3 minutes. DAB chromogen substrate (3,3-diaminobenzidine tetrahydrochloride) was added and incubated for 5-10 minutes on ambient temperature and rinsed again using PBS. Counterstain was done using Hematoxylin for 5 minutes on ambient temperature and subsequently washed with flowing water, before dried. It was then mounted with entellan and observed under light microscope BX-53 with 600 times magnification. Calculation method was done using Axio-vision ratio, of which can be seen at http://153.1.200.58:8080/immunoratio/.

Normal data distribution of all research variables included in the study was tested. After data had been known to distribute normally, a mean difference of BDNF, AIF levels, as well as the difference between apoptosis rate between treatment, negative control, and positive control groups were measured and analysed by using one way ANOVA. Meanwhile, the neurological score was analysed in two phases, i.e. the first phase was to evaluate the mean difference of neurological score between control and treatment group over time (head-to-head) which was analysed using independent t-test. The second phase was to evaluate the mean difference of neurological score over time in one group (treatment and control group were analysed separately) by using one way ANOVA test. All data were tested under 95% confidence interval with a p-value of less than 0.05 considered being significant.

Results

In the control group, there were differences of the neurological score between the first day (first, second, and third hour), second, and seventh day **(table 2).** There was no observable difference of neurological score between the first, second, and third hour in the first day (mean difference = 0). However, neurological score differed significantly between the first and third day by 1.4 (95% CI 1.11 – 1.69; $p < 0.001$). Similarly, the mean neurological score day 1 also differed significantly from day 7 by 3 (95% CI 2.71 – 3.29; $p < 0.001$). This trend was also observed among a mean neurological score of day 3 when compared with day 7 which differed by 1.6 (95% CI 1.31 – 1.89; $p < 0.001$) (Table 2).

Table 2: Mean Difference and Significance of Neurological Score based on Timing

Parameter	Time Indicator	Mean Difference	P Value	95% Ci
Control Group				
Day-1 (1st Hour)	Day-1 (2nd Hour)	0	1.0	-0.29 – 0.29
	Day-1 (3rd Hour)	0	1.0	-0.29 – 0.29
	Day-3	1.4	< 0.001	1.11 – 1.69
	Day-7	3	< 0.001	2.71 – 3.29
Day-3	Day-7	1.6	< 0.001	1.31 – 1.89
Treatment Group				
Day-1 (1st Hour)	Day-1 (2nd Hour)	0	1.0	-1.06 – 1.06
	Day-1 (3rd Hour)	0.6	0.50	-0.46 – 1.66
	Day-3	2	< 0.001	0.94 – 3.06
	Day-7	2.6	< 0.001	1.54 – 3.66
Day-3	Day-7	0.6	0.50	-0.46 – 1.66

Neurological score of the first hour vs. third hour on the first day did not differ significantly among treatment group (mean: 0.6; 95% CI 0.46-1.66; $p = 0.50$). Nevertheless, neurological score of day-1 vs day-3 differed markedly by 2 (95% CI 0.94-3.06; $p < 0.001$). Meanwhile, neurological score on day 1 also differed significantly when compared with day 7 by 2.6 (95% CI 1.54-3.66; $p < 0.001$). Neurological score between day 3 and 7 also differed though statistically insignificant by 0.6 (95% CI -1.66-0.46; $p < 0.50$) (Table 2).

Table 3: Mean difference and Significance of Neurological Score between Control and Treatment Group over time (*head-to-head*)

Parameter	Control Group	Treatment Group	Mean difference	95% CI	P value
1st hour score	3	2.60	0.40	-0.16 – 0.96	0.15
2nd hour score	3	2.60	0.40	-0.16 – 0.96	0.17
3rd hour score	3	2	1	0.17 – 1.83	0.023
Day 3 score	1.6	0.6	1	0.33 – 1.67	0.006
Day 7 score	0	0	0	0	0

Neurological score difference from day to an hour between control and treatment group

demonstrated that 1st-hour score on day 1 between control and treatment group differed by 0.4, although statistically insignificant (95% CI -0.16-0.96; p = 0.15). Whereas 2nd-hour score of both groups also differed by 0.4 but statistically insignificant (95% CI -0.16-0.96; p = 0.17). In contrast, neurological sore on the 3rd hour began to show gap by 1 and was statistically significant (95% CI 0.17-1.83; p = 0.023) (Figure 1).

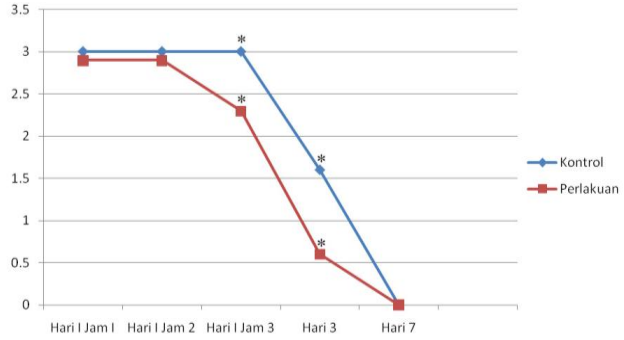

Figure 1: Mean neurological score difference between treatment and control group over time (indicates significance, p < 0.05)*

Mean BDNF levels between treatment and positive control differed markedly by 965.45 (95% CI 909.44 – 1,021.46; p < 0.001).

Table 4: BDNF, AIF, and Apoptosis

Parameter	Reference Indicator	Comparator	Mean difference	P value	95% CI
BDNF	Treatment	Positive control	965.45	< 0.001	909.44 – 1,021.46
		Negative control	-916.99	< 0.001	-973.00 – -860.99
	Negative control	Positive control	1.882.45	< 0.001	1,826.44 – 1,938.46
AIF	Treatment	Positive control	-4.02	< 0.001	-4.06 – -3.98
		Negative control	2.30	< 0.001	2.26 – 2.34
	Negative control	Positive control	-6.32	< 0.001	-6.36 – -6.28
Apoptosis	Treatment	Positive control	-17.86	< 0.001	-26.04 – -9.69
		Negative control	1.99	0.821	-6.18 – 10.16

Meanwhile, average BDNF levels between treatment and negative control group were also found to differ significantly by -916.99 (95% CI -973.00 – -860.99; p < 0.001). Mean BDNF levels between positive and negative control group also differed significantly by 1,882.45 (95% CI 1,826.44 – 1,938.46) [Figure 2 (left)].

According to the one-way ANOVA test, AIF levels differed markedly between negative control, positive control, and treatment group. The mean AIF levels between treatment and positive control differed markedly by -4.02 (95% CI -4.06 – 03.98; p < 0.001), while AIF levels between treatment and negative control group differed significantly by 2.30 (95% CI 2.26 – 2.34; p < 0.001). In addition, mean AIF levels between negative and positive control group also differed by -6.32 (95% CI -6.36 – 6.28; p < 0.001) [Figure 2 (right)].

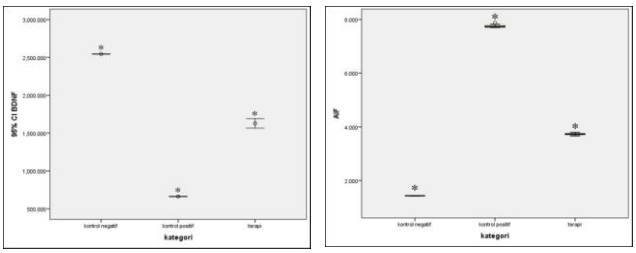

Figure 2: Mean difference of BDNF levels between treatment, negative and positive control group (indicates significant results at p < 0.05) (left); Mean difference of AIF levels between treatment, negative and positive control group (* indicates significant results at p < 0.05) (right)*

The number of cells which underwent apoptosis between treatment and negative control group was found to differ insignificantly with mean of 1.99 (95% CI -6.18 – 10.16; p = 0.82), whereas the mean apoptosis rate between treatment and control group was 17.86 and significant statistically (95% CI -26.04 – -9.69; p < 0.001) (Figure 3).

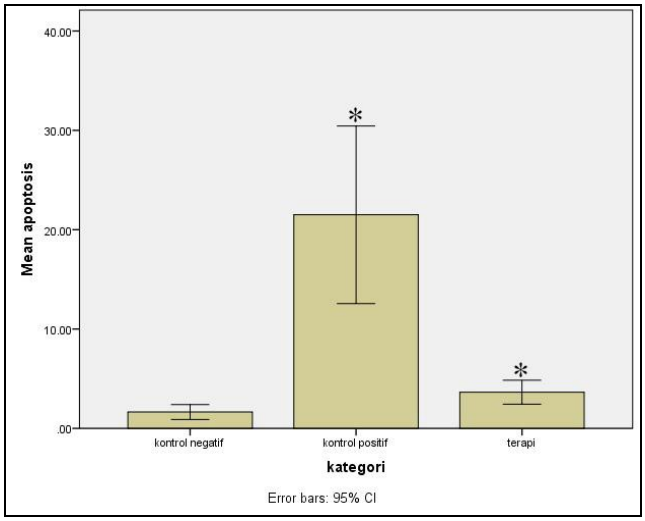

Figure 3: Mean rate of apoptosis between negative and positive control, and treatment group (indicates significant results at p<0.05)*

Discussion

Middle cerebral artery occlusion leads to the neurological deficit, infarction, and apoptosis which were regarded as important indicators for brain damage after ischemia [13]. Cerebral artery occlusion induces cerebral ischemia with subsequent mitochondrial damage that stimulates ROS production. ROS is a key factor in the underlying pathophysiology of ischemia, trauma, and degenerative disease. ROS induces macromolecular damage, including lipid peroxidation, protein oxidation, and DNA oxidation, all of which lead to cellular

damage and death (necrosis and apoptosis) [14]. Apoptosis occurs both via extrinsic and intrinsic pathway. The intrinsic mechanism involves mitochondria after receiving stimulus from Bcl-2 protein family, BIH only protein, such as Bid, Ba, and Bim. Stimuli from these proteins will induce oligomerisation of pro-apoptotic proteins, including Bax and Bak. Bak oligomerisation and Bax will induce changes in the outer mitochondrial membrane permeability which in turn releases proapoptotic protein, including cytochrome c, endonuclease G, apoptosis induction factor (AIF), a second mitochondrial activator of caspase/direct IAP binding protein with low pl (Smac/diablo). Cytochrome c will bind to apoptosis platelet activating factor (APAF) which then forms apoptosome which further activates procaspase 9 into caspase 9, and subsequently activates caspase-3 as an executor caspase. AIF can induce caspase-independent apoptosis which spontaneously triggers DNA condensation and fragmentation [3], [4].

A pharmacological drug with antioxidant properties that can inhibit ROS production has a role in preventing brain tissue destruction) [15]. Anthocyanin belongs to polyphenolic class compound easily found in fruits, leaves, and vegetables. Both epidemiological data and clinical studies have confirmed anthocyanin efficacy in preventing multiple types of diseases. For instance, individuals who consume high levels of anthocyanin were linked with lower arterial stiffness rate [16]. Also, young women who consume high anthocyanin concentration also has a lower risk of getting a myocardial infarction [16] and Parkinson's disease [17]. Anthocyanin can be commonly derived from purple sweet potato and possess a neuroprotective effect by inhibiting ASK1-JNK/p38 and neutralise ROS, thus preventing apoptosis [7]. Balinese cultivar of purple sweet potato possesses anthocyanin and thus antioxidant and anti-inflammatory properties [9]. Antioxidant activity will suppress ROS production, hence minimising mitochondrial damage and apoptotic cascade. This was proven by the significantly lower AIF levels ($p < 0.05$) among the treatment group. Lower AIF levels also responsible for the significantly lower apoptosis rate among the treatment group ($p < 0.05$) (Figure 4). The lower rate of apoptosis also reduces neurological score among anthocyanin-treated mice (Figure 1).

BNDF is a neurotrophic factor which plays a central role in the recovery phase after stroke. BDNF is a signal molecule which is crucial in neuronal adaptation and plasticity post-stroke [18]. BNDF is involved in neuronal viability, synaptic plasticity, learning, memory, and neuronal plasticity. BDNF treatment in animal models or rehabilitation has been shown to increase BDNF and improve neuronal recovery after injury/trauma. Neuronal improvement after cerebral trauma depends on BDNF levels because it is a critical neuroprotectant against cerebral ischemia which on *in vivo* study acts as anti excitotoxicity and inhibits inflammation, thus reducing apoptosis [19]. BDNF maintains neuronal viability via activating 2 surface receptors, i.e. tropomyosin-related kinase B (TrkB) and p75 neurotrophin (p75NTR). BDNF will, in turn, activates intracellular signalling pathway such as PI3K/Akt using TrkB receptor, which influences central nervous system development and function [20]. PI3K/Akt signalling pathway promotes cellular growth and viability by suppressing the activities of proapoptotic protein family of Bcl-2 [21]. Also, Yao et al., [22] found an increase of BDNF expression via TrkB and Akt activities, thus ameliorating apoptotic rate among mice models with focal cerebral ischemia treated with one of the flavonoid groups, i.e. quercetin. It is known that pharmacological therapy can modify the BDNF signalling pathway, including cAMP response-element binding protein (CREB) which facilitates cortical reorganisation and functional recovery after ischemic stroke [23]. We found significantly higher BDNF levels among treatment compared to positive control groups ($p < 0.05$), thus in line with lower apoptosis levels found among treatment groups and better neurological score on day-3 among treatment as opposed to control groups ($p < 0.05$).

AIF is a proapoptotic protein released from mitochondria in the cytosolic and nucleus along with EndoG. Its released level is highly dependent on familial proapoptotic protein activity Bcl2 on mitochondria. Also, AIF is capable of inducing caspase-independent apoptosis by directly binds with DNA chromosome and induce chromatin condensation and remodelling, all of which facilitates DNA fragmentation by nuclease enzyme such as Endo G [24]. Apoptosis via mitochondria is induced by increased ROS production and inhibiting ROS as a representative of oxidative stress using antioxidant (such as anthocyanin in the form of cyanidin-3-O-glucoside) has been proven to inhibit mitochondrial AIF release, thus ameliorate apoptosis among mice with focal cerebral ischemia [25]. Furthermore, AIF was found significantly different between negative and positive controls ($p < 0.01$). AIF levels between positive controls and treatment group were also found to be significantly different ($p < 0.001$).

Apoptosis can occur via both extrinsic and intrinsic pathway. Apoptosis in ischemic stroke is one mechanism of cellular death among others, as a consequence of excessive ROS production which impairs mitochondrial outer membrane permeability, thus triggering Bax translocation from the cytoplasm to mitochondria and subsequent release of cytochrome c into the cytoplasm. Proapoptotic protein translation is governed by the proapoptotic protein family of Bcl-2 [26], [27].

Apoptosis via the intrinsic pathway consists of two mechanisms, i.e. caspase-dependent and caspase-independent. Caspase-dependent apoptosis occurs due to Bcl-2 proapoptotic protein family signalling, e.g. BH3 only, which then activates Bax

and Bak protein to form oligomer in the mitochondrial outer membrane, thus changing its permeability which triggers the release of proapoptotic proteins including AIF and Endo G to the cytoplasm which then activates apoptotic cascade [28].

Anthocyanin is capable of suppressing excessive ROS production, thus ameliorating ROS damaging effects, including the activation of apoptosis cascade [29], [30]. Anthocyanin derived from Balinese cultivar of purple sweet potato also possesses antioxidant properties as well as inducing endogenous antioxidant production [9], [10], thus limiting apoptosis cascade as demonstrated by lower AIF levels among treatment group ($p < 0.05$), Figure 4.

In conclusion, anthocyanins derived from Balinese cultivar of purple sweet potato possesses antioxidant activity, thus able to inhibit ROS damaging effects among ischemic stroke model of Wistar rats. We have proven the efficacy of antioxidant activity of anthocyanin derived from Balinese cultivar of purple sweet potato by elevated AIF levels, lower apoptosis rate, improved neurological score on day-3 to day-7 post-stroke, as well as increased BDNF levels.

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ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2019 Jan 15; 7(1):45-49.
https://doi.org/10.3889/oamjms.2019.018
eISSN: 1857-9655
Clinical Science

Serum Interleukin-27 Level in Different Clinical Stages of Lung Cancer

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Citation: Soleimani Babadi A, Kiani A, Mortaz E, Taghavi K, Khosravi A, Marjani M, Seifi S, Emami H, Abedini A. Serum Interleukin-27 Level in Different Clinical Stages of Lung Cancer. Open Access Maced J Med Sci. 2019 Jan 15; 7(1):45-49. https://doi.org/10.3889/oamjms.2019.018

Keywords: Interleukin 27; Lung Cancer; cytokine; NSCLC; SCLC; Serum

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Received: 27-Oct-2018; **Revised:** 28-Nov-2018; **Accepted:** 29-Nov-2018; **Online first:** 05-Jan-2019

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

Abstract

BACKGROUND: Advanced lung cancer is indicated with rapid disease development. Interleukin 27 (IL-27) is regarded as a cytokine with anti-tumour activities.

AIM: Since, the impact of type of lung cancer on the level of IL-27 in patient's serum has not yet been investigated; current study evaluated the clinical stages according to American Joint Committee on Cancer (AJCC) criteria, Tumor-Node-Metastasis (TNM) stage and the lung cancer spread (localized or widespread) and it's correlation with serum IL-27.

MATERIAL AND METHODS: Thirty patients with confirmed histopathological lung cancer and 30 cancer-free healthy individuals as the control group were included in the current study. Patients group were assigned to either small cell lung cancer group (SCLC) or non-small cell lung cancer (NSCLC) according to the clinical features and the results of lung biopsy specimens. Level of IL-27 was quantified with enzyme-linked immunosorbent assay (ELISA) test in serum samples.

RESULTS: A significant increase in serum IL-27 level was noticed in individuals with lung cancer in comparison with the control group. The level of serum IL-27 in the NSCL squamous carcinoma (NSCLC-Sc) type was significantly greater than in the NSCLC adenocarcinoma (NSCLC-Ad) type, and in both groups, this variable was more than the control group. The serum IL-27 content level was greater in stage III versus stage IV.

CONCLUSION: The current research confirmed the existence of the anti-tumour components in patients with NSCLC. IL-27 can be utilised in diagnosis and screening in early stages of lung cancer along with the management of patients. Different levels of IL-27 in different types of lung cancers in the current study can lead to design more comprehensive studies in the future.

Introduction

Cancer is still a global critical public health issue. Lung cancer is one of the most prevalent cancer types, leading to almost 18% of all cancer-related mortality across the world. Most patients show a poor prognosis. Therefore, diagnosis of a remarkable number of patients happens at advanced stages of the disease [1], [2]. In Iran, after car accidents and cardiovascular diseases, lung cancer is the third leading cause of death [3]. The aetiology of lung cancer remains inadequacy explained. The pathogenesis of lung cancer, however, is multifactorial. Lung cancer is divided into two main types: Small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), which is subdivided into NSCLC adenocarcinoma (NSCLC-Ad), NSCL squamous carcinoma (NSCLC-Sc), and NSCLC large cell carcinoma (NSCLC-LC) [4].

NSCLC accounts for nearly 80% of cases, with an overall 5-year survival rate of 13% [5], [6].

SCLC is an aggressive tumour that is known for it's extensive and rapid metastatic dissemination and recurrence the following chemotherapy with a poor prognosis [7]. Surgery, chemotherapy and radiotherapy are the primary treatments for advanced NSCLC [8].

A definitive diagnosis is confirmed by tissue sampling that is often carried out by bronchoscopy [9]. Delay in diagnosis or treatment of lung cancer results in dissemination into the lung through metastasis. Early diagnosis of cancers, especially lung cancer, can significantly reduce mortality. Diagnosis of lung cancer is difficult in the early stages. Cell and chest mucus pathological tests, chest radiography (CTX) and CT-scan are some diagnostic methods for lung cancer. Non-invasive markers that are cost-effective and the new molecular and immunological diagnostic methods are crucial in the early stages of the disease [10].

Accumulating evidence shows that inflammation plays an important role in developing of the lung cancer, mainly in the induced cases by cigarette smoke and other noxious particles and gases [11], [12]. Immunoregulatory cytokines may play a fundamental role in tumour growth and metastases. The association of pro- and anti-inflammatory cytokines with histology type- or smoking-independent lung cancer risk has been demonstrated [13], [14]. Interleukin (IL)-27, which is a recently discovered member of the IL-12 family of cytokines, has been reported to exhibit anti-tumour activity in different preclinical test models because of anti-angiogenic, antiproliferative, and immune-enhancing effects [1], [15]. IL-27 is a heterodimer cytokine containing the Epstein-Barr virus-induced gene 3 (EBI3) and IL-27p28, which engages a receptor including gp130 and the IL-27Rα that activates activator of transcription (STAT) and Janus kinase (JAK)-signal transducer [16].

Few studies have been conducted to investigate the effect of IL-27 on lung cancers, with conflicting results, while most of them have been conducted on the mouse or in vitro and few of them have been conducted in vivo [17], [18], [19]. Given the importance of detecting lung cancer in the early stages and imprecision of some available diagnostic methods, it will be helpful to find a simple diagnostic procedure in which the serum is used.

In this regard, due to the non-toxicity of IL-27 in previous clinical studies and the discovery of its anticancer properties in other examined tumours [20], the present study investigated the use of IL-27 as an approach to the diagnosis of lung cancer. This study is also the first one to examine the association between IL-27 concentration and lung cancer according to the type of tumour. The concentration of IL-27 in various stages of lung tumour will also be investigated. The association between IL-27 in the circulatory system and lung cancer can be helpful in screening, evaluating and treating patients with pulmonary involvement symptoms.

Material and Methods

In this study, from September 2018 to September 2019, inclusion criteria were suffering from lung cancer with unknown causes and the age of over 18 years.

Definitive diagnosis of lung cancer for the referred patients was made based on clinical findings, the diagnosis made by an oncologist, and also histopathological examination on biopsy or tissue samples removed in lung surgery. Individuals with inflammatory or infectious diseases were excluded from the study. In the control group, individuals with a history of autoimmune or malignant disorders and those with a family history of lung cancer were not included in the study.

The sample size was calculated by using a cross-sectional study sample size calculation formula (Standard effect size = 1.1, α = 5%, β = 20%, and confidence interval = 95%). Then, 30 patients with lung cancer and 30 healthy non-cancer patients, as a control group [21], were selected by convenience sampling. The participants of the two groups were selected in a way that they were matched by age and sex.

The study protocol was confirmed at the Ethics Committee. After obtaining written consent to participate from the participants, demographic data were collected. Before treatment, 4 ml of complete venous blood samples of patients were collected in anticoagulant free tubes. In the laboratory, a serum sample was prepared and stored in a refrigerator at -20°C. The serum level of IL-27 of the samples isolated by enzyme-linked immunosorbent assay was evaluated using a kit (R & D Co.).

Patients, who were enrolled in the study, were diagnosed as either NSCLC or SCLC according to the results of their clinical specimens and a sample of lung biopsy. Clinical stages of patients were performed according to American Joint Committee on Cancer (AJCC) criteria, Tumor-Node-Metastasis (TNM) stage and the disease spread (localised or widespread) [22].

Data were statistically analysed by Statistical Package for Social Sciences (SPSS) (ver. 22.0; SPSS Inc. Chicago, IL, USA) software. Statistical analyses were conducted following international statistical standards. Continuous variables were expressed as mean ± standard deviation. Differences between two groups were analysed using the Mann – Whitney test for continuous variables. A p-value of < 0.05 was considered to be statistically significant.

Results

In this study, 30 patients with lung cancer were studied to investigate the relationship between cytokine IL-27 and lung cancer. 13 lung cancer patients had NSCL squamous carcinoma, and 11 had NSCLC adenocarcinoma (Figure 1).

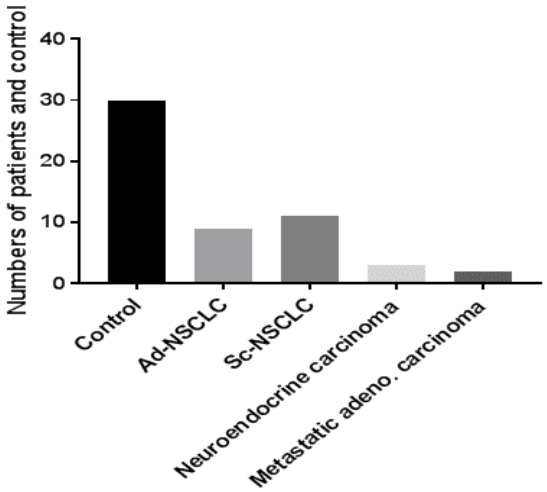

Figure 1: IL-27 values in lung cancer patients according to TNM classification. Bars express mean IL-27 value ± SD, pg/ml

An increase in serum IL-27 level was observed in patients with lung cancer in comparison with the control group with the Mann Whitney test. This study showed that the serum level of IL-27 in NSCLC-Sc type was higher than that in NSCLC-Ad type ($P < 0.05$). There was a significant difference between IL-27 level in control groups, NSCLC-Ad, and Sc-NSCLC ($P < 0.05$). There was also a significant difference between NSCLC-Ad group and NSCLC-Sc group ($P < 0.05$) regarding IL-27 level.

As illustrated in Figure 2, serum IL-27 levels are higher in the squamous cell carcinoma group than in the adenocarcinoma groups, and also are higher in both groups than in the control group.

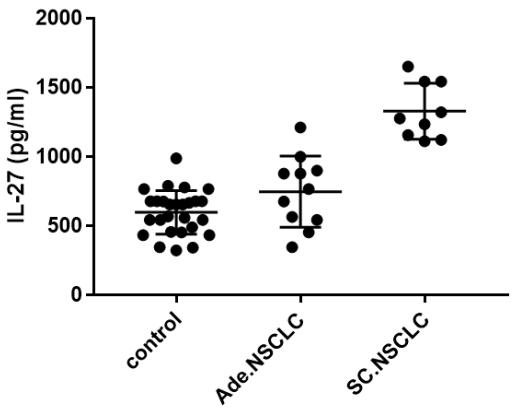

Figure 2: Serum level of Interleukin-27 in patients with lung cancer in comparison with the control group ($p < 0.05$, Mann-Whitney test)

In the next step, the IL-27 levels in the two groups were examined with the Mann-Whitney test according to different stages of the disease. Patients with III clinical stage showed a higher IL- 27 level in comparison with patients with clinical stage IV (Figure 3).

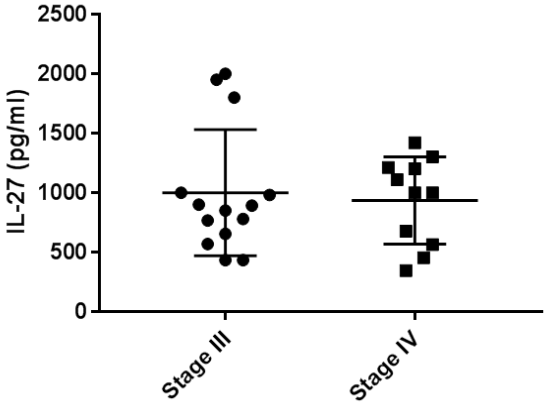

*Figure 3: IL-27 levels of lung cancer patients classified according to the AJCC. Values are expressed as mean ± SEM; *p < 0.05; **p < 0.01; c–controls*

Discussion

Several studies have recently suggested the role of IL-27 polymorphisms in the risk of developing different types of cancer such as nasopharyngeal carcinoma, hepatocellular carcinoma, oesophagal cancer, ovarian cancer, cervical cancer, and glioma with conflicting results [15], [23] but its role in lung cancer has not yet been studied. In this study, 30 patients with lung cancer and 30 healthy individuals participated. The primary aim of this study was to investigate the serum level of IL-27 in these two groups. The current study showed that the overall level of IL-27, irrespective of the type of cancer, was higher in patients with lung cancer than in the control group. NSCLC-Sc was the most frequent type of lung cancer among the samples. This type of lung cancer seems to have a higher prevalence in society so that in a study in China, most patients with NSCLC had squamous cell carcinoma (58.2%), and the rest were found to have adenocarcinoma (41.8%) [1], which is consistent with the present study.

It has recently been found that IL-27 produces strong antitumor effects against different tumour models through a variety of mechanisms in various cancer cell lines and in vitro, not only by exerting direct effects on the tumor cells but also by activating anti-tumor immune responses [20], including IL-27 antitumor, anti-inflammatory and antiangiogenic effects-mediated activities and activation of natural killer (NK) cells antimetastatic activities [21].

IL-27 antitumor components were also observed in lung cancer in another study. IL-27 seems to increase IFN-γ production that is necessary to differentiate TH0 to T-helper-1 (Th1) cells and induce cellular immune system [22].

Another aim of the present study was to investigate the level of IL-27 in various stages of lung cancer. In this regard, an increase in serum IL-27 level was observed in stage III of the disease compared to stage IV. This could indicate the importance of a tumour being concentrated in a part of the lung and metastasis has not been developed. In previous studies, it was also found that IL-27 will be very high if a pulmonary tumour is small and at the earlier stages [19].

Naumnik et al. did not observe any differences in IL-27 concentrations between patients with late-stage lung cancer and controls, and they also noted an insignificant increase in IL-27 in serum samples of patients of stage IIIB compared to those of stage IV [17]. Barrera et al. studied the association of cytokine profile with survival prognosis in serum samples of 110 NSCLC patients. Out of the investigated cytokines, IL-27 was significantly higher in non-smoker patients, the patients of IIIB stage, patients without CNS metastases, and those with positive pleural effusion [13], which is consistent with our study.

Karlicic et al. found that mean IL-27 concentration was significantly higher in healthy control people than in lung cancer patients and the severity of the disease dissemination was significantly correlated with IL-27 levels [19].

In the current study, IL-27 was indicated as a beneficial prognosis parameter in the early diagnosis of lung cancer. IL-27 was shown as a beneficial indicator in early diagnosis of lung cancer. Several previous studies have reported an increased level of serum IL-27 in association with several diseases, such as Sarcoidosis, allergic alveolitis and mainly pulmonary tuberculosis, which is also very life threatening and may result in death due to hemoptysis. Therefore, it is obligated to differentiate lung cancer from other diseases with similar manifestations. Tuberculosis and most of lung cancer types differ significantly in scientific signs, paramedical diagnostic strategies, direct sputum smear and the Ziehl–Neelsen stain, chest X-ray, CT [5], [7], [9], [10], [23].

Another aim of the study was to investigate the relationship between the level of IL-27 and the type of a lung tumour. To the best of our knowledge, this aim has not yet been taken into account in previous studies. In this study, examinations of serum samples and histopathology examinations of damaged tissues showed that the level of IL-27 was higher in Sc-NSCLC type than in NSCLC-Ad, which was not shown before. However, in contrast to the results of this study, Duan et al. observed a reduction of serum IL-27 concentration in NSCLC patients in comparison with the healthy controls [20]. The differences in the results can be due to the difference in the stage of cancer in the cited studies.

Also, the important findings of the current study and the higher levels of IL-27 in NSCLC-Sc than in NSCLC-Ad could be the reason for inconsistencies in the results of preceding studies, especially because the type of cancer was not addressed in past studies. Due to the role of lung cancer in serum IL-27 levels, discovered here, current study could be followed by greater comprehensive studies with larger sample size. However, a combination of different cytokines seems to play a role in the development of complex anti-inflammatory and anti-tumour properties in lung cancer and determine the prognosis of the disease.

In conclusion, the current study suggests that IL-27 can be used as an early diagnostic and screening tool for lung cancer. IL-27 seems to be a good indicator of for treatment response in lung cancer patients. In the current study, the differences in the level of IL-27 in different types of lung cancer was observed for the first time, which could be the basis for designing more comprehensive studies with larger sample sizes.

Acknowledgement

The current study was obtained from a subspecialty thesis of pulmonology. At this moment, we like to express our grateful appreciation.

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ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2019 Jan 15; 7(1):50-52.
https://doi.org/10.3889/oamjms.2019.011
eISSN: 1857-9655
Clinical Science

Vaspin in Developing Obesity (Vande-Ob); the Correlation of Waist Circumference and Visceral Fat Percentage with Vaspin Levels in Patients with Type II Diabetes Mellitus

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Citation: Dwipayana IMP, Semadi IMS, Gotera W, Saraswati MR, Suastika K. Vaspin in Developing Obesity (Vande-Ob); the Correlation of Waist Circumference and Visceral Fat Percentage with Vaspin Levels in Patients with Type II Diabetes Mellitus. Open Access Maced J Med Sci. 2019 Jan 15; 7(1):50-52. https://doi.org/10.3889/oamjms.2019.011

Keywords: Vaspin; Waist circumference; Visceral fat percentage; Type II diabetes mellitus

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Received: 11-Sep-2018; **Revised:** 21-Nov-2018; **Accepted:** 22-Nov-2018; **Online first:** 04-Jan-2019

Funding: This study funded by the Research and Development Unit, Medical Faculty of Udayana University, Denpasar, Bali, Indonesia

Competing Interests: The authors have declared that no competing interests exist

Abstract

BACKGROUND: Vaspin concentration was thought to be associated with obesity, impaired insulin sensitivity, and fitness level. The correlation of vaspin and leptin supports the theory of vaspin associated with body fat mass.

AIM: To determine the correlation between visceral fat distributions and serum vaspin level in type II DM patients.

METHODS: We conduct an observational, analytical cross-sectional study. Sixty subjects with type II diabetes mellitus who came to Diabetes Center of Sanglah General Hospital were included consecutively. Each subject has to sign an informed consent before physical and laboratory examination took place. Spearman correlation test was used to analyse the correlation between waist circumference and visceral fat percentage with serum vaspin level since the data were not distributed normally.

RESULTS: Mean laboratory results in all subjects of vaspin levels was 2.389 ± 3.586 ng/ml, mean waist circumference was 94.95 ± 11.78 cm and mean visceral fat percentage was 18.05 ± 23.63%. We found we found no significant correlation between between vaspin with waist circumference (r = -0.044; p = 0.738) and visceral fat percentage (r = -0.103; p = 0.435).

CONCLUSIONS: The vaspin level did not significantly correlate with waist circumference and visceral fat percentage in type II diabetes patients.

Introduction

Diabetes mellitus (DM) is one of medical and social health problems both in developed and developing countries. The prevalence is increasing, and approximately 4% of the world population suffered from DM. This disease is closely related to obesity and endocrinal activity of adipose tissue [1].

The association between the increase of body weight and waist-hip ratio (WHR) with the incidence of impaired glucose tolerance, dyslipidemia (primarily hypertriglyceridemia) and hypertension firstly reported in detail at various population-based studies in the early 1980s. The combination of symptoms, which are known as metabolic syndrome, is reported as a major cause of the global epidemic and death caused by DM and cardiovascular disease. However, those studies have not comprehensively described the involvement of adipose tissue with glucose metabolism [1], [2], [3].

The visceral fat tissue is not only involved as fat-storage but also as an active endocrine organ, in which the occurrence of obesity is causing hyperplastic changes in this tissue [3]. Vaspin (visceral adipose tissue-derived serine protease inhibitor) is the most recent adipocytokine exclusively expressed by rat visceral fat tissue, which is Otsuka Long-Evans Tokushima Fatty (OLETF), an experimental animal

model for obesity and types II DM. Vaspin is a family of serine protease inhibitor. In human, the expression of vaspin found in adipose, gaster, liver and pancreatic tissue. Vaspin also found at the hypothalamus of db/db and C57BL/6 mouse [4].

The increase of serum vaspin concentration associated with obesity, impaired insulin sensitivity, and fitness level, and insulin resistance. Serum vaspin also significantly correlated with leptin, thus support the theory that vaspin associated with body fat mass. However, other studies found no association between serum vaspin with insulin sensitivity or obesity parameters and fat distribution. In one study, serum vaspin levels significantly correlated with fasting insulin, HOMA-IR and ratio of visceral and subcutaneous vaspin expression however, if compared to obese patients with sensitive insulin and resistance after matching with BMI, age and gender, no significant difference of vaspin levels were found in both groups. This suggests that the association between serum vaspin levels, fat distribution and insulin sensitivity is far more complex than expected [5].

According to the data above, to date, the association between the distribution of visceral fat with vaspin serum remains a controversy and their association in type II DM patients remain unclear, hence a study to determine the correlation between visceral fat distribution (measured with waist circumference and visceral fat percentage) with serum vaspin level in type II DM patients is needed.

Methods

This was an observational, analytical cross-sectional study. This study was approved by the Ethics Commission for Research at Medical Faculty of Udayana University, Denpasar Bali. The subjects were 60 type II diabetes mellitus patients who came to Diabetes Center of Sanglah General Hospital, Denpasar Bali. Samples were taken consecutively based on the order of the patients which came to Diabetes Center until minimal sample required achieved.

Table 1: Characteristic of Subjects

No	Variables	Mean	Standard deviation	Unit
1	Vaspin	2.389	3.586	ng/ml
2	Waist circumference	94.95	11.78	Cm
3	Visceral fat percentage	18.05	23.63	%

Each subject was asked to sign an informed consent. The identity of the patients was recorded and followed by physical examination and laboratory examination. History of diabetes, duration of having diabetes, medication history (including diabetic drugs) were asked — physical examination including vital signs, abdominal circumference and visceral fat percentage measurement with bioelectric impedance. Blood samples then collected to measure serum vaspin concentration, performed with "Human/Mouse/rat SERPINA12/Vaspin (Competitive EIA) kit" from Life Span Bio Sciences, Inc. Spearman correlation test was used to analyse the correlation between waist circumference and visceral fat percentage with serum vaspin level since the data were not distributed normally.

Results

Mean vaspin levels in all subjects was 2.389 ± 3.586 ng/ml, mean waist circumference in all subjects was 94.95 ± 11.78 cm and mean visceral fat percentage was 18.05 ± 23.63%.

Table 2: Correlation of Vaspin with Waist Circumference and Visceral Fat Percentage

No	Variables	R	P
1	Waist circumference	-0.044	0.738
2	Visceral fat circumference	-0.103	0.435

No significant correlation was found between vaspin with waist circumference (r = -0.044; p = 0.738) and visceral fat percentage (r = -0.103; p = 0.435).

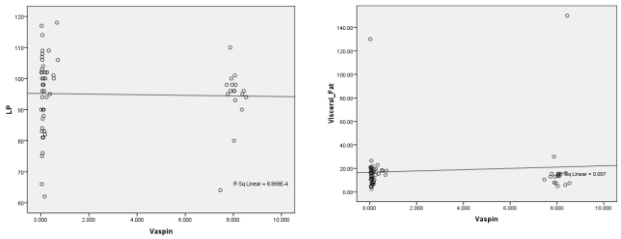

Figure 1: Scatter Plot Correlation between Waist Circumference and Serum Vaspin (left); Scatter Plot Correlation between Visceral Fat Percentage and Vaspin Levels (right)

Discussion

Vaspin (*visceral adipose tissue-derived serine protease inhibitor*) is the most recent adipocytokine exclusively expressed by visceral fat tissue of Otsuka Long-Evans Tokushima Fatty (OLETF) mouse, an experimental animal model for obesity and type II DM. Vaspin is a family of serine protease inhibitor. Serum vaspin concentration decreases significantly along with an increase in age and severe hyperglycemic condition. This process could reduce with insulin therapy or pioglitazone in human, the expression of vaspin found in adipose tissue, gaster, liver, and pancreas. The expression of vaccine also found at the

hypothalamus of db/db and C57BL/6 mouse. The administration of vaspin recombinant on the obese mouse may improve the glucose tolerance and increase insulin sensitivity by affecting the expression of gene candidate for insulin resistance and rapidly decrease food intake [4].

The exact mechanism on how vaspin associated with worsening of glucose homeostasis and insulin sensitivity remains unclear. Based on existing data regarding vaspin mechanism of action, it has been suggested that vaspin inhibit proteases which involved in hormone or molecule degradation which decreased glucose either direct or indirect — the increase of serum vaspin levels associated with obesity, impairment in insulin sensitivity and fitness level. Serum vaspin also significantly correlated with body fat mass. In a female with polycystic ovary syndrome and insulin resistance, the administration of metformin may decrease serum vaspin levels and improve insulin sensitivity.

The result of the current study obtained that mean vaspin levels was 2.389 ± 3.586 ng/ml. This result was higher compared to other studies in Asia. One study in Bangladesh found that mean levels of serum vaspin was 0.83 ± 0.28 ng/ml, while a study in Turkey and China found 0.18 ± 0.10 ng/ml and 0.69 ± 0.31 ng/ml, respectively. These shows that vaspin levels differ in various populations [5].

This study obtained no significant correlation of vaspin with parameters of adipose tissue, e.g., waist circumference can visceral fat percentage. The correlation between vaspin and parameters of adipose tissue remain controversial. Some research found no association between serum vaspin with insulin sensitivity or parameters of obesity and fat distribution. In one study, serum vaspin levels significantly correlated with fasting insulin, HOMA-IR and ratio of visceral and subcutan vaspin expression. However, if compared to insulin-sensitive and insulin-resistance obese patients after matching with BMI, age, and gender, no significant difference was found in both groups. This suggests that the association between serum vaspin levels, fat distribution and insulin sensitivity is far more complex than imagined [5].

The different result was also affected by the type of adipose tissue which produces vaspin. The expression of vaspin mRNA detected at visceral and subcutan adipose tissue. Visceral vaspin mRNA correlated with body mass index, body fat percentage, and plasma glucose levels 2 hours after an oral glucose tolerance test, but not correlated with waist circumference or waist-hip ratio. While subcutaneous vaspin mRNA correlated with the waist-hip ratio, fasting insulin plasma concentration, and glucose delivery. But linear regression analysis showed body fat percentage as the most powerful predictor of visceral vaspin, while insulin sensitivity as the most powerful predictor for subcutaneous vaspin [6]. Visceral and subcutan vaspin were not distinguished in the current study. Thus the relationship between both with adipose tissue parameters could not be depicted through this study.

In conclusion, vaspin did not significantly correlate with waist circumference and visceral fat percentage in diabetes patients. Further study with more samples to determine characteristics of vaspin in various ethnicity and race is needed and differentiate the subcutaneous, and visceral vaspin is needed.

Acknowledgements

We acknowledge the Research and Development Unit, Medical Faculty of Udayana University, Denpasar, Bali, Indonesia.

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ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2019 Jan 15; 7(1):53-55.
https://doi.org/10.3889/oamjms.2019.020
eISSN: 1857-9655
Clinical Science

Correlation between Serum Leptin Level with Type and Number of Lesion Skin Tag

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Citation: Putra IB, Siregar R, Jusuf NK, Ginting O, Nurhayati R. Correlation between Serum Leptin Level with Type and Number of Lesion Skin Tag. Open Access Maced J Med Sci. 2019 Jan 15; 7(1):53-55. https://doi.org/10.3889/oamjms.2019.020

Keywords: Skin tag; Serum leptin level; Type and number of skin tag

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Received: 01-Nov-2018; **Revised:** 28-Nov-2018; **Accepted:** 29-Nov-2018; **Online first:** 08-Jan-2019

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

Abstract

BACKGROUND: Skin tag is a benign tumour of connective tissue in the skin, sessile or pedunculated, skin-like to brownish coloured and often arises in the flexure area. Etiopathogenesis of skin tag is still unclear, but one of the aetiology is associated with leptin hormone.

AIM: To determine the correlation between leptin serum level with type and number of the lesion skin tag.

METHODS: This study is an observational analytic study with a cross-sectional design involving 33 skin tag patients. Diagnosis of skin tag was based on history and clinical examination; we conducted blood sampling and measurement of serum leptin level to the patients.

RESULTS: We found the mean serum leptin level of skin tag patients were highest on the type of lesion mixed (31.54 ± 12.85 ng/ml). The mean number of skin tag lesions was 13.6 ± 5.8 lesions. There is a very high positive correlation between serum leptin level with a number of skin tag ($r = 0.86$) with $p < 0.05$ and significant correlation between serum leptin level with the type of lesions ($p = 0.037$).

CONCLUSION: Serum leptin level has a very high positive correlation to a number of skin tag and significant correlation between serum leptin level with the type of lesion.

Introduction

A skin tag is a papule-shaped benign skin tumour with soft consistency, skin-like to brownish coloured, protruding on the skin surface. The prevalence of skin tag in the general population varies considerably with the occurrence of males and females generally the same and can occur in various age ranges [1], [2].

The cause of skin tag is not known with certainty even though several factors are thought to have a role in the pathogenesis of skin tag. Until now there have been many theories explaining the pathogenesis of skin tag, including the process of repeated scratching or friction on the skin, hereditary factors in the family, hormonal factors, and obesity [3], [4], [5]. Also several studies also revealed that metabolic disorders of carbohydrates and insulin, as well as metabolic disorders of lipids and leptin hormone, play a role in the pathogenesis of skin tag [6], [7], [8], [9], [10].

Leptin is an obese gene product that can stimulate growth factors, differentiation and proliferation of epithelial cells in the dermis and epidermis [11]. Binding of leptin and its receptors on the skin can trigger the proliferation and differentiation of keratinocyte and fibroblast cells, and this is thought to play a role in the pathogenesis of skin tag [12], [13].

Methods

This research was an analytic observational study with a cross-sectional design involving 33 skin tag patients aged 20-70 years in the Tumor and Skin Surgery Division of the Department of Dermatology and Venereology, Faculty of Medicine, Universitas Sumatera Utara Medan. Each subject of the study who had signed the informed consent was included in this study. Exclusion criteria are pregnant and lactating women, history of using insulin hormone and glucocorticoid drugs, and diabetes mellitus.

Ethical permission is given by the Health Research Ethics Committee, Faculty of Medicine Universitas Sumatera Utara Medan. In all study subject, we conducted blood sampling and measurement of serum leptin level to the patients. The result was analysed statistically by Spearman correlation test to investigate the relationship between the level of serum leptin with a number of skin tag and analysed statistically by Mann Whitney to investigate the relationship between the level of serum leptin with the type of skin tag.

Results

Characteristic of skin tag lesion in this study showed majority type of lesion is the mixed type (54.5%) (Table 1), 28 (84.8%) subjects had a multiple lesion (Table 2).

Table 1: Distribution of skin tag patients based on the type of lesion

Type of lesion	n	(%)
Pedunculated	8	24.2
Non pedunculated	7	21.2
Mixed	18	54.5
Total	33	100

In this study, we found the mean serum leptin level of skin tag patients were highest on the type of lesion mixed (31.54 ± 12.85 ng/ml). The mean number of skin tag was 13.6 ± 5.8 lesions.

Table 2: Distribution of skin tag patients based on the number of lesions

Number lesions	n	(%)
Multiple	28	84.8
Single	5	15.1
Total	33	100

There is a very high positive correlation between serum leptin level with a number of skin tag ($r = 0.86$) with $p < 0.05$ and significant correlation between serum leptin level with the type of lesions ($p = 0.037$). Statistically, there is a very high positive correlation ($r = 0.86$) between serum leptin levels and a number of skin tag with $p < 0.05$ (Table 3).

Table 3: Correlation between serum leptin level with the type of lesion skin tag

	Serum leptin level (ng/ml)			
Type of lesion	n	Mean	SD	p
Pedunculated	8	21.22	11.37	
Non-pedunculated	7	20.98	9.64	
Mixed	18	31.54	12.85	0.037

Discussion

In this study the majority of skin tag patients had mixed type of skin tag lesions 18 subjects (54%), and there is a significant correlation between serum leptin level with type of lesions ($p = 0.037$), authors have not found a literature discussing the correlation between serum leptin level with type of lesions but in study by Jusuf et al., found there was majority type of lesion is mixed type (56.2%) [2]. Based on a number of skin tag the majority of skin tag patients had multiple lesions. The results of this study according with the study conducted by Jusuf et al., who found 28 subjects (87.5%) had a multiple lesion [2]. The number of skin tag is associated with lipid metabolic disorders, one of which is affected by leptin [14].

Leptin is a product of the obese gene which can stimulate growth factors, differentiation, and proliferation of epithelial cells in the dermis and epidermis [11].

Statistically, there is a very high positive correlation ($r = 0.86$) between serum leptin levels and a number of skin tag with $p < 0.05$ and significant correlation between serum leptin level with the type of lesions ($p = 0.037$). The results of this study are by the research of Erkek et al., which found a correlation between serum leptin levels and a number of skin tag ($r = 0.62$) with $p < 0.001$ [13]. Gautama also obtained a positive correlation between serum leptin levels and with values of $r = 0.94$ and number of skin tag with $p < 0.001$ [15].

A strong correlation between serum leptin levels and the number of skin tag lesions is associated with a high mean body mass index in skin tag patients. A high body mass index is associated with a greater amount of lipid and then there is secreted a large amount of leptin that affects various organs including the skin. As a result of the presence of a high level of leptin and leptin ob R receptors on keratinocytes and fibroblasts cells can trigger cell proliferation and differentiation into skin tag lesions [12].

The conclusion in this study we investigated possitive correlation between serum leptin level with number of skin tag and significant correlation between serum leptin level with type of lesions very highly.

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Open Access Macedonian Journal of Medical Sciences. 2019 Jan 15; 7(1):56-64.
https://doi.org/10.3889/oamjms.2019.032
eISSN: 1857-9655
Clinical Science

Surgical Treatment of Meningiomas - Outcome Associated With Type of Resection, Recurrence, Karnofsky Performance Score, Mitotic Count

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Citation: Sumkovski R, Micunovic M, Kocevski I, Ilievski B, Petrov I. Surgical Treatment of Meningiomas-Outcome Associated With Type of Resection, Recurrence, Karnofsky Performance Score, Mitotic Count. Open Access Maced J Med Sci. 2019 Jan 15; 7(1):56-64. https://doi.org/10.3889/oamjms.2018.503

Keywords: Outcome of surgically treated meningiomas; Simpson; Karnofsky; Mitotic count

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Received: 08-Nov-2018; **Revised:** 17-Dec-2018; **Accepted:** 18-Dec-2018; **Online first:** 11-Jan-2019

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

BACKGROUND: Meningiomas are the type of central nervous system tumours, derived from the cells of the arachnoid membrane that are well constrained from surrounding tissues, mainly no infiltrating neoplasm with benign features. Meningiomas consist about 15-20% of all primary intracranial neoplasms.

AIM: The evaluation of the outcome of the operatively treated meningiomas in relation with the Karnofsky performance score, survival, recurrence, type of the surgical excision, histological type, mitotic count (MC), localisation and volume of the lesion

METHODS: In this article 40 operatively treated patients are reviewed for the outcome of the operation about the Karnofsky performance score, survival, recurrence, type of the surgical excision, histological type, mitotic count (MC), localisation and volume of the lesion.

RESULTS: Association/interconnection between the mitotic count grade I and the regrowth of meningioma have been verified. Association/interconnection between the mitotic count grade I and the regrowth of meningioma have been verified. Association/interconnection between the mitotic count grade I and the regrowth of meningioma have been established.

CONCLUSION: Gender, age and Karnofsky performance score have predictive value in the treatment of different types of meningiomas. The magnitude of surgical resection is associated with the regrowth of a tumour. The mitotic count in different types of meningiomas presents significant feature in the appearance of meningioma recurrence. The surgical resection and the quality and quantity of patient's survival have a significant relation to the mitotic count of the meningiomas. There is no connection between the size and the localisation of a tumour related to different values of the mitotic count.

Abstract

Introduction

Meningiomas are slow-growing tumours of the central nervous system that are well-limited from surrounding tissues, mainly non-infiltrative neoplasms with benign characteristics. Among the meningiomas, some of the histologically described are malignant forms, which include 1.7% of all meningiomas, as well as fast-growing variants that act as malignant tumours [1]. The origin of these tumours is derived from the arachnoid cells. Usually, meningioma's are single, solitary, but can also be multiple - up to 8% of cases, which is more common in neurofibromatosis [2]. The growth of meningiomas is expansive, followed with displacement and compression of the surrounding structures, the brain, blood vessels, cranial nerves, etc., but there are also forms of diffusional thickening of the brain membranes, a type of meningioma called meningioma en plaque [3]. Meningiomas can grow everywhere that arachnoid membrane is found (between the brain and the cranium, in the ventricles, down the spinal canal). The ectopic meningiomas may be found growing in the inside of the bone which limits the cranial box-as primary intraosseal meningeomas.4 Meningiomas account for 14.3-19% of all primary

intracranial neoplasms. They are the second most common primary tumour of the central nervous system, just behind the gliomas (43%), and the pituitary adenomas (17%) in the third place. The peak of incidental occurrence of this group of neoplasms is 45 years of age. The gender distribution of meningiomas is almost twice as high in females (men: women = 1: 1.8). Meningiomas in childhood and adolescence account for 1.5% of the total number [3]. Meningioma's most frequent distribution is as follows: parasagittal (20.8%), then convexity (15.2%), and tuberculum sellae (12.8%), followed by localization: sphenoid ridge, olfactory groove, falx, lateral ventriculi, tentorium, the middle fossa, the orbit, the spinal channel, the Sylvian fissure, extracalvarial, multiple localization, the pontocerebral angle, the sphenoidal plane, and the foramen magnum. Around 60-70% of all meningiomas have falcian distribution (including parasagittal distribution), sphenoid wing (including tuberculum sellae), or on the convexity of the brain. Meningiomas rarely occur in the pediatric population, and 28% of them have intraventricular and posterior fossa localisation [4], [5].

Histopathological variables that determine the characteristics of meningiomas include tumour gradient, histological subtype, proliferative index and invasiveness of a tumour to the brain [3], [6], [7]. For our paper, the WHO Classification of Tumours of CNS, 2016 was used:

1. Plain X-ray – Tumor calcifications can be verified (about 10%), then hyper/dysostosis or changes in configuration, the thickness of the skull (including the floor of the anterior cranial fossa in olfactory groove meningioma), an increase in vascular fissures (especially in the middle meningeal artery);

2. Computerised tomography-The presentation of a tumour with this diagnostic tool shows a homogeneous dense mass with a wide base attached to the dura. The presence of psammomatous calcifications can be presumed through the measured 60-70 Hounsfield units in a non-contrast series. Intraventricular meningiomas in 50% of patients produce extraventricular oedema;

3. Nuclear Magnetic Resonance-The signal that a tumour gives in T1WI and T2WI may be isodense, but it is often intensified with contrast gadolinium. The brain's oedema may, but it does not have to be present. Nuclear magnetic resonance provides information about dural venous sinuses (accuracy in the presumption of dural sinus invasion is > 90%). A common sign on NMR is the dural tale;

4. Angiography-The classical finding: "appears rapidly, remains long-lasting" explained that a tumour is visualised in the early arterial phase and remains visible after the completion of the venous phase. It is characteristic that the meningioma's have arteries that arise from the external carotid artery, excluding the olfactory groove meningioma, whose nutrient vessels originates from the internal carotid artery (ICA), or the ethmoidal branches of the ophthalmic arteries, suprasellar meningiomas may be vascularized from the long branches of the ophthalmic artery and parasellar meningioma's that are largely nourished by the internal carotid artery (ICA). The secondary vascular supply may come from the pial branches of the anterior, the middle and posterior cerebral artery. In cases of tentorial meningiomas the artery of Bernasconi & Cassinari or the Italian artery is of interest, or the artery of the tentorium (a branch of the meningohipophysal stem) that is enlarged in lesions, tumours that envelop the tentorium. Angiography gives excellent information on the involvement of the dural venous sinuses and their occlusion, especially in the parasagittal/falx meningiomas. At the same time, angiography may provide preoperative embolisation of a tumour [8].

Surgical treatment is a method of choice for symptomatic meningiomas. Incidental meningiomas without tissue oedema, vascular compromise, or those having clinical presentation only with epileptic seizures that are easily controlled with anti-epileptic medications, can be managed with an expectative approach. In general, the meningioma surgery is very bloody which would imply a great deal of attention to the hemostatic techniques during the intervention, as well as the possible use of autologous blood for transfusion donated preoperatively and also preoperative embolisation of the dominant tumour feeding artery [9]. Radiotherapy is often estimated not to be effective as a primary model of treatment [10].

The outcome in the treatment of meningiomas: 5 years of survival for patients with meningiomas is generally 91.3% [3], [11].

When conducting surgical removal of meningiomas, a system of stratification of the degree of excision is used, and this can describe the radically in the performed surgical treatment. The system for removing meningiomas according to Simpson covers 5 grades:

0) macroscopically complete removal of a tumour, excision of its dural attachment within 2 cm of no-affected dura:

I) macroscopically complete removal of the tumour, with excision of its dural attachment, and of any abnormal bone (including resection of the venous sinus when involved);

II) macroscopically complete removal of the tumour and its visible extensions with endothermic coagulation (Bovie, laser) of the dural attachments;

III) macroscopically complete removal of an intradural tumour, without resection or coagulation of its dural attachments or its extradural extensions (hyperostotic bone);

IV) partial removal, leaving intradural tumour in situ;

V) simple decompression (± biopsy) [12].

The study assesses the outcome through the disability of patients classified according to Karnofsky score, which evaluates psycho-physical (dis)ability as a percentage expressed in dozens starting from 0% (death) to 100% (normal patient, without symptoms and signs of illness) which are grouped into three groups:

0-40% - the patient is not able to care for himself, institutional or hospital care is necessary, in which the patient can progress rapidly;

50-80% - the patient is not able to work, but can live at home and to cope with most individual needs;

80-100% - the patient can continue with everyday activities and work-related obligations, and no special care is required [3].

The most important prognostic factor for treating meningiomas is predicting possible recurrence and survival for malignant or transient types of meningiomas [13], [14]. Negative prognostic factors include non-extensive resection, brain invasion and high mitotic activity which are the most significant predicting factors. The extensiveness of surgical excision is the most important factor in the prevention of recurrent meningiomas. Simpson's grading system [12] is a valuable tool for evaluation for possible tumor recurrence [15], [16]. In addition to this principle, the basic element for the prevention of recurrent meningiomas, the Mitotic index is a factor with great significance for assessment, anticipation, planning of further treatment and outcome in meningiomas [12], [15], [16], [17].

By definition, the mitotic index (MI) is the sum of the mitotic "figures" (MF) in 10 consecutive HPF (High Power Field) in the zone of the highest mitotic activity relative to the remaining cells [18], [19]. This defining factor is dominant in the classification for meningioma’s according to the World Health Organization, with 3 degrees of the mitotic index being defined as a risk factor for local recurrence, according to criteria of histological analysis with prognostic significance [17], [20].

According to Perry et al., [3], [18], [19] and accepted by the World Health Organization, as an objective grading criterion, there are: benign/WHO Grade 1 meningioma’s that do not exceed 4 mitosis per 10 consecutive HPF, atypical/WHO Grade 2, with 5-20 mitoses on HPF and anaplastic/WHO Grade 3, meningioma’s in which mitotic activity exceeds 20 mitoses of 10 consecutive HPFs in the zone of the highest mitotic activity [3], [15], [17], [18], [19].

This classification (determining the mitotic index (MI)) has its weakness. Namely, the selection of zones with the "highest mitotic index" is subjective and subject to bias and the heterogeneity of mitotic activity in various areas of a tumour. Further factors of "error" are variations in the size of the sampled tissue samples, as well as the resected samples and their cellularity, both factors affecting the number of cells that can be evaluated [21].

To specify this method, the mitotic activity can be determined by introducing an immunohistochemical method to objectify this procedure. Protein Ki-67 is a nuclear protein that is closely related and essential in cellular proliferation. This protein is associated with the ribosomal RNA transcription [21], [22].

The H & E (hematoxilyn & eosin) mitotic index, as well as the mitotic indices determined according to immunohistochemistry, can be identified as independent prognostic factors for the poor outcome (recurrent tumor and/or death), adjusted to the patient's age and extensiveness to the tumor resection (with STR (subtotal resection) as an independent factor) [17].

Numerous studies have shown that the mitotic index (in the repeat-MI text) isolated or synchronously, is in harmony with the occurrence of recurrence of meningiomas [15]. Namely, it is one of the most probable prognostic factors in meningiomas. The identification of the mitotic "figures" (MF) [23] and the zone of the most intensive mitotic activity in histological H & E coloured slides is a representative, cost-effective, necessary, but also a subjective task.

The motives were the evaluation of the outcome of the operatively treated meningiomas through objective parameters, to improve the results of the treatment, to reduce the complications, disability, improving quality, and survival, as well as re-socialisation in correlation with the mitotic index.

The purpose of this study is evaluation of the relation of the mitotic index to various groups of meningioma’s and tumour regrowth, assessment of the impact of tumour size over the time of tumour recurrence, assessment of the impact of tumour localization over the time of tumour recurrence, correlation between the mitotic index and the outcome of surgical treatment, followed by disability and survival evaluated according to Karnofsky Score, correlation between the mitotic index and the size and localization of the tumour, and also determination of the time of tumour recurrence in surgically treated subjects with meningiomas.

Material and Methods

In a retrospective study, 40 successive randomly selected patients with an initial diagnosis of intracranial supra- and infratentorial meningioma, treated at the Neurosurgery Clinic-Skopje, were processed.

The subject of the analysis were previously operated patients, without limitation in terms of location, gender, age, which are subject to standard micro-surgical excision, with a tendency to achieve "gross total resection" [12], i.e. maximum, radical surgical excision, Grades 1 and 2 according to Simpson, as well as patients undergoing "subtotal resection", respectively Grade 3, 4 and 5 according to Simpson. For this purpose, the technology for micro-surgical excision was used-microinstumentarium, operative microscope, bipolar coagulation, ultrasonic aspirator.

For surgical excision grading, and the radicality of the operative treatment, the previously mentioned evaluation system for removing meningiomas according to Simpson was used [12].

The study assesses the outcome through the disability of patients classified according to previously mentioned Karnofsky performance status, which assesses psycho-physical condition as a percentage expressed in dozens starting from 0% (death) to 100% (normal patient without symptoms or signs of a disease) that are grouped together into three groups [3].

The pathohistological diagnostics was performed at the Institute of Pathology, Medical Faculty, Ss Cyril and Methodius University of Skopje, Skopje, Republic of Macedonia, using histological and immunohistochemical analyses (diagnosis, mitotic count, subtype-specific immunohistochemistry).

Inclusion criteria were patients of our population with an initial diagnosis of intracranial supra- or infratentorial meningioma and exclusion criteria were patients with meningioma in the spinal canal.

Statistical processing of data is performed in statistical programs STATISTICA 7.1 and SPSS 13.0. The following methods apply: In series with attributive marks percentages per structure (%) are determined; The association, or the differences in the analyzed parameters in relation to the mitotic index, tumour regrowth, Karnofsky score, excision grade according to Simpson, were tested with Pearson Chi-square (χ^2); In series with numerical records determined are: Descriptive statistics (Mean ± Std. Dev., ± 95% CI, Min., Max.); The ratio between the mitotic index and the recurrence period; Karnofsky score; tumour size; Determined with Spearman Rank Order Correlations (R); The influence of size and tumour localization as independent variables on the occurrence of tumour growth as a dependent variable; the influence of the mitotic index and surgical excision as independent variables on the occurrence of tumour recurrence as a dependent variable was determined using Logistic regression analysis (Chi Square, Wald, Exp (B)); The re-emergence of a tumour, tumour size, years, patient age, the time of occurrence of the first symptoms as independent variables, on the independent living as a dependent variable, was determined using Logistic regression analysis (Chi Square, Wald, Exp (B)).

Results

Of the 40 enrolled patients 13 (32.5%) were male, 27 (67.5%) were female. Mean age was 58.58 ± 8.91 years of age, with minimal age of 42 and maximal age of 78.

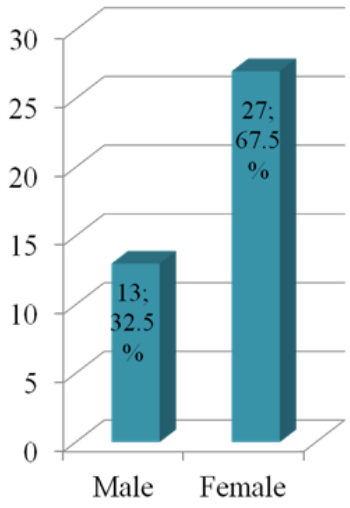

Figure 1: Distribution of the patients according to the gender

Average lesion volume was estimated to be 40.23 ± 16.39 cm, with a minimal volume of 7 ccm and maximal volume of 80.00 ccm.

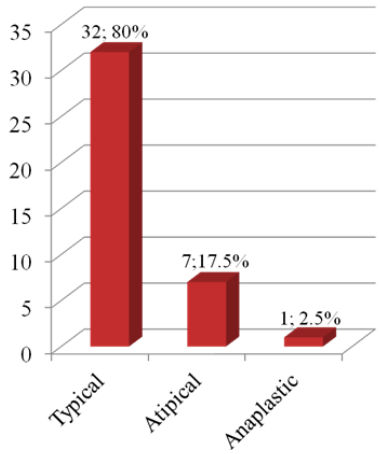

Figure 2: Distribution of the patients according to the histological type

According to the histological type 32 patients or 80% had a typical meningioma, 7 patients or 17.5% had an atypical meningioma, and one patient (2.5%) had anaplastic meningioma.

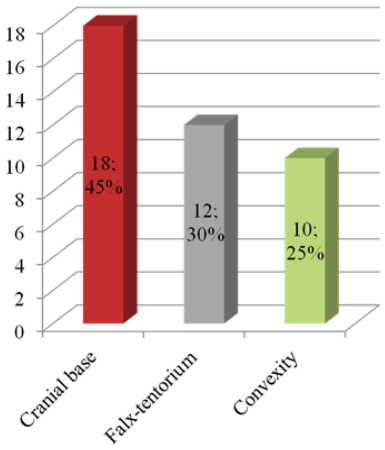

Figure 3: Distribution of the patients according to the localisation of a tumour

The localisation of a tumour was divided into three sections: convexity, cranial base and falx-tentorium. In 18 patients (45%) the meningioma was localised on the cranial base, 12 patients (30%) had a meningioma localised on falx-tentorium, 10 patients (25%) had a convexity meningioma.

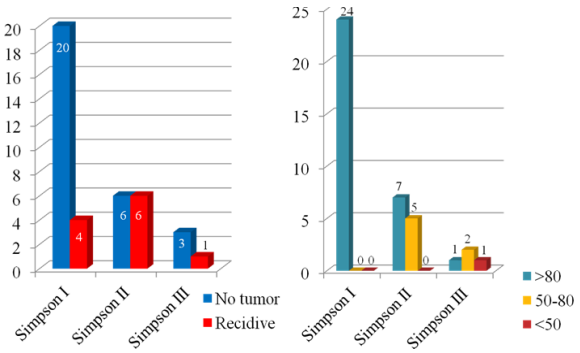

Figure 4: Distribution of the patients according to the surgical excision/tumour recurrence (left); according to the surgical excision/Karnofsky scale (right)

According to the mitotic count 34 patients (85%) had a Grade I, four patients (10%) had a Grade II and 2 patients (5%) had a Grade III mitotic count.

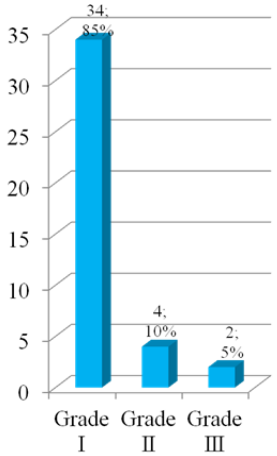

Figure 5: Distribution of the patients according to the mitotic count

Disability and survival were evaluated according to Karnofsky Performance Scale with the following results: 32 patients (80%) had a score of 80% or higher, 7 patients (17.5%) had a score from 50-80% and 1 patient (2.5%) had a score of 50% or lower.

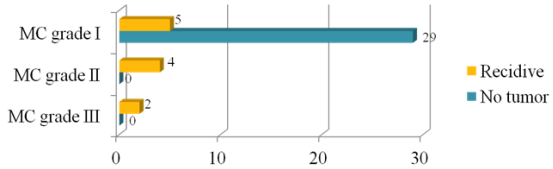

Figure 6: Mitotic count (MC) recidive of a tumour

Simpson scale was used for grading of the degree of excision: 24 patients (60%) had a Simpson I resection, 12 patients (30%) had a Simpson II resection, and 4 patients (10%) had Simpson III resection.

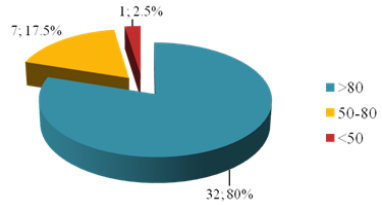

Figure 7: Karnofsky performance scale

In 29 patients (72.5%) no tumour regrowth was noted after the first operation, and in 11 patient (27.5%) tumour regrowth was diagnosed.

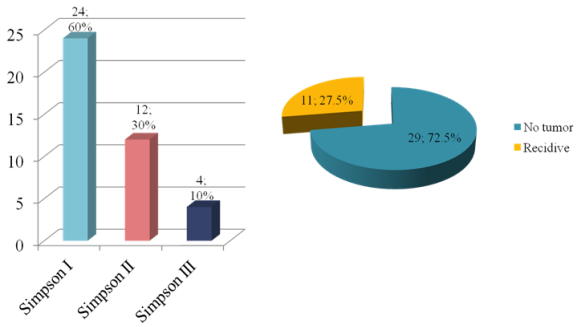

Figure 8: Surgical excision (Simpson) (left); Outcome after the 1st operation (right)

Discussion

Meningiomas represent 13 to 26% of all intracranial tumours with an annual incidence of 6 cases per 100 000 people [24]. Although these tumours behave like benign tumours, they are still associated with significant morbidity and mortality

[18], [25]. In 1993, the World Health Organization (WHO) proposed a classification of meningioma's in three groups: classic or typical (WHO Group I), atypical (WHO Group II) and malignant (WHO Group III) [27]. Depending on the diagnostic criteria used in different studies, the atypical meningiomas are represented with 5 to 17%, whereas the malignant meningiomas range from 1 to 3%, while the rest remains on the classic, typical meningiomas [8], [18], [24], [26].

In this study, according to the use of the mitotic index and the pathophysiological diagnosis, typical meningiomas were represented with 80% (32/40) of cases, atypical meningiomas in 17% (7/40) of cases and one case of anaplastic, malignant meningioma, or 2.5% (1/40), which is in accordance with the representation of the subgroups of meningiomas published in the professional literature [3], [8], [9], [18], [24], [27].

The pathophysiological criteria used for classification of meningiomas include the following estimates: the mitotic index, the presence of atypical mitosis, necrosis, significant pleomorphism, macronucleus, disrupted histoarchitectonics, hypercellularity, the presence of mosaic tissue, the presence of brain invasion, even the minimum presence of a focally clear malignant behaviour (cancer-like, sarcoma or melanoma) [28].

According to the classification of brain tumours published by the World Health Organization in 2007, all meningioma's that exhibit a brain tissue invasion were set in the higher group, WHO Group II [8]. Usability of the brain invasion as a marker of the aggressiveness of the tumour is limited, since the brain tissue can be identified in a small number of surgical specimens, and is rarely recorded by the pathologists [18], [24], [26]. Using these criteria, Andrej Vranic, Mara Popovic, Andrej Cör, Borut Prestor and Joze Pizem published a series of patients with the representation of atypical meningiomas in 76 cases (88%) and 10 patients with malignant meningioma (12%). In this paper, 7 patients had atypical meningioma (17%), and one case of anaplastic meningioma (13%) was enrolled. In the professional literature [26], the ratio of atypical and malignant meningioma's in the interrelated correlation is 1.5:1 to 6:1. In the study of Slovenian authors, this ratio equals 7.6:1, while in our study 7: 1. Benign meningiomas are more common in the female population (female: male ratio is approximately 2:1), while atypical and malignant meningiomas are more common in the male population (the ratio of women to men is 0.8-1.4: 1). In the series of Slovenian colleagues, the ratio of women to men is 1.1:1, while in our study this ratio is 1.6:1, while in the benign form, the ratio according to the gender female: male equals 2.2:1. In the previously cited study of the Slovenian authors, during the patient's follow-up, tumor regrowth was noted in 31 patients (36%), in the period from 4 to 76 months (median of 31), while in our study there were 11 patients, that is, 27.50% without tumour regrowth in the interval of 14.36 ± 7.10, i.e. the minimum re-occurring time from 8 months to the maximum re-occurrence time of 28 months.

The significance of the extensiveness of surgical resection is equally important for all histological subtypes of meningiomas [18], [25]. Sometimes it is difficult to compare the results between studies because the tumour resection in some studies is graded according to Simpson [11], [29] while in the other it is graded as a total or subtotal resection (gross total vs subtotal resection, GTR vs STR) [18], [25], [30]. In the study of Slovenian authors, Simpson I excision was achieved in 47% of patients, and total resection (as GTR) (including Simpson I and II) was achieved at 90%. The non-recurring period of patients with Simpson I excision was longer compared to patients with Simpson II-IV excision, but there were no statistically significant observations.

In the study of Donald Simpson of the Department of Neurological Surgery, Radcliffe Infirmary, Oxford, 90 operations were made according to Simpson I, in which beside tumour extirpation, the dural attachment and extradural extensions were removed so that 8 cases of relapse occur later, or 9% [12]. Of the 114 operations performed according to Simpson II, in which the visible tumour was completely removed, while the dural adherence was coagulated with the endotherm coagulation, 18 relapses or 19% were observed. For operations that are considered incomplete (Simpson III, IV, V), the rate of recurrence is more significant, but it is important to note that a large number of patients treated with limited excision gain a long period of relief [12].

In our study, of the 40 patients with meningioma treated surgically, in 24 patients (60.0%) maximally radical surgical excision was achieved, grade I according to Simpson, of which 4 patients (10.0%) had a tumour recurrence. In 12 patients (30.0%), surgical excision grade II was performed by Simpson, and 6 of them (15.0%) had a tumour recurrence. Four patients (10.0%) performed surgical excision grade III according to Simpson, 1 patient (2.5%) had a recurrence of the tumour, and for all within the defined period of follow-up. There is no significant difference in the reported distribution of data on the degree of surgical excision graded by Simpson and the incidence of tumour regrowth, for $\chi^2 = 4.47$ and $p > 0.05$ ($p = 0.11$).

When patients are grouped according to the histological type of the meningioma, than those with benign form-typical meningioma, have a 5-year survival of 75%, while those with atypical and anaplastic meningioma have a rate of a 5-year survival rate of 70% and 56%, respectively. The prognostic factors for survival in benign tumours

include the patient's age in the time of setting the diagnosis, tumour size, institution (the manner/standard of treatment) in which the patient was treated; for malignant tumours the age at the time of diagnosis is important. The five-year recurrence rate of the symptoms (regardless of the choice of treatment of the disease) equalled 18.2% for benign tumours and 27.5% for patients with malignant meningiomas. In patients with benign meningioma, who were susceptible to complete eradication, the 5-year reappearance rate equalled 20.5%.

It was shown that women have a higher prevalence of all three histological types of meningiomas, compared with men, with no racial difference in the representation of meningiomas. Age is an important predictor of mortality in patients with benign, atypical and malignant meningiomas. As the age of the diagnosis increases, the survival rate decreases dramatically, although the exact cause of death in patients is not taken into consideration, depending on or regardless of the primary illness. Age and race are considered as individual prognostic factors for benign and malignant meningiomas.

According to the study, the 5-year tumour recurrence rate that was previously completely surgically eliminated equalled 20.5%, which is higher than that quoted in the literature [3], [15], [20], [26], [27], [31], [32].

Mirimanoff et al., [32] recognised that the rate of re-emergence of benign meningiomas previously operatively treated was 7% for 5 years and 20% for 10 years. The recurrence rate in the Mirimanoff et al., [20] study accounted for 2% throughout 5 years. In this study, the recurrence time for medium-term time is 14.36 ± 7.10 months, that is, at least 8 months and a maximum of 28 months.

Complete surgical tumour excision is referred to as gross total resection (GTR), which is defined as Simpson grade I or II, which corresponds to a macroscopically performed tumour resection with bipolar coagulation of the dural insertion. Any other type of tumour resection would be understood as subtotal resection (or STR). Pathologists in the study assessed the mitotic activity of the tumour through the determination of the mitotic index with classical H & E colouration, immunohistochemistry with monoclonal anti-PHH3 (phosphohistone H3) antibody and immunohistochemistry technique with a monoclonal anti-Ki-67 antibody [17], [21].

The following results were obtained in the determination of the mitotic index (MI): 251 WHO grade I, 45 atypical meningioma's (WHO grade II) and 5 malignant (anaplastic) meningioma's (WHO grade III). In operative treatment, 238 cases had GTR, while 27 patients had STR. The recurrence rate in the study equaled 12.5% (i.e. 33 patients) with a relative risk for tumor regrowth significantly higher in patients treated with subtotal resection (STR), (10/27; RR = 37%) compared with those treated with radical resection (GTR) (23/238; RR = 9.7%). The average recurrence time was 45 months (48 months after GTR and 36 months per STR). The meningioma recurrence rate with WHO grade I level equalled 6.0% for tumour GTR, or 9.8% in the total cohort. Reproducibility was recorded at 18% for meningiomas with the WHO grade II mitotic index, while for meningiomas with grade III mitotic index, according to the WHO, recurrence equalled 80%. In the pathophysiological assessment of tumours, in addition to cell proliferation and cellular abnormalities, the potential of the tumor for invasion in the brain parenchyma was considered, with the type of tumour being labelled as possibly invasive meningioma. Of the examined series, brain invasiveness was registered in 14 tumours with the following distribution-12 tumours in grade II and 2 in grade III of the WHO (of which, 4 brain tissue-invasive recurrent meningioma's with a relative risk of 29%). The study compares the importance and efficiency of the mitotic index measured by the traditional way of histological preparations according to H&E, as well as the use of monoclonal anti-phosphohistone H3 (PHH3) antibodies and monoclonal anti-Ki 67 antibodies in immunohistochemistry of the preparations. The MI (MI) H & E and MI PHH3 can be identified as independent predictors (predictors) of poor prognosis (recurrence and/or death) adjusted to the age and extensiveness of surgical tumour resection (and STR as an independent variable).

Following the WHO-recommended mitotic indexing guidelines, a lower threshold for mitotic activity, i.e. a mitotic index of 4 and more mitotic figures of 10 large-scale magnifications (HPF) was proposed, at the point of high risk of tumour recurrence and / or death. MI according to H & E is an independent indicator of unfavourable prognosis, as proposed by Perry et al., [18], [33].

In our study, we have the following data on the significance of the mitotic index in the prognosis: out of the total extirpated meningioma's (40 in all), 34 meningiomas, (85%) pathohistologically classified based on mitotic index in grade I according to the WHO, 4 meningioma's (10%) are classified according to the mitotic index in grade II according to the WHO and 2 meningiomas (5%) classified according to the mitotic index in degree III according to the WHO.

As for the examined correlation between the mitotic tumor index and the localization of the tumor, 34 (85, 00%) patients registered a mitotic index of grade I, of whom 15 (37, 50%) patients had a localized tumour on the skull base, 10 (25, 00%) patients had a tumor localized to falx-tentorium, and in 9 (22, 50%) patients the tumor was localized to convexity. In 4 (10.00%) patients a mitotic index of grade II was registered, of which 2 (5.00%) patients had a tumour localized to the skull base, 1 (2.50%) the patient had a tumor localized to the falx-tentorium, and 1 (2.50%) the patient had a localized convexity tumour. Two patients (5.00%) had a mitotic index of grade III, of which 1 (2.50%) the patient had a tumor

localised to the skull base, and 1 (2.50%) patient had a tumor localized to the falx-tentorium. For $\chi^2 = 0.87$ and $p > 0.05$ ($p = 0.93$), there is no significant difference in the displayed distribution between the level of the mitotic index and the localization of the tumour.

Grading of work capacity and disability was done according to the Karnofsky Performance Score, which as an average for patients in Akagami Ryojo's study, Napolitano Mario, Sekhar Laligam equalled 83 ± 10%. Ninety-seven per cent of patients were satisfied with the treatment, expectations were fulfilled in 90%, and according to the Karnofsky index, 83% of patients were labour-efficient. In this study, the comparison is as follows: 40 patients, of which 13 men and 27 women were studied, at a mean age of 58.58 ± 8.91 years. Gross total resection (Simpson grade I) was achieved in 24 patients (60%) and Simpson grade II was achieved in 12 patients (30%) in a total of 90% of patients. The mean tumour volume was 40.23 ± 16.39 cc. The work capacity assessment and the capacity for independent living, conducted according to Karnofsky, 80% of the patients were able to live independently with the possibility of returning to their jobs, 17.5% cannot fulfil their previous working capacity, but can independently function and can fulfil their own individual need, while 2.5%, that is, one patient, is not able to care for himself, that is, he needs an institutional type of care [34], [3].

In conclusion, the results have shown that gender, age and Karnofsky performance score have predictive value in the treatment of different types of meningiomas. Also, the magnitude of surgical resection is associated with the regrowth of a tumour. The mitotic count in different types of meningiomas, as an independent predictor, represents a significant feature in the appearance of meningioma recurrence. The surgical resection and the quality and quantity of patient survival have a significant relation to the mitotic count of the meningiomas. Results have also demonstrated that there is no connection between the size and the localisation of a tumour related to different values of the mitotic count.

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ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2019 Jan 15; 7(1):65-67.
https://doi.org/10.3889/oamjms.2019.022
eISSN: 1857-9655
Clinical Science

Correlation between Serum 25-Hydroxyvitamin D Levels with Keloid Severity

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Citation: Damanik VI, Putra IB, Ginting O. Correlation between Serum 25-Hydroxyvitamin D Levels With Keloid Severity. Open Access Maced J Med Sci. 2019 Jan 15; 7(1):65-67. https://doi.org/10.3889/oamjms.2019.022

Keywords: Serum 25-hydroxyvitamin D level; Keloid severity

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Received: 01-Nov-2018; **Revised:** 28-Nov-2018; **Accepted:** 29-Nov-2018; **Online first:** 12-Jan-2019

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

Abstract

BACKGROUND: Keloids are dermal fibroproliferative tumours characterised by excessive deposition of extra cellular matrix components. The active form of vitamin D is known to inhibit the proliferation of keloid fibroblasts by inhibiting extracellular matrix production induced by transforming growth factor ß (TGF-β) and increasing matrix metalloproteinase (MMP) activity. Vitamin D derivatives are thought to be an early preventive treatment strategy for keloid.

AIM: To determine the correlation between serum 25-hydroxyvitamin D level with keloid severity.

METHODS: This is a cross-sectional analytic study involving 32 keloid patients. Keloid patients were diagnosed by history and clinical examinations. Then an assessment of the severity was conducted using the Vancouver Scar Scale (VSS). We conducted blood sampling and measurement of serum 25-hydroxyvitamin D level to the patients. This study has been approved by the Health Research Ethics Commission of the Faculty of Medicine, Universitas Sumatera Utara, H. Adam Malik General Hospital Medan.

RESULTS: There is negative correlation between serum 25-hydroxyvitamin D level with severity in keloid patients ($p = 0.0001$; $r = -0.737$). There is no significant correlation between serum 25-hydroxyvitamin D level with gender ($p = 0.271$), age ($p = 0.201$; $r = -0.232$), duration of keloid ($p = 0.505$; $r = -0.122$) and family history ($p = 0.262$).

CONCLUSION: Lower level of plasma 25-hydroxyvitamin D, the severity of keloid became an increasingly heavy. There is no significant difference between serum 25-hydroxyvitamin D level with gender, age, duration of keloid and family history in keloid patients.

Introduction

Keloid is dense fibrous tumours that develop from abnormal responses to skin injuries restoration. Keloid is a pathologic scar that extends beyond the boundary of the wound, benign and has no potential for malignancy [1], [2]. Keloid is dermal fibroproliferative tumours characterised by excessive deposition of extracellular matrix components [2].

The 25-hydroxyvitamin D level which is the main form of vitamin D in the circulation and this molecule is a measure for assessing vitamin D status in the body [3]. It is known that vitamin D has a beneficial role in slowing the progression of tissue fibrosis [4], [5], [6]. The active form of vitamin D is known to inhibit the proliferation of keloid fibroblasts by inhibiting extracellular matrix production induced by TGF-β and increasing MMP activity and acting as an anti-inflammatory mediator [7].

Vancouver Scar Scale is one of the most widely used scarring scoring systems in clinical research. This scale assesses four variables: vascularisation, pigmentation, consistency, and height/thickness [8], [9]. It is known that each of this variable is relevant to untreated keloid scar [9].

Methods

This study was conducted from January 2018 to September 2018. This study was a cross-sectional analytical study involving 32 keloid patients who had been diagnosed with keloid by history and clinical examination at the Universitas Sumatera Utara, Medan, Indonesia, with an age of 16-40 years. Every subject who had signed informed consent was included in this study. Exclusion criteria were pregnancy, hormonal disorders including thyroid and parathyroid disease; kidney and liver disease, systemic diseases including cardiovascular disease, diabetes mellitus, and tuberculosis and malignancy; autoimmune diseases including psoriasis, systemic lupus erythematosus, and scleroderma; long-term history of anti-seizure medication, antibiotic and antiviral drugs such as phenobarbital, phenytoin, carbamazepine, rifampicin, and antiretroviral; there is a history of conventional treatment for keloids such as triamcinolone acetonide injection, topical corticosteroids, cryosurgery, radiation, laser, occlusive dressings, compression therapy and interferon in the past two months; and consuming any vitamin D supplementation in the past 1 month. This study has been approved by the Health Research Ethics Committee of the Faculty of Medicine, Universitas Sumatera Utara. In all study subjects, we assessed the severity of keloid using VSS and blood samples were taken for measurement of serum 25-hydroxyvitamin D level.

The results were analysed statistically by the Spearman correlation test. This test was used to investigate the relationship between the level of serum 25-hydroxyvitamin D with keloid severity.

Results

Most of the subjects involved in this study were women, aged 21-25 years, suffered from keloid for 1-5 years, had no family history of keloid, and moderate keloid severity (Table 1).

Table 1: Subjects and characteristic

Variable	n	%
Gender		
Male	8	25.0
Female	24	75.0
Age		
16-20 years	6	18.8
21-25 years	13	40.6
26-30 years	2	6.3
31-35 years	3	9.4
36-40 years	8	25.0
Duration of disease		
1-5 years	23	71.9
6-10 years	4	12.5
11-15 years	1	3.1
16-20 years	4	12.5
Family history		
Father	1	3.1
Mother	2	6.3
Sibling	9	28.1
None	20	62.5
Severity		
Mild	2	6.3
Moderate	16	50.0
Severe	14	43.8

Statistically, there was a negative correlation between serum 25-hydroxyvitamin D level, and keloid severity ($p = 0.0001$, $r = -0.737$), the lowest level of serum 25-hydroxyvitamin D in this study subject was in severe-degree (VSS 10-14) is 12.34 ± 2.61 ng/mL (Table 2).

Table 2: Serum 25-hydroxyvitamin D level based on the severity

Severity	Serum 25-hydroxyvitamin D level (ng/mL)				
	n	Mean	SD	Min	Max
Mild	2	26.95	0.78	26.40	27.50
Moderate	16	20.98	5.19	6.90	27.40
Severe	14	12.34	2.61	6.60	16.60

In Table 3, there was no significant relationship between serum 25-hydroxyvitamin D level with gender ($p = 0.271$), age ($p = 0.201$; $r = -0.232$), duration ($p = 0.505$; $r = -0.122$), and family history ($p = 0.262$).

Table 3: Relationship between serum 25-hydroxyvitamin D level with gender

	Serum 25-hydroxyvitamin D level (ng/dL)			
	n	Mean	SD	p
Gender				
Male	8	19.73	4.73	0.271
Female	24	16.85	6.70	
Family history				
Present	12	19.22	6.83	0.262
None	20	16.60	5.95	

Discussion

The active form of vitamin D is known to inhibit the proliferation of keloid fibroblasts by inhibiting extracellular matrix production induced by transforming TGF-β and increasing MMP activity [7].

In this study, there was a negative correlation between serum 25-hydroxyvitamin D level with keloid severity. The negative correlation indicates that the lower the serum 25-hydroxyvitamin D level, the more severe the degree of the keloid. The results of this study are by the research conducted by Medikawati et al., which found a negative correlation between plasma 25-hydroxyvitamin D level and the severity in keloid subjects ($r = -0.584$; $p < 0.001$) [10]. Study on the correlation of serum 25-hydroxyvitamin D level in keloid is still scarce, but in the study by Zhang et al., found the presence of VDR in keloid fibroblasts culture and incubation of keloid fibroblasts with 1.25-dihydroxy vitamin D suppresses TGF-β1 which induces type I collagen, fibronectin, and α-SMA expression [11]. Also, several studies have shown that the Nuclear Transcription Factor-kB (NF-kB) signalling pathway is activated in keloid fibroblasts, whereas it is known that vitamin D has a role in regulating inflammation through inhibition of NF-kB. This contributes to the idea that vitamin D can inhibit the inflammatory process as occurs in keloid [12].

There was no significant relationship between serum 25-hydroxyvitamin D level and the gender of keloid patients in this study. The authors have not found a study discussing the relationship between serum 25-hydroxyvitamin D level with gender in keloid patients, but in a study by Yu et al., it was found that gene polymorphisms from vitamin D receptors affected the level of serum 1.25-hydroxyvitamin D and on stratification analysis based on gender found that this tendency only exists in the female subject but not in the male subject [4].

In this study, there was no significant relationship between serum 25-hydroxyvitamin D level and the age of keloid patients. According to Hagenau et al., study of the global serum 25-hydroxyvitamin D status in the general population, it was found that the lowest serum 25-hydroxyvitamin D level was at an age group of ≤ 15, and the highest was at the age group of 66-75 years, but there was no significant relationship between the level 25-hydroxyvitamin D with age [13].

In this study, there was no significant relationship between the level of serum 25-hydroxyvitamin D and the duration of the keloid. It is known that there is a role for vitamin D in the pathogenesis of keloid, but the authors have not found literature discussing the relationship between the level of serum 25-hydroxyvitamin D and the duration of the keloid. To determine whether there is a role for vitamin D in the course of the duration of the disease requires further research.

This study also found no relationship between the level of serum 25-hydroxyvitamin D with a family history. In a study by Lu et al., which analysed the role of family history of keloid, the highest keloid severity was observed in subjects with a positive family history, and the risk for the occurrence of keloid in the first-degree relative was 72.45%. These results indicate that genetic factors play an important role in the occurrence of the keloid [14].

In conclusion, the lower level of serum 25-hydroxyvitamin D, the greater the severity of keloid, there was no correlation between serum 25-hydroxyvitamin D level with gender, age, duration, and family history of the keloid. Further studies are needed to determine the benefits of vitamin D derivatives administration to keloid patients, as a basis for consideration of additional therapy in the management of keloid in health services.

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ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2019 Jan 15; 7(1):68-72.
https://doi.org/10.3889/oamjms.2019.015
eISSN: 1857-9655
Clinical Science

Sonographic Diagnosis and Clinical Correlates of Gallbladder Stones in Patients with Sickle Cell Disease in Calabar, Nigeria

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Citation: Inah GB, Ekanem EE. Sonographic Diagnosis and Clinical Correlates of Gallbladder Stones in Patients with Sickle Cell Disease in Calabar, Nigeria. Open Access Maced J Med Sci. 2019 Jan 15; 7(1):68-72. https://doi.org/10.3889/oamjms.2019.015

Keywords: Sickle cell disease; Cholelithiasis; Ultrasonography

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Received: 02-Jul-2018; **Revised:** 19-Oct-2018; **Accepted:** 20-Oct-2018; **Online first:** 12-Jan-2019

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

Abstract

BACKGROUND: Sickle Cell Disease (SCD) causes chronic haemolysis which is a risk factor for cholelithiasis.

AIM: To determine the prevalence and clinical correlates of cholelithiasis in SCD patients in steady state treated at the University of Calabar Teaching Hospital.

METHODS: This was a prospective study that took place at the Haematology and sickle cell disease clinics, University of Calabar Teaching Hospital, Calabar, Nigeria between January and June 2018. The study population were aged between 1.5-5.5 years and confirmed to have SCD through haemoglobin electrophoresis. A brief history was obtained, and all the patients had a physical examination. Ultrasound examination was performed using a B-mode mind-ray ultrasound machine using a 3.5-5.0 MHz probe after an overnight fast. A Calculus is diagnosed when a highly echogenic structure casting a concrete shadow is detected in the lumen of the gallbladder.

RESULTS: One hundred and twenty confirmed SCD patients aged between 1.5-55 years were recruited in the study, 69 (57.5%) were males, while 51 (42.5%) were females. The overall prevalence of cholelithiasis was 10%, and it increased with age. The youngest patient with cholelithiasis was 13 years old. All the patients were asymptomatic at the time of examination. At the multivariate level, age, gender, weight and gallbladder volume were associated with gallbladder stones.

CONCLUSION: The prevalence of cholelithiasis in patients treated at the Sickle Cell Clinic at the University of Calabar Teaching Hospital, Calabar is fairly high. The patients were largely asymptomatic, and cholelithiasis is more common in females than males. This study showed a weak association between blood transfusion and gallbladder stone. It is recommended that routine abdominal ultrasound scan for gallbladder be done for SCD patients from the second decade of life in our environment.

Introduction

Sickle cell disease (SCD) is the commonest haemoglobinopathy in people of African racial origin [1]. Sickle cell haemoglobin (HBS) has its highest prevalence in West Africa where it is reported to have originated and is also present in black Americans of African descent, Indians and those from the eastern Mediterranean region [2] [3] [4]. Nigeria by her population is the most sickle cell disease (SCD) endemic country in the world with over 40 million people (30% of its population) being carriers of the haemoglobin `s` gene while the homozygous SS is found in about 3% of the population [5].

Sickle cell disease (SCD) can occur as a homozygous form (HbSS) or heterozygous form, such as HbSC or HbSD among other variants. However, the homozygous variant HbSS has the severest clinical manifestation [6] [7] [8].

Cholelithiasis is a frequent complication of chronic haemolysis due to sickle disease [9]. It is sometimes revealed by digestive symptoms difficult to distinguish from painful abdominal vaso-occlusive crises (recurrent abdominal pain sometimes similar to biliary colic, nausea, vomiting). However, cholelithiasis is often asymptomatic and can lead to serious complications (cholecystitis, cholangitis, pancreatitis, septicaemia starting in the bile) which can jeopardise patients` lives [10] [11].

Many studies show that the prevalence of cholelithiasis in patients with sickle cell disease increases with age and affects 6% of patients before 15 years of age and more than 50% of young adults [9] [12] [13]. It is thought that the prevalence of cholelithiasis is substantially lower in African patients than Jamaican or north American patients [14]. This difference is attributed to differences in dietary cholesterol and/or fibre, but other factors (genetic or environmental) could have an influence. Gallstones treatment is equivocal, but most studies recommend cholecystectomy in the symptomatic cases and regular ultrasonography in other cases [11] [15].

Although there have been reports of the use of ultrasound in the diagnosis of cholelithiasis, the clinical correlates of cholelithiasis in the people with SCD is under-reported [9] [16]. Unlike most of the modern imaging modalities, ultrasound provides a widely available, non-invasive, inexpensive method for evaluating the gallbladder without the use of ionising radiation [17]. These factors are of particular importance in young patients with chronic diseases who require recurrent follow-up imaging. An ultrasound scan can be performed on routine clinic visits as it provides accurate pre-treatment diagnosis essential to plan appropriate management of this pathology.

This study was designed to sonographically determine the prevalence and clinical correlates of gallbladder stones in patients with sickle cell disease in southern Nigeria.

Subjects and Methods

This was a prospective study carried out to determine the prevalence and clinical correlates of gallbladder stones in patients with homozygous sickle cell disease in southern Nigeria, between January 2018 and April 2018. During the study period 120 patients between the ages of 1.5-55 years attending the sickle cell clinics (both children and adult) at the University of Calabar Teaching Hospital, Calabar, Nigeria were consecutively recruited into the study. A brief history was taken with emphasis on some blood transmissions, number and type of crises, chronic abdominal pain and nature of stools. Weight and height of participants were documented.

All the patients had a physical examination including anthropometry, under conditions of privacy with the following being examined:

General examination for pallor, jaundice and clubbing as well as digestive system examination were carried out with emphasis on organ enlargement (liver and spleen), tenderness and Murphy's sign.

Ultrasound examination was performed on all the SCD patients without a history of cholecystectomy. All patients were examined with a B-mode MINDRAY ultrasound machine using a 3.5-5.0 MHz probe after an overnight fast. The examination was performed in supine and decubitus positions on a couch. Calculi were diagnosed when highly echogenic structures with acoustic shadowing were detected in the lumen of the gallbladder.

Informed consent was obtained from all participants and strict confidentially ensured. Ethical clearance was obtained from the Research Ethics Committee of UCTH Nigeria.

Those excluded from the study were SCD patients who had any other coexisting morbidities like HIV or malignancies, SCD patients with a history of cholecystectomy and SCD patients who did not give consent for the examination.

The data were analysed with the Statistical Package for Social Sciences (SPSS) software version 18 for the window. Simple proportions, percentages and graphs were used to analyse the data. Chi-square test was used to test the difference between categorical variables. Student's t-test was used to compare continuous variables. Odds ratio and multiple regression analysis were used to identify predictors of gallbladder stones, a p-value of < 0.05 was regarded as significant.

Results

One hundred and twenty confirmed sickle cell disease patients were recruited into the study. The age range of the study group was between 1.5 to 55 years with a median age of 14.5 years; IQR 6-25years (Figure 1). There was a slight male preponderance; 69(57.5%) were males, while 51(42.5%) were females (male: female ratio of 1.3:1). The mean age for females was 18.4 ± 12.3 years (95% CI 14.9-21.9 years). The mean age for males was 14.6 ± 11.0 years (95% CI 12.0-17.2 years). The males were slightly younger than the females ($t = 1.8$; $p = 0.03$).

Cholelithiasis was reported in 12 patients giving a general prevalence of gallbladder calculi as 10.0% (95% CI 5.3-16.8%). No individual had developed gallbladder calculi in the first 10 years of life. The youngest SCD patient with gallbladder calculi was 13 years old.

At the univariate level, increasing age was significantly associated with the prevalence of gall bladder stones (OR = 15.17; $p = 0.0001$). Gender had no effect ($x^2 = 0.47$; $p = 0.50$). Weight showed a trend (OR = 2.8; $p = 0.09$; 95% CI 0.99-1.04). Height was significantly associated with gall bladder stone (OR 1.02, $p = 0.04$, 95% CI 1.00-1.05).

The body mass index (BMI) had no association with gallstones (OR = 1.06; p = 0.17; CI = 0.98-1.14).

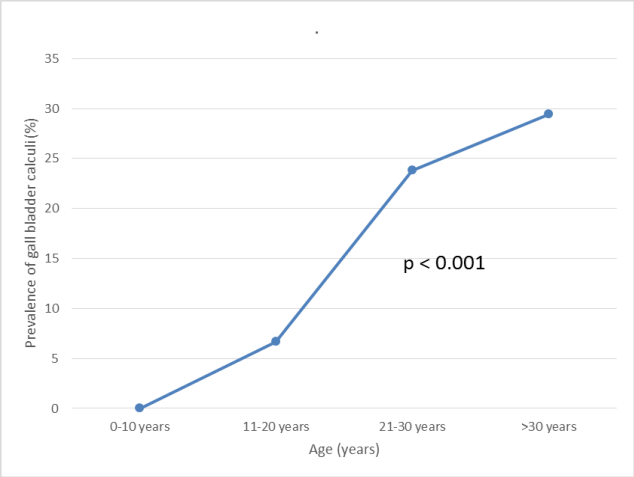

Figure 1: Line graph showing the prevalence of gall Stones with age

At the multivariate level age, gender, weight and gallbladder volume were associated with gallbladder stones (table). Area under the ROC curve was 0.96, indicating a very good model. No subject had positive Murphy's sign.

Table 1: Logistic Regression Model for Predictors of Cholelithiasis in SCD Patients

VARIABLE	UNIVARIATE ANALYSIS ODDS RATIO (95% CI, p-VALUE)	MULTIVARIATE ANALYSIS ODDS RATIO (95% CI, p-VALUE)
Age	1.10 (1.04 – 1.17, 0.001)	1.18(1.05 – 1.33, 0.010)
Gender	1.54(0.44 – 5.42, 0.50)	11.77(1.02 –1.34, 0.05)
Weight	1.02(0.99 – 1.04, 0.09)	0.90(0.82 – 0.99, 0.03)
Height	1.02(1.00 – 1.04, 0.04)	0.99(0.98 – 1.02, 0.97)
Number of blood transfusion	1.40(1.00 – 1.96, 0,05)	1.22(0.76 – 1.98, 0.40)
Gall bladder volume	1.03(1.012 – 1.03, 0.00)	1.04(1.01 – 1.06, 0.00)
Liver span	1.45(1.09 – 1.90, 0.01)	1.50(0.87 – 2.44, 0.15)

Area under ROC curve = 0.9567.

Discussion

Chronic haemolysis with its accelerated bilirubin turnover leads to a high incidence of pigment gallstones, and gallbladder sonography has become the dominant method of examining the gallbladder [18] [19] [20]. This is so because ultrasonography is convenient, safe and does not use ionising radiation. It has 88% sensitivity and 80% specificity for the diagnosis of gallstones [21].

Previous studies on the prevalence of cholelithiasis on SCD patients show a wide variation in the prevalence [12] [22] [23] [24]. The prevalence of cholelithiasis is reported to be substantially lower in African patients than in Jamaican or North American patients.[14]This difference could be attributed to differences in dietary cholesterol and/or fibre, but other factors (genetic or environmental) could have an influence.

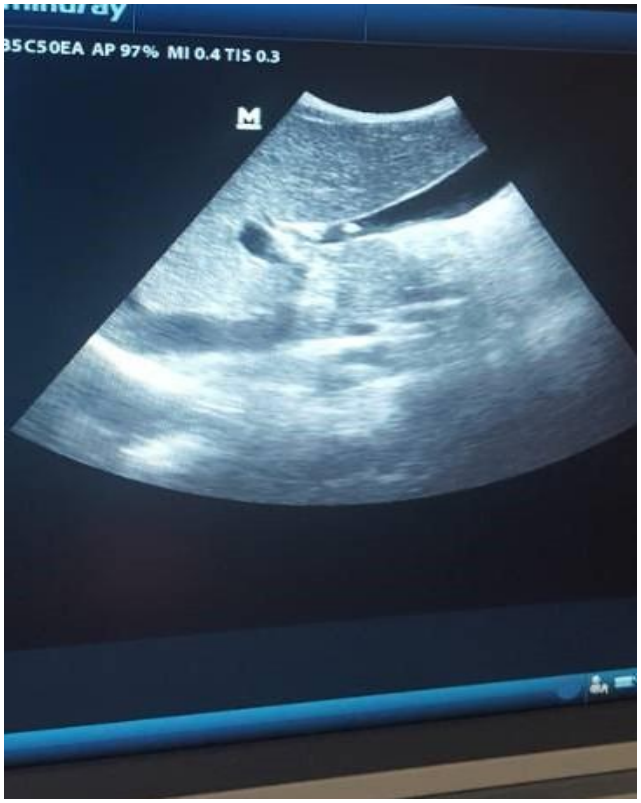

Figure 2: Cholelithiasis in a 32-year-old female SCD patient

In this study, the overall prevalence of gallstones was 10%. The low prevalence of cholelithiasis compared to other studies such as that reported by Agholor CA et al., [16] who reported 16.0% among 150 patients aged between 10 months and 51 years and Durosimi MA et al., [25] who documented 24.2% among 18 to 56 years old Nigerians with SCD could be attributable to dietary factors and access to specialized care. A low prevalence of cholelithiasis of 6% in patients younger than 15 years who were examined by oral cholecystography was reported by Akinyanju and Ladapo in Ibadan, Nigeria [12]. This is similar to the 5% prevalence of cholelithiasis observed byAkamaguna et al., [26] among sickle cell disease patients at the University of Benin Teaching Hospital Nigeria using oral cholecystography and the 50% reported by Odunvbun ME et al., in a study population of 101 aged 1-18years.

The prevalence of gallstones is usually increased with increase in age, and by the age of 18 years, 30% of SCD are expected to have developed gallstones [27] [28]. The youngest SCD patient with gallbladder calculus was 13 years old. This finding was at variance with the study by Akamaguna et al., who reported 5.5-year-old boy as the youngest with gallbladder stones and the study by Odunvbun et al., [29] reported the youngest child with gallstones to be

5 years old. A Ghanaian study reported gallstone in a 2.5-year-old SCD child [22], who was symptomatic. The high age of onset of gallbladder stone in our study may be attributed to dietary and environmental factors. Age, gender, weight and gallbladder volume are predictors in this study. This trend was also reflected in other studies when they were categorised into age groups [24] [30] [31] [32].

This study showed a weak association between blood transfusions and gallbladder stones and no association with chronic abdominal pain, gallbladder wall thickness and change in bowel habit. We studied patients in the steady state and patients with gallstones in this study were asymptomatic. This finding is the same as what has been reported by Barrett-Connor [30], Webb et al., [23] and Attalla et al., [22] where most their patients were asymptomatic. While Sarnaik [28], Cameron et al., [32] Karayalcin [33], and Bond et al., [24] reported that symptoms of typical biliary tract disease were common in patients with gallstones.

With the earliest incidence of gallstones seen in a 13-year-old in this study, routine screening with abdominal ultrasound scan is recommended for people with SCD from the second decade of life for early diagnosis and treatment to prevent complications.

In conclusion, the prevalence of cholelithiasis in patients treated at the sickle cell clinic at the University of Calabar Teaching Hospital, Calabar (1 m 10%). The patients were largely asymptomatic. It increases with increasing age, weight, gallbladder volume, and is more common in females than in males. It is recommended that routine abdominal ultrasound scan for gallbladder be done for sickle cell patient from the second of life in our environment,

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ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2019 Jan 15; 7(1):73-76.
https://doi.org/10.3889/oamjms.2019.024
eISSN: 1857-9655
Clinical Science

Dexmedetomidine versus Magnesium as Adjuvants to Bupivacaine-Induced Caudal Block in Children: A Randomized, Double-Blinded, Placebo-Controlled, Trial

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Citation: Refaee HH, Abo Elela AH, Hanna MG, Ahmed Ali MA, El Khateeb AM. Dexmedetomidine versus Magnesium as Adjuvants to Bupivacaine-Induced Caudal Block in Children: A Randomized, Double-Blinded, Placebo-Controlled, Trial. Open Access Maced J Med Sci. 2019 Jan 15; 7(1):73-76. https://doi.org/10.3889/oamjms.2019.024

Keywords: Caudal block; Children; Magnesium; Dexmedetomidine

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Received: 08-Nov-2018; **Revised:** 14-Dec-2018; **Accepted:** 15-Dec-2018; **Online first:** 12-Jan-2019

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

Abstract

BACKGROUND: Caudal block remains fundamental in pediatric anaesthetic practice. It is very useful in a wide range of surgical procedures and has proved to have a remarkable safety record, But one of the major limitations of the single-injection technique is the relatively short duration of postoperative analgesia. Prolongation of caudal analgesia using single-shot technique has been achieved by the addition of various adjuvant.

AIM: This work aims to compare magnesium and dexmedetomidine as adjuvants to bupivacaine-induced caudal block in children undergoing lower limb orthopaedic surgery.

STUDY DESIGN: Randomized, double-blind trial.

SETTINGS: Pediatric or of a tertiary care centre.

METHODS: A double-blinded, randomised controlled trial included 36 children, aged between 1 and 7 years, scheduled for lower limb orthopaedic surgery. Patients received general anaesthesia in addition to the caudal block. Patients were divided into three groups: Dexmedetomidine group (n = 12): received 0.5 mL/Kg bupivacaine + 2 mcg/Kg dexmedetomidine, Magnesium group (n = 12): received 0.5 mL/Kg bupivacaine + 50 mg magnesium, and control group (n = 12): received 0.5 mL/Kg bupivacaine + normal saline. Patients were compared according to the duration of analgesia, pain scores, sedation scores, mean arterial pressure, and heart rate.

RESULTS: Both magnesium group and dexmedetomidine group showed better analgesic profile (duration of analgesia and pain scores) compared to the control group without significant difference between the two former groups. Dexmedetomidine group showed higher sedation score, lower mean arterial pressure and lower heart rate compared to other groups.

CONCLUSIONS: Both magnesium (50 mg) and dexmedetomidine (2 mcg/Kg) improved the analgesic profile of bupivacaine-induced caudal block in children. Dexmedetomidine administration was accompanied with higher sedation score and negative hemodynamic profile.

Introduction

Caudal block remains fundamental in pediatric anaesthetic practice. Single-injection technique for the caudal block is usually limited by short duration of postoperative analgesia [1]. Addition of adjuvants to local anaesthetics could prolong the duration of the caudal block.

Dexmedetomidine (Dex) is a highly selective α2-adrenoreceptor agonist with various uses in anaesthesia and intensive care units [2], [3]. Dex offers a unique pharmacological profile because it is a sedative, sympatholytic, analgesic with minimal respiratory depression. Dex had shown good results when used as an adjuvant to caudal block [4]. Although dexmedetomidine is a highly promising adjuvant to local anaesthetics, its use is limited by the negative hemodynamic profile and the high cost.

Magnesium (Mg) is another drug which has

analgesic and antinociceptive effects. In a recent systematic review, Kawakami et al. had reported that Mg improved the analgesic profile of caudal block in children [5]. To the best of our knowledge, no data is available for comparison of Dex and Mg as adjuvants to bupivacaine-induced caudal block in children.

We conducted this study to compare the analgesic and hemodynamic effects of dexmedetomidine and magnesium sulphate when added to bupivacaine in the caudal block in children.

Methods

This double-blinded, randomised, controlled trial was conducted in Cairo University hospital after approval of the research ethics committee. Written informed consent was obtained from the patient's parents or guardians.

Thirty-six patients aged between 1-7 years, ASA I-II scheduled for infra-umbilical orthopaedic surgeries were included in the study. Patients were randomly assigned into 3 groups using an online randomisation program (http://www.randomizer.org). Concealment was achieved using opaque envelopes. Exclusion criteria include allergy to the study drugs, suspected coagulopathy, local infection, history of developmental delay, neuromuscular disorders, skeletal deformities, and magnesium therapy, Patients in whom caudal block failed were excluded from our study.

On arrival to the preparing room, patients were sedated by midazolam (0.2 mg/kg IM).On arrival to the operating room, patients were monitored using five-lead electrocardiography (ECG), automated non-invasive blood pressure monitor (NIBP), pulse oximetry and temperature probe.

Inhalational induction of anaesthesia was achieved by sevoflurane in 100% oxygen, and an appropriate-sized cannula was inserted. The endotracheal tube was inserted.

Patients were randomly assigned into 3 groups:

a. Group D (n = 12): received caudal block bupivacaine (0.25%) at dose of 1 mL/Kg plus dexmetedomidine (2 µg/kg diluted into 0.5 mL) mL/Kg.

b. Group M (n = 12): received caudal block with bupivacaine (0.25%) at a dose of 1 mL/Kg plus magnesium sulphate (50 mg in 0.5 mL)

c. Group C (n = 12): received caudal block withbupivacaine 0.25% diluted in normal saline at a dose of 1 ml/kg plus 0.5 mL normal saline.

At the end of the operation, sevoflurane was discontinued. The endotracheal tube was removed, and patients were discharged in the post-anesthesia care unit. Rescue analgesia in the form of oral paracetamol (10-15 mg/kg/dose) was taken and had to be repeated every 6 hours if needed (the time at which FLACC score 4 or more).

The patients were transferred to the ward after spending two hours in the recovery room after the surgery.

Duration of analgesia (defined as the time of caudal block was performed to the time at which FLACC score 4 or more)

Hemodynamic measures: mean arterial blood pressure (MAP) (mmHg) and heart rate (bpm): were measured at the baseline (before induction of anaesthesia), after induction of anaesthesia, and every 5 minutes till the end of the operation. We analysed three readings: baseline reading, post-caudal block reading, and the average of all other intra-operative readings.

Pain score (FLACC score) [6] (ranging from 0 to10): was assessed at the end of surgery and 1, 2, 6, 8, and 12 hours postoperatively,

Ramsay score [7]: (ranging from 1 to 8)was recorded after PACU arrival

Total (12-hours) postoperative paracetamol requirements were recorded

Complications secondary to test drugs (postoperative nausea, vomiting, bradycardia (HR < 80 BPM) and hypotension (SBP < 70 mmHg+ age in years * 2) were also reported.

Our primary outcome was the duration of analgesia. The previous study showed a mean duration of analgesia286.4 ± 47.8 minutes in children who received bupivacaine in caudal block [8]. We calculated our sample size to detect a difference of 20% (58 ± 47.8 minutes) between study groups. A minimum number of 9 patients per group were calculated to have a study power of 80% and an alpha error of 0.05. The number was increased to 12 patients per group to compensate for dropouts.

Data were statistically described regarding mean ± standard deviation (± SD), median and range, or frequencies (number of cases) and percentages as deemed appropriate. Comparison of numerical variables was made using one-way analysis of variance (ANOVA) test with post-hoc multiple 2-group comparisons in normally distributed data, and Kruskal-Wallis test with post-hoc multiple 2-group comparisons in skewed data. For comparing categorical data, Chi-square (χ^2) test was performed. The exact test was used instead when the expected frequency is less than 5. A p value less than 0.05 was considered statistically significant. All statistical calculations were done using computer program SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) release 15 for Microsoft Windows (2006).

Results

Forty-four patients were screened for eligibility. Six patients were excluded, and 36 patients were available for final analysis (Figure 1).

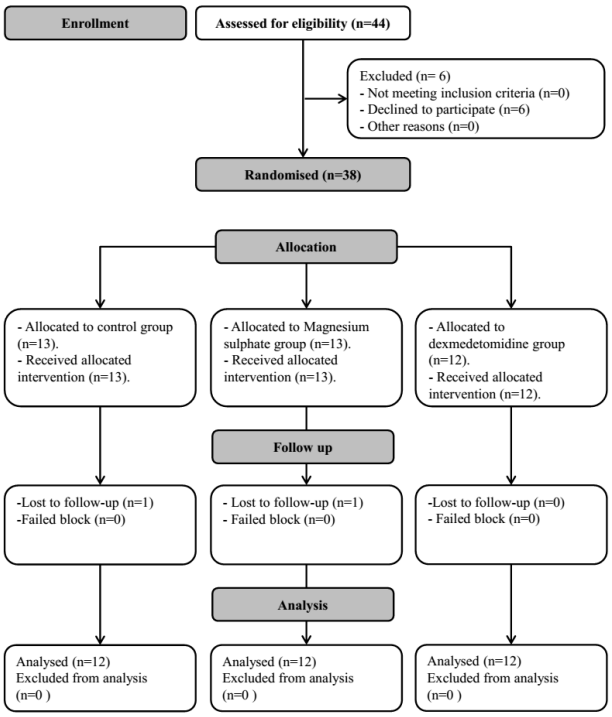

Figure 1: Consort chart for patient enrolment

Demographic data were comparable between the three groups (Table 1).

Table 1: Patient characteristics. Data are presented as mean ± standard deviation and frequency (%)

	Group C (n = 12)	Group D (n = 12)	Group M (n = 12)
Age (years)	4.11 ± 1.7	3.9 ± 2.2	3.1 ± 1.4
Weight (kg)	17.3 ± 4.1	16.8 ± 4.9	15.2 ± 4.1
Male gender	8 (67%)	6 (50%)	3 (25%)

Data are presented as mean ± standard deviation and frequency (%).

FLACC score was lower in both Group M and Group D when compared to Group C at all postoperative readings (Table 2).

Table 2: Mean arterial pressure. Data are presented as mean ± standard deviation

	Group C (n = 12)	Group D (n = 12)	Group M (n = 12)
Baseline reading (mmHg)	70 ± 11	63 ± 9	63 ± 10
Post-block reading (mmHg)	63 ± 8	55 ± 9	66 ± 8
Mean intraoperative reading (mmHg)	60 ± 15	48 ± 6*	52 ± 7

*denotes significance compared to group C.

Group D showed lower FLACC score compared to group M at the first postoperative hour; while both groups were comparable in the subsequent readings (Table 3).

Table 3: Heart rate. Data are presented as a mean ± standard deviation

	Group C (n = 12)	Group D (n = 12)	Group M (n = 12)
Baseline reading (mmHg)	136 ± 13	136 ± 20	138 ± 16
Post-block reading (mmHg)	124 ± 11	118 ± 10	119 ± 13
Mean intraoperative reading (mmHg)	115 ± 13	103 ± 7 *	111 ± 10

*denotes significance compared to group C.

Ramsay score was comparable between group D and group M; while it was higher in both groups compared to group C (Table 4).

Table 4: FLACC score. Data are presented as median (range)

	Group C (n = 12)	Group D (n = 12)	Group M (n = 12)
One-hour postoperative	1.5 (1-3)	*0 (0-1)	*1 (0-2)
Two-hour postoperative	2 (2-4)	*1 (0-2)	*2 (1-2)
Six-hour postoperative	4 (2-6)	*2 (0-4)	*2 (1-4)
Eight-hour postoperative	5 (4-6)	*4 (1-5)	*4 (2-6)
Twelve-hour postoperative	6 (5-6)	*4 (4-4)	*4 (4-6)

FLACC: face, legs, activity, cry, insolubility scale; *denotes significance compared to group C; † denotes significance compared to group M.

Time of 1st rescue analgesia was longer in the group M, and group D compared to group C without significant difference between the two former groups (7.83 ± 1.19 hours, 7.75 ± 1.35 hours, and 4.92 ± 1.16 respectively) (Table 5).

Table 5: Postoperative data. Data are presented as mean ± standard deviation, median (range), and frequency (%)

	Group C (n = 12)	Group D (n = 12)	Group M (n = 12)
Ramsay score	2 (2-2)	4 (4-5) *†	4 (2-5) *
Time to rescue analgesia (hours)	4.9 ± 1.2	7.8 ± 1.4 *	7.8 ± 1.2 *
Number of rescue analgesic boluses (1 bolus / 2 boluses)	4/8	12/0 *	12/0 *

*denotes significance compared to group C. † denotes significance compared to group M.

Hemodynamic measures (MAP and heart rate) were comparable between the three groups at the baseline and post-block reading. Post-hoc analysis showed no difference between Group M and the other two groups in the intraoperative hemodynamic readings; while Group D had lower mean intraoperative readings compared to group C (MAP: 60.33 ± 14.9 vs 47.58 ± 5.6, p = 0.029), and (114.83 ± 13.5 vs 102.92 ± 7.2, p = 0.012)(Table 2 and 3).

Discussion

We reported that both dexmedetomidine and magnesium improved the analgesic properties of the caudal block when added to bupivacaine. No difference was observed between both drugs according to the duration of analgesia and pain score at most of the postoperative reading; however, dexmedetomidine was associated with lower intraoperative MAP and heart rate. Dexmedetomidine had been frequently reported as one of the most promising additives in neuraxial as well as peripheral nerve blocks [2], [9]. In a recent review, Trifa et al. had

suggested that there is sufficient evidence to recommend adding dexmedetomidine to local anaesthetics in the caudal block in children [4]. Dexmedetomidine is an alpha-2 adrenoreceptor agonist with unique pharmacological properties overall response to α_2 adrenoreceptors agonists is related to the stimulation of α_2 adrenoreceptors located in the CNS and spinal cord. These receptors are involved in the sympatholysis, sedation, and antinociception effects of α_2 adrenoreceptors [10]. Although dexmedetomidine is a potent adjuvant to local anaesthetics, its use had some limitations such as high cost and negative hemodynamic profile.

Magnesium is another drug with various clinical uses. Magnesium is a non-competitive NMDA receptor antagonist that promotes intracellular signalling culminating in long-term synaptic plasticity and the 'wind-up' phenomenon. Blocking the channel inhibits the development and maintenance of central sensitisation [5]. Thus, magnesium is characterised by useful analgesic effects when used as an adjuvant to local anaesthetics. In a recent systematic review and meta-analysis, Kawakami et al. had [5)] reported that magnesium might improve the analgesic properties of the ropivacaine-induced caudal block in children. Kawakami et al. had [5] recommended more randomised controlled trials to affirm their findings. No study to the best of our knowledge had investigated magnesium as an adjuvant to bupivacaine in the caudal block in children. We reported that using dexmedetomidine was associated with negative cardiovascular profile compared to magnesium; this finding is in line with most of the available data for using dexmedetomidine in neuraxial and peripheral nerve blocks [3], [9], [11]. Systemic absorption of dexmedetomidine had been well reported after extra-vascular injection [12]. This systemic absorption is associated with linear dose-dependent plasma level [13].

Pain management in children is essential and challenging. Caudal analgesia is the commonest regional block performed in the pediatric population. To the best of our knowledge, this is the first trial which compares magnesium and dexmedetomidine in the caudal block. It is also the first study to investigate both drugs as adjuvants to bupivacaine. Our findings suggest that the use of both magnesium and dexmedetomidine was associated with similar analgesic properties. This favours the use magnesium as a reasonable choice in the caudal block which is efficient, economic, and safe.

Our study has the advantage of being a well-powered, double-blinded, randomized controlled trial. However, it had some limitations: 1- It is a single centre study. 2- We used a single dose for each drug. 3- We performed as a single shot caudal block; thus, we could not extrapolate our findings in continuous blocks. We recommend future studies to evaluate the optimum doses and to investigate both drugs in continuous blocks.

In conclusion, both magnesium (50 mg) and dexmedetomidine (2 mcg/Kg) improved the analgesic profile of bupivacaine-induced caudal block in children. Dexmedetomidine administration was accompanied with higher sedation score and negative hemodynamic profile.

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ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2019 Jan 15; 7(1):77-81.
https://doi.org/10.3889/oamjms.2019.035
eISSN: 1857-9655
Clinical Science

Effect of Early Breast Milk Nutrition on Serum Insulin-Like Growth Factor-1 in Preterm Infants

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Citation: Elzaree FA, AbuShady MM, Atti MA, Fathy GA, Galal EM, Ali A, Elias TR. Effect of Early Breast Milk Nutrition on Serum Insulin-Like Growth Factor-1 In Preterm Infants. Open Access Maced J Med Sci. 2019 Jan 15; 7(1):77-81. https://doi.org/10.3889/oamjms.2019.035

Keywords: Breastfeeding; Growth; IGF-1; Premature infants

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Received: 05-Nov-2018; **Revised:** 04-Dec-2018; **Accepted:** 22-Dec-2018; **Online first:** 12-Jan-2019

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

Abstract

BACKGROUND: Insulin-like growth factor 1 (IGF-1) is one of the essential intrauterine hormonal mediators of growth, and its serum values are often low after preterm delivery.

AIM: To evaluate the influence of immediate breast milk feeding on serum IGF-1 in preterm newborns.

SUBJECTS AND METHODS: This prospective, observational cohort study included 60 premature infants born < 32 weeks of gestation, divided into group A and B regarding breastfeeding or formula feeding. Growth measurements were taken at birth. The standard deviation of each measurement was calculated. Serum IGF-I was measured one day postnatal and at a time equivalent to 40 weeks of gestation.

RESULTS: Significant higher level of mean serum IGF-1 was detected in group A than B at postnatal age equivalent to 40 weeks of gestation. In group A, the higher significant level was detected in mean serum IGF-1 at an age equivalent to 40 weeks of gestation than at birth (25.21 ± 6.69 and 20.13 ± 5.46 $p < 0.05$). Multiple linear regression analysis showed that high birth weight, increased age of gestation and breastfeeding were correlated to the elevated serum level of IGF-1 at a postnatal age corresponding to 40 weeks gestational age.

CONCLUSION: Immediate breast milk feeding was accompanied by elevated IGF-1 in the serum of preterm infants.

Introduction

IGF-1 is an anabolic hormone with mitogenic, differentiating, antiapoptotic and metabolic effects [1]. A plethora of genetic and experimental researches suggest that IGF-1 is an essential factor of intrauterine growth of the fetus and after birth [2]. The placenta secretes IGF-1 throughout gestation which encourages the transmission of important nutrients from the mother to the fetus via the placenta [3]. During gestation, fetal circulating IGF-1 increases and at term birth, levels of cord serum IGF-1are positively related to the size of fetus and fat mass [4]. Fetal serum IGF-I constantly increases during last trimester, which coincides with the interval of the most rapid increase in fetal weight [5]. Late in gestation, circulating IGF-1 is mainly derived from the liver, although all fetal tissues express IGF-1 from an early stage of development [6]. The amniotic fluid contains higher IGF-1 concentrations than cord blood during gestation and at delivery and is swallowed by the fetus, and this source is missing after premature delivery [7]. Insulin is the main determinant of fetal and neonatal hepatic IGF-1 secretion, and via insulin, IGF-1 is magnified by the direct and indirect influences of nutrients like glucose and protein. Also, insulin reciprocally regulates hepatic production of insulin-like growth factor binding protein (IGFBP-1),

which in turn inhibits IGF-1 bioactivity [8]. There is 6 binding proteins (IGFBP) control IGF-1 action. About 80% of IGF-1 is combined with IGFBP-3 which together with an acid-labile subunit maintains a reservoir of circulatory IGF-1 [9]. After fasting and during hypoxia serum levels of IGFBP-1 increase, which restrict growth through lowering the IGF-1 bioavailability [10]. Mothers' milk should be advised for preterm infants by direct breastfeeding and/or by mother's own expressed milk [11]. Some studies on Holder pasteurisation have demonstrated partial or total inhibition of bioactive components as immunological, growth and antioxidant factors. These constituents are available in huge quantities in preterm infants' mothers' milk and are significantly essential immediately after delivery [12]. In particular, the premature breast milk contains high levels of IGF-1which are lowered by approximately 40% upon Holder pasteurisation [13]. Milk-borne IGF-1 acts as a growth factor for intestinal maturation; while, some animal researches have demonstrated possible absorption into circulation with a dose-dependent elevation in orally administered IGF-1 and protective action of milk proteins on IGF-1 degradation [14].

The current study aimed to evaluate the influence of immediate breast milk feeding either expressed breast milk (EBM) or direct breastfeeding on levels of serum IGF-1in preterm newborns.

Subjects and Methods

This is a prospective, longitudinal, observational cohort study; included all preterm infants of < 32 weeks gestational age admitted into neonatal intensive care unit of Children Hospital, Faculty of Medicine, Ain Shams University between December 2016 and January 2018. The study was approved by the medical ethical committee of National Research Center, Cairo, Egypt by the code of ethics of the world medical association (Declaration of Helsinki) for experiments in humans, 1975. Approval No. (18113).

Exclusion criteria were conditions that influence IGF-1 plasma levels: intrauterine growth retardation, small for gestational age, insulin or steroid therapy; also, infants with cerebral lesions or any gestational congenital anomaly were excluded. The study design and clinical and laboratory assessments were explained, and informed consent was obtained from parents and/or legal guardians of infants before enrolment in the study.

Subjects of the study were divided into 2 groups regarding breast milk intake (direct or expressed breast milk) shortly after birth: Group A, 30 newborns fed on breast milk ≥ 50 mL/Kg/day and glucose infusion (10%), without any parenteral supplementation with fat or amino acids from the first days of life; Group B, 30 infants received glucose infusion (10%), plus milk formula without any parenteral supplementation with fat or amino acids due to insufficient mother's breast milk or the illness of the mother by any condition that prevents normal breastfeeding as infection of the mother by HIV or Hepatitis B. Parenteral feeding was started on the first day of life in all infants with a birth weight < 1250 g. Fluid intake was started with 60 mL/Kg/day to guarantee sufficient intake of calories (120-130 Kg/day) [15]. Before administration, the milk bottles were shaken to integrate any remains, and the needed amount was aseptically separated. Fresh breast milk was not given to infants of mothers with HIV, HBV, HCV, CMV, typhoid, paratyphoid, brucellosis, pertussis, active pulmonary tuberculosis, or syphilis, or under medications unsuitable for breastfeeding. Also, breastfeeding was interrupted temporarily in the case of mastitis, nipple mycosis, breast or chest herpes simplex or varicella zoster infections. To estimate feeding tolerance, the daily gastric residual volume (GRV) and episodes of daily emesis were recorded. The criteria for decreasing enteral feeding were GRV > 4 mL/Kg after one meal or > 2 mL/Kg after 3 consecutive meals, or > 3 consecutive episodes of vomiting. For a total withdrawal of enteral feeding were GRV > 5 mL/Kg after one meal, abdominal distension with an increase in abdominal circumference > 2 cm in 24 hours, metabolic acidosis with pH < 7.20 for > 2 hours, hypoxia with paO2 < 50 mm/Hg for > 2 hours, or hypotension. Full enteral feeding was defined by an intake of 150 mL/kg/day. Total parenteral and enteral caloric and protein intakes were calculated. Despite the diversity of macronutrients, with breast milk given over the first two weeks of life, it was assumed that the raw milk energy and protein contents were 78 kcal/100 mL and 2.2 g/100 mL, respectively [16].

IGF-I in serum was measured by chemiluminescence immunoassay (Liaison, DiaSorin, Saluggia, Italy) at 1-2 days postnatal and 40 weeks gestational age. The intra-assay coefficient of variation is 8% at both 10.3 nmol/l and 17.5 nmol/l and 9% at 23.8 nmol/l. Inter-assay coefficient of variation is 10% at 6.9 nmol/l, 7.4% at 30.8 nmol/l and 16% at 59.4 nmol/l.

Standardised measurements of weight, length, and head circumference (HC) were recorded within the first 24 h after birth and then weekly on the same weekday as blood sampling for IGF-I and continued until discharge. Most of the growth measurements were performed by one examiner. Weight was measured on a digital scale (Tanita TL-150MA, Tanita Corporation, Tokyo, Japan). Crown to heel length was measured using a portable length scale instrument with increments in millimetres developed at the NICU enabling measurements within the incubator as on the nursing table. HC was measured in the maximum front-occipital plane using

an individual non-extensible plastic-coated tape with increments in millimetres. Z-score (Standard deviation score, SDS) was calculated for all measurements of each respective growth parameter. SDS signifies how many SD is > or < the mean of a reference population. SDS length and SDS HC were computed from a gender-specific growth reference in the Egyptian population, whereas SDS weight was calculated from an intrauterine growth curve based on ultrasonically estimated fetal weights in Egypt (17).

It was done by statistical package for social sciences (SPSS) version 21 for Windows (IBM Corp., Armonk, NY, USA). Continuous data were expressed as mean ± standard deviation and were compared by using student's t-test and paired t-test. Categorical data were expressed as numbers and analysed with the two-tailed chi-square test. Multiple linear regression analysis was done to find predictors of serum IGF-1 at birth and at age correspond to 40 weeks gestational age. P < 0.05 was accepted as statistically significant.

Results

A group of 70 preterm newborns were included in the study. We excluded 6 neonates small for gestational age and 4 with cerebral lesions. The remaining 60 were divided into 2 groups: group A (N = 30) fed breast milk either direct or expressed and group B (N = 30) receiving formula till the age corresponding to 40 weeks of gestation. Table 1 shows the baseline features of both groups.

Table 1: Neonatal characteristics of preterm infants in both groups

	Group A (N = 30)	Group B (N = 30)	
	Mean ± SD	Mean ± SD	P
Gestational age (weeks)	30.13 ± 1.07	30.17 ± 1.02	0.902
Weight (kg)	1.45 ± 0.25	1.44 ± 0.24	0.813
Length (cm)	37.43 ± 1.49	37.87 ± 1.55	0.274
Head circumference (cm)	27.47 ± 1.61	27.22 ± 1.31	0.518
Weight SDS	0.26 ± 0.55	0.21 ± 0.41	0.700
Length SDS	-0.59 ± 0.47	0.42 ± 0.37	0.122
Head circumference SDS	0.10 ± 0.65	-0.01 ± 0.50	0.335
Apgar score (1 minute)	5.04 ± 1.43	5.07 ± 1.36	0.929
Apgar score (5 minutes)	7.46 ± 0.95	7.46 ± 0.92	0.991
	No	No	
Sex (Male/Female)	10/20	10/20	1.000
Mode of delivery (CS/SVD)	24/6	25/5	0.739

P < 0.05 is significant; SDS = Standard deviation score; CS = Cesarean section; SVD = Spontaneous vaginal delivery.

No apparent differences were seen in both groups as regards gestational age, weight, height, head circumference and SDS of height, weight and head circumference (p > 0.05). Both groups included 10 males and 20 females. In group A, 24 were delivered by section and 6 by normal labour, while in group B, 25 were delivered by section and 5 by normal labor with no marked difference among both groups (p > 0.05).

However, mean plasma level of IGF-1in group A was not significantly different from that in group B at birth (p > 0.05), a significantly higher level of mean serum IGF-1 was detected in group A than in Group B at age equivalent to 40 weeks of gestation measurement (p < 0.05) (Table 2).

Table 2: IGF-1 in the studied groups at birth and at 40 weeks gestational age

	Group A (n = 30)	Group B (n = 30)	p
	Mean ± SD	Mean ± SD	
IGF-1 at birth	20.13 ± 5.46	20.19 ± 6.21	0.970
IGF-1 at 40 weeks GA	25.21 ± 6.69	21.11 ± 4.59	0.008*

*p < 0.05 is significant; GA = Gestational age.

In Table 3, mean serum IGF-1 in each separate group was compared at birth and 40 weeks GA using a paired t-test. In group A, a higher significance level was detected in mean serum IGF-1 at equivalent 40 weeks of gestation than that at birth (p < 0.001) while in group B, no evident difference was shown (p > 0.05).

Table 3: IGF-1 at birth and 40 weeks gestational age in each group

	IGF-1 at birth	IGF-1 at 40 weeks GA	p
	Mean ± SD	Mean ± SD	
Group A (n = 30)	20.13 ± 5.46	25.21 ± 6.69	0.000*
Group B (n = 30)	20.19 ± 6.21	21.11 ± 4.59	0.111

*p < 0.05 is significant; GA = Gestational age.

Table 4, and 5 shows the predictors of IGF-1 as the dependent variable at birth and equivalent 40 weeks of gestation respectively. At birth, birth weight and gestational age were the main predictors of serum IGF-1. High birth weight and high gestational age were associated with high serum IGF-1 at birth.

Table 4: Multiple linear regression analysis for the predictors of serum IGF-1 at birth

	Unstandardized Coefficients		Standardised Coefficients	t	P
	B	Std. Error	Beta		
Gestational age (weeks)	3.585	1.663	.645	2.156	0.036*
Weight (kg)	11.557	5.287	0.467	2.186	0.034*
Length	-1.511	0.810	-0.406	-1.867	0.068
Apgar score 1 min	0.342	0.630	0.078	0.542	0.590
Apgar score 5 min	1.760	0.956	0.278	1.841	0.072

*P < 0.05 is significant.

At the age equivalent to 40 weeks of gestation, predictors of serum IGF-1 were birth weight, gestational age and breastfeeding during the early months of postnatal life. Increased birth weight, high GA and breastfeeding were accompanied by high serum level of IGF-1 at a postnatal age corresponding to 40 weeks GA.

Table 5: Multiple linear regression analysis for the predictors of serum IGF-1 at 40 weeks gestational age

	Unstandardized Coefficients		Standardised Coefficients	t	Sig.
	B	Std. Error	Beta		
Gestational age (weeks)	3.560	1.630	0.603	2.184	0.034*
Weight (kg)	11.396	5.173	0.433	2.203	0.033*
Length	-0.200	0.802	-0.051	-0.249	0.804
Apgar score 1 min	0.890	0.617	0.191	1.442	0.156
Apgar score 5 min	0.253	0.936	0.038	0.270	0.788
Group	-4.822	1.501	-0.391	-3.213	0.002*

*P < 0.05 is significant.

Discussion

It is known that fetus ingests significant amounts of amniotic fluid during the intrauterine third trimester, which contains higher values of IGF-1 than cord blood during pregnancy or at delivery. Human milk also, particularly colostrum, contains IGF-1. Although preterm infants may be fed on maternal milk/colostrum, the amounts are often very scanty, and maternal expression of colostrum could be inconvenient after birth immediately. Therefore, in preterm newborns, lack of IGF-1 resources in amniotic fluid and colostrum/milk may lead to less IGF-1 levels [18]. After very premature delivery, IGF-1 serum values decrease significantly to about 10 ng/ml while it is about > 50 ng/ml intrauterine at 23 to 30 weeks GA. Continuous low levels of IGF-1 after premature delivery are shown to be accompanied by a poor general and brain development as well as neonatal complications as intraventricular haemorrhage, retinopathy of prematurity (ROP), bronchopulmonary dysplasia (BPD)and necrotising enterocolitis (NEC) [19]. Levels of IGF-1were shown to be scanty at time of delivery of low birth weight babies, but increased gradually over the first 8 weeks of life and were positively associated with body weight, body length and body mass index at all time points. Also, IGF-I was shown to be accompanied by satisfactory growth at early postnatal age than by feeding and influence of nutrition on values of IGF-I may be limited to the period of settled catch-up growth [20]. In the current study, mean serum IGF-1 in preterm infants feeding breast milk (from birth till age corresponding to 40 weeks of gestation) was markedly more than that in preterm newborns feeding formula milk. Early breastfeeding was associated with increased serum value of IGF-1in preterm newborns.

In 2013, de Zegher et al. compared the influence of breastfeeding vs formula feeding after birth (small for gestational age) on weight and endocrine markers in late infancy (at birth, 4 and 12 months) [22]. In contrast to the results of our study, they found that formula-fed infants had increased adiposity and higher IGF-I values than breastfed babies. That can be explained by the difference in the age at which IGF-1 was measured, and type of infants included in the study at birth at an age corresponding to 40 weeks of gestation and premature infants in the present study [21], [22]. Hansen-Pupp et al., 2011 studied the interaction between feeding, IGF-I, and growth in 64 of premature infants. They concluded that IGF-I is related to growth at an earlier postnatal age more than feeding and influence of feeding on IGF-I levels may be limited to the phase of settled catch up growth [23]. Giapros et al., 2012 investigated in a prospective study the 1st year of life's IGF serum levels in 112 premature babies born equivalent to 32-36 weeks of gestation and their correlation with weight at birth and early neonatal growth. The average values of IGF-I at 2 and 6 weeks, was shown to be 82 ± 44, 100 ± 31 ng/ml, respectively [24]. In the current study, mean serum IGF-1, at the start and end point was 20.13 ± 5.46 and 25.21 ± 6.69 in group A respectively which were below than that in Giapros et al. 's results. That can be explained by the difference in GA of preterm babies enrolled at the start point of both studies [24].

Serrao et al., 2016 studied 52 premature newborns with GA < 31 weeks. They were divided into two groups as regards intake of expressed breast milk (< or ≥ 50 mL/Kg/day) till 32 weeks of gestation when sampling of blood for IGF-1 analysis was performed. In contrast to our study, they found that early expressed breast milk did not influence IGF-1 plasma values in preterm newborns [25]. The difference in results could be explained by the end point of measurement of IGF-1 which was 32 weeks of gestation in their study and 40 weeks of gestation in our study.

In conclusion, early breast milk nutrition, either expressed or direct feeding was correlated with a high serum IGF-1 in premature babies who may act to diminish general growth abnormalities, metabolic disorders, lung and retinal immaturity and brain developmental abnormalities which results in abnormalities in cognitive function. This study encourages maximum efforts to support immediate breast milk nutrition in the neonatal intensive care unit.

Further clinical trials are needed to study pitfalls and advantages of IGF-1 replacement in very premature newborns to keep postnatal IGF-1 to near fetal levels.

The essential restriction of this study is a small number of infants. The results have to be confirmed in larger groups of preterm infants to ensure high statistical power. Second, the study recruited subjects born in one hospital, and this limited sample makes it hard to be generalised on other populations. Finally, residual confounding factors not measured in our study may have affected the correlation between breastfeeding and IGF-1.

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ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2019 Jan 15; 7(1):82-87.
https://doi.org/10.3889/oamjms.2019.023
eISSN: 1857-9655
Clinical Science

Analysis of Treatment Results of the Thoracic Part of Oesophageal Cancer

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Citation: Orazbayev BA, Musulmanbekov K, Bukenov A. Analysis of Treatment Results of the Thoracic Part of Oesophageal Cancer. Open Access Maced J Med Sci. 2019 Jan 15; 7(1):82-87. https://doi.org/10.3889/oamjms.2019.023

Keywords: Esophageal cancer; Radiation therapy; Survival; Regional metastases

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Received: 04-Nov-2018; **Revised:** 29-Nov-2018; **Accepted:** 01-Dec-2018; **Online first:** 13-Jan-2019

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

Abstract

AIM: This study aims to evaluate the methods of treatment of the thoracic part of oesophagal cancer and to predict the results of treatment depending on the factors of the prognosis.

MATERIALS AND METHODS: The results of treatment of 366 patients with cancer of the thoracic part of the oesophagus for 10 years (2007-2016) by the department of thoracic oncology of the Karaganda oncological dispensary were studied.

RESULTS: The overall five-year survival rate, regardless of the method of treatment, was only 8.72% (28 of 321), and in the 6-10 year period 8.41% (27 of 321) lived, $p < 0.05$. Analysis of the overall survival of patients with cancer of the thoracic part of oesophagus showed that the method of treatment does not have a significant effect on life expectancy. A multivariate analysis of 19 grades that affect the prognosis of the disease was carried out.

CONCLUSION: Radical type of treatment of the middle and lower thoracic oesophagus is surgical, in which the median of cumulative survival is 19 months. Traditional radiotherapy should be used in a limited way, as it is palliative, with a median survival of no more than 9 months. The leading factor in the prognosis for thoracic part of oesophagal cancer is the presence of regional metastases, on which the choice of method of treatment depends.

Introduction

The incidence of oesophagal cancer in Kazakhstan remains high, amounting to 7.7 per 100 thousand, which is 1.2 times more than in the Russian Federation. Even though the population of the Russian Federation is 10 times more than in Kazakhstan. Mortality from this pathology in Kazakhstan takes the 4th place, accounting for 5.8%. The incidence of oesophagal cancer in the Karaganda region is 8.8 per 100 thousand populations, which is 1.3 higher than in the Republic of Kazakhstan.

The main cause of high mortality up to one year after diagnosis is late detection of oesophagal cancer; mortality is 65% [1]. Many researchers [2], [3] note that the majority of patients at the beginning of treatment have stage III-IV of the tumour process in 65-75% of cases. The presence of common forms of oesophagal cancer at the time of treatment creates certain difficulties in the choice of therapy. Many patients are unresectable due to the high prevalence of the tumour process [4], competing comorbidities. In 20% of cases after radical treatment, local recurrences of a tumour in the anastomosis was detected.

P.A. Herzen (Moscow) [5] has developed the following program for the treatment of oesophagal cancer. At stage I-II and in the absence of metastases, an organ-preserving treatment is proposed: electro- and laser destruction of the tumor with the administration of the “Photochem” and “Photosens” preparations, with locally advanced esophageal cancer, i.e. in stage III, in view of the severity of the initial condition (significant loss in weight, severe dysphagia, and dehydration), at the first stage, gastrostomy is performed according to our

own method with revision of paracardial, paraaortal metastasis areas and their removal. According to reports [5], the imposition of gastrostomy allows for adequate rehabilitation of the patient and to prepare for a radical operation. In the presence of a decompensated initial state, simultaneous resection of the oesophagus with esophagoplasty by an isoperistaltic stem from the greater curvature of the stomach is recommended. In doubtful cases, when the initial condition of the patient is assessed as serious, then dobromyslov's two-stage operation-Toreka is performed. Delayed esophagoplasty is used 6 months after the first. According to L.D. Roman et al., [3], the surgical method of treating cancer of the oesophagus remains the main method, despite the success of chemoradiotherapy. So R.Kube et al., [6] consider the results of 5-year survival during chemoradiation therapy of oesophagal cancer to be doubtful. According to D. Karpov et al., [7], with the common and neglected forms of oesophagal cancer, palliative surgical and endoscopic methods must be supplemented with radiation therapy. At the same time, Chissov V.I., Daryalova S.L. [8] believe that chemotherapy and radiation methods rarely regress a tumour, and the life expectancy after it does not exceed 13 months.

Other researchers [9], on the contrary, note that neoadjuvant radiochemotherapy is a promising method, and in 10–20% of cases complete regression can be achieved. Orringer M.B. et al. used a complex method of treatment of common forms of oesophagal cancer, and in 52% of patients in the postoperative period, no complications were noted [9].

Thus, the prospects for chemoradiation therapy for cancer of the thoracic oesophagus are extremely limited if they are not complemented by surgical intervention.

The purpose of this study is to evaluate the treatment of cancer of the thoracic oesophagus and to predict the results of treatment depending on the prognostic factors.

Material and Methods

The results of treatment of cancer of the ore oesophagus for 10 years (2007-2016) were analysed based on the materials of the Department of Thoracic Oncology of the Karaganda Oncologic Dispensary. The results of treatment of 366 patients with thoracic part of oesophagal cancer were studied. In the analysed material (Figure 1) men prevailed than women, the ratio was 1.6: 1 ($p \leq 0.05$).

Among the patients, the inhabitants of rural areas prevailed, 70.21 ± 2.4% (257), compared with urban areas, 29.78 ± 2.4% (109) $p \leq 0.05$.

According to the ethnic composition (Figure 2) of the patients, it turned out that the majority were ($p \leq 0.05$) of the indigenous people of Kazakhs 71.58 ± 2.36% (262), slavs-only 19.39 ± 2.07% (71) while others were identified at 9.03 ± 1.5% (33).

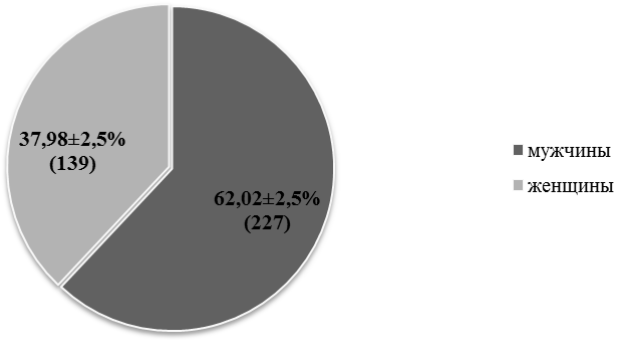

Figure 1: Sex composition of patients

Most often, tumours were localised in the middle part of thoracic part of the oesophagus - 58.74% (215), then in the lower part, 34.15 (125). According to the prevalence of the tumour process (Table 1), patients were distributed according to the international classification of staging (TNM, 2011) as follows, stage IIa, T3N0M0, 59.56% (218), then stage IIIa, T4aN0M0, 16.93% (62) and Ib stage T2N0M0 was only 10.65% (39) $p < 0.05$.

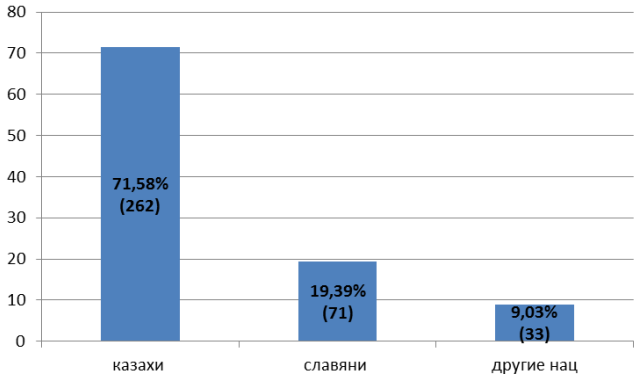

Figure 2: Ethnic composition of patients

Among the histological forms, squamous cell carcinoma without keratinisation with keratinisation dominated, respectively -65.30% (239) and 27.05% (99). Rare forms such as adenocarcinoma and undifferentiated cancer did not exceed, respectively, 5.46% (20) and 2.19% (8) $p < 0.05$.

Table 1: Stage cancer of the thoracic part of the oesophagus

№	Stage	TNM stage	Abs. number	Per cent, %	Mistake, m
1	I a	T1N0M0	4	1.09	0.54
2	I b	T2N0M0	39	10.65	1.61
3	II a	T3N0M0	218	59.56	2.57
4	II b	T1-2N1M0	9	2.46	0.81
5	III a	T4aN0M0, T3N1M0, T1-2N2M0	62	16.94	1.96
6	III b	T3N2M0	30	8.20	1.43
7	III c	T4aN1-2M0, T4bN1-3M0, T1-4N3M0	4	1.10	0.54

Regional metastases were detected in paraesophageal lymph nodes in 13.11% (48) cases, paracardial-12.57% (46). Without metastases, there were 35.25% (129) patients, and metastases in 39.07 (143) patients were not verified, which, based on X-ray data and computed tomography, enlarged lymph nodes were detected, and they were subjected to radiation therapy. All patients were distributed according to the treatment plan, taking into account the prognosis factors for 3 groups:

Group 1-surgical treatment of 45.36% (166);

Group 2-radical radiation therapy 41.53% (152);

Group 3-combined method (preoperative course of radiation therapy + surgery) 13.11% (48).

Surgical intervention was carried out in two ways-Subtotal resections of the mid-thoracic part of the oesophagus with bizonal lymph dissection-Lewis operation-46.45% (170), Garlock operation-resection of the lower thoracic part of the oesophagus with resection of the proximal stomach with bizonal lymph dissection-12.02 % (44).

With the traditional method of operation according to the Lewis method, after laparotomy, mobilisation of the stomach with the intersection of the right ligament of the diaphragm with preservation of the right gastroepiploic artery, a turn is made to the left side. The right-sided thoracotomy in the V intercostal space and after revision of the pleural cavity, clarification of the extent of the tumour process, mobilises the oesophagus above the aortic arch with the intersection and ligation of the unpaired vein. At the same time, lymph node dissection is carried out, all mediastinal lymph nodes are removed to the cardiodiaphragmatic angle. After that, the stomach, along with the oesophagus, is pulled into the pleural cavity. After skeletonisation of the lesser curvature of the stomach, we produce a proximal resection of the stomach using UO-60, UO-40 devices. The mechanical suture is covered with grey-serous sutures. After resection of the oesophagus, proximal to a tumour 5 cm impose the oesophagal-gastric submersible anastomosis. In view of the high resection of the esophagus with its significant mobilization, graft tension, extended lymph node dissection, 7 (3.27%) cases of oesophagal anastomosis insolvency were noted for 214 radical operations and 2 (0.93%) for transplant necrosis, in connection with what they decided to improve the method of imposing oesophagal anastomoses.

Starting in 2012, they began to produce esophagoplasty for cancer with a “solid” stomach (patent of the committee for Intellectual property rights of the Ministry of Justice of the Republic of Kazakhstan No. 1449905 dated 04.24.2017, “Method of esophageal plasty for cancer with a “whole” stomach during resection of the thoracic part of esophagus”).

After a right-sided thoracotomy during Lewis type surgery, pulling the oesophagus with the stomach into the pleural cavity, the oesophagus is resected 5 cm above a tumour. Then, only the cardinal part of the stomach is resected, immediately below the cardiac sphincter, a small part of the fundus of the stomach is partially resected, some 5 cm from the extreme short fundal artery. The resection of the stomach is performed using the apparatus of УО-60, УО-40. The mechanical suture is covered with grey-serous sutures with the immersion of the nodes of the stomach stump into semi-set sutures, which later, after the imposition of the oesophagal anastomosis, act as a cardiac sphincter. Then an oesophagal-gastric immersion anastomosis of the “end to side” type is formed. In this way, 113 operations were performed, 2 cases with complications (1.76 ± 1.23%), in one case, there was an insufficiency of the oesophagal anastomosis 0.88 ± 0.87%, and 1 graft necrosis 0.88 ± 0.87%, which have been lethal.

The prevailing postoperative complication was congestive pneumonia, which was noted in 6.55% (24) cases. Postoperative mortality was 6.54% (14) for 214 operations, of which in 7 cases (50.00%) there was a failure of the esophageal anastomosis, 5 (35.71%)-cardiovascular disorders, 2 (14.29%), cardiopulmonary insufficiency ($p < 0.05$).

The long-term results of treating patients with the thoracic part of oesophagal cancer were studied depending on the method of therapy and prognostic factors using survival.

Statistical analysis was performed using Statistica version 10 software. All values are expressed as mean ± mistake or medians and interquartile ranges for continuous factors and frequencies for categorical factors. The one-dimensional analysis was performed using Chi-square and Fisher probability tests for continuous variables. Student t-test was used for continuous factors. Survival 5-year and overall survival were compared between groups using Kaplan-Meier analysis and log-ranking criteria. In a one-dimensional analysis of overall survival, variables with value were statistically rethought to adjust for concomitant factors. The coke model was used for multivariate regression analysis. A p-value less than 0.05 was considered statistically significant.

Results

The overall five-year survival, regardless of the method of treatment, was only 8.72% (28 of 321), and in 6-10 years lived 8.41% (27 of 321), $p < 0.05$. Analysis of the overall survival of patients with cancer of the thoracic part of oesophagus showed that the method of treatment does not have a significant

impact on life expectancy.

Table 2: The total lifetime of patients with oesophagal cancer after radical treatment

The number of observed patients	Lifetime in years									
	1		2		3		5		>5	
321	Abs. number	%	Abs. number	%	Abs. number	%	Abs. number	%	Abs. number	%
	128	39.88	86	26.79	52	16.20	28	8.72	27	8.41

From the analysis excluded patients who as of 01/01/2018, after treatment, the follow-up period was less than 2 years, 321 (87.7 ± 1.7) cases were analysed.

A multifactor analysis of 19 gradations that affect the prognosis of the disease was carried out. Analysis of cumulative survival showed that (Figure 3) the best and 5-year survival was among patients in group 1, which was 26.53% (out of 147 treated, 5 years lived 39). The median survival is 19 months.

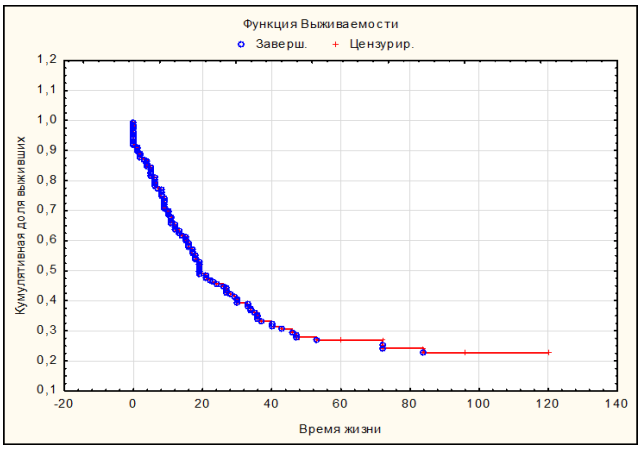

Figure 3: Lifetime with surgical treatment

In the second group (Figure 4), the 5-year survival rate did not significantly exceed 8.57% (out of 140 treated, 5 years survived 12).

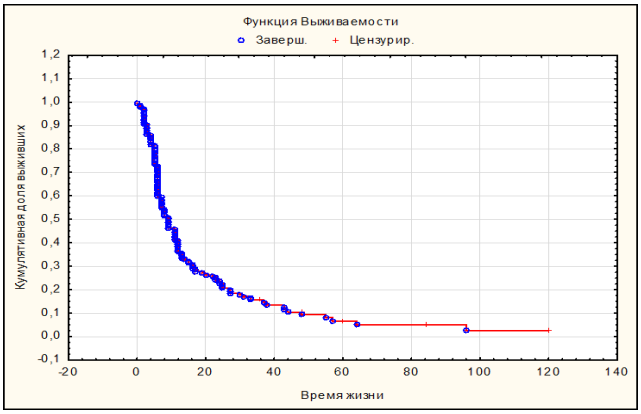

Figure 4: Radical radiation therapy

The patients who underwent combined treatment (Figure 5) lived 5 years in 17.65% (out of 34 treated, 5 years lived 6) cases. At the same time, it should be noted that the median of survival was the same with the surgical treatment group, and was 19 months.

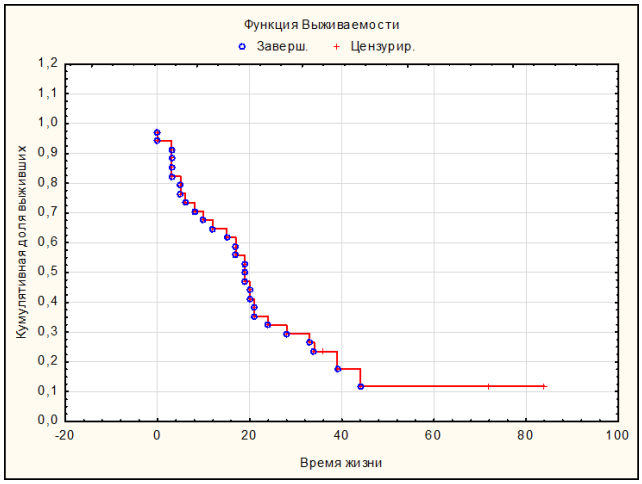

Figure 5: Lifetime with combined treatment

However, a comparative analysis of cumulative survival by Kaplan-Meier (Figure 6) showed that the best survival was reliably observed with surgical treatment ($p < 0.05$) than with radiation and combined treatment, the median survival was 13.5 months.

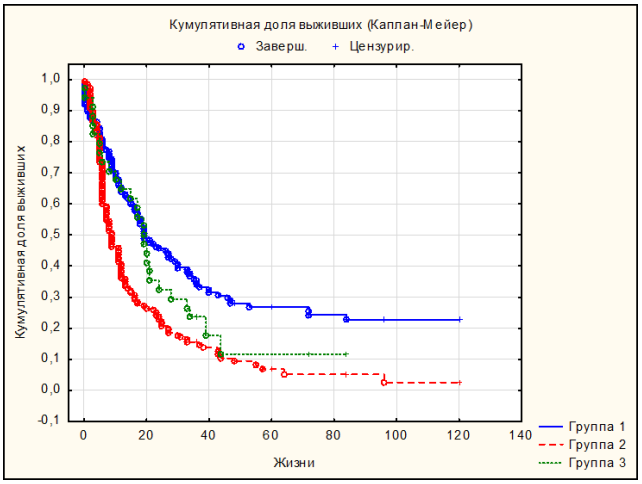

Figure 6: Cumulative survival depending on the method of treatment

Such a low cumulative survival among patients of the 2nd and 3rd groups is because patients with high operational risk were subjected to radiation treatment. The leading cause of failure in surgical treatment was old age (over 70 years of age was 46.7 ± 4.05%) and cardiopulmonary insufficiency was 11.84 ± 2.62. Whereas, in the 1st group, patients aged 51-60 years (46.38 ± 3.87%) with cardiopulmonary pathology prevailed in only 5.42% ± 1.76% of cases. In the group of patients after combined treatment, 33.33 ± 6.8% of cases aged 51-60 years with a cardiopulmonary pathology of 6.25 ±

3.49% ($p < 0.05$) prevailed.

A mathematical model for assessing the risk of death in cancer of the oesophagus showed (Figure 7), regardless of the method of treatment of cancer of the thoracic oesophagus, the risk of mortality increases in the first 2 years and increased 4 years after treatment.

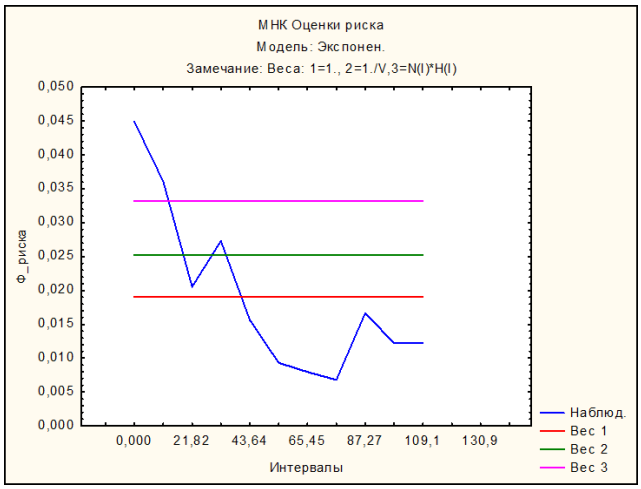

Figure 7: The risk of mortality in the treatment of oesophagal cancer

Discussion

According to most researchers [1], [2], [4], [5], [7], the prospects for the use of radiation and chemotherapeutic care for patients with distributed forms of esophageal cancer are limited in view of its resistance, and basically these types of treatment are of an auxiliary nature. The leading treatment for oesophagal cancer is surgical.

According to reports [10], [11], oesophagal cancer has a high potential for lymphogenous metastasis and in case of a lesion of the submucosal layer, i.e. T1 metastases in the lymph nodes are detected in 40%, and during germination of adventitia, 90%.

According to Stilidi I. et al., so-called “jumping metastases” in regional and distant nodes are detected in 20%, and it is extremely difficult to determine the direction of lymphatic drainage. Therefore, a prerequisite, i.e. the standard for the surgical treatment of oesophagal cancer should be considered extended 2-hzonalny lymph node dissection [11].

One of the important prognostic factors affecting life expectancy is the frequency of damage to the lymphatic catch and their number [12]. The 3- and 5-year survival in the presence of N1 was 47.7% and 12.9%, and at the stage of N0, it was 68.6% and 54.8%, respectively ($p < 0.05$). Equally important for the forecast is the number of affected lymph nodes. The “critical” number is 7 affected lymph nodes [5], [12]. According to Napier K.J. et al., [12], and Stilidi I.S. et al., [11] none of the patients with 7 or more lymph nodes did live up to 3 years.

Japanese surgeons from the 80s [13] used a three-zone lymphadenectomy, arguing that in 40% of patients with squamous cell carcinoma of the oesophagus after radical surgery, metastasis to the cervical lymph nodes is detected in the long term. According to the authors of [14], the 5-year survival rate after the above operations was 65% for squamous cell carcinoma and 46% for adenocarcinoma. The same data confirm Altorki N. et al., [14], who proved that 25% of patients experienced a 5-year follow-up, despite the presence of cervical metastatic lymph nodes.

One of the most promising methods of combined treatment, according to N.V. Dengina [15] is neoadjuvant chemotherapy, in which resectability and patient survival are increased. The effectiveness of neoadjuvant chemotherapy followed by surgery, the author, estimates in 45-70% of cases. With radiation therapy in a total dose of 45-75 Gray in patients with small tumours, the 5-year survival rate does not exceed 10 to 15%. The author notes that radiation and chemotherapy are palliatives in nature. Therefore the use of the above methods as an independent method of treating oesophagal cancer is an alternative solution.

The data obtained confirm the correlation analysis of Spearman, conducted in pairs with each group of patients, depending on the method of treatment and the presence of prognostic factors.

According to the findings, affecting the outcome of treatment of esophageal cancer, factors such as tumour localization ($rs = -0.089776$), histological form ($rs = -0.055125$), regional lymph node metastases ($rs = -0.0342697$) effect , the length of the oesophagus tumour ($rs = -0.248513$) with a confidence interval of 95%. All of the above factors relate to the concept of the stage of the disease. However, among all the prognostic factors, the presence of regional metastases plays the most important role in the choice of treatment and the prognosis of a long-term outcome. For patients under 60 years of age with regional metastases, the long-term outcome in the combined degree ($rs = 0.277693$) is determined, as well as the tumour localization in the bronchial and retrocardial segment, it is equal to ($rs = 0.243261$), the histological type is squamous cell cancer with keratinization ($rs = 0.164874$), the length of the tumour is not more than 3 cm ($rs = 0.311595$), invasion of the muscle layer tumour ($rs = 0.320403$). Also, a lifetime in the long term depends on the factors of cancer recurrence and the progression of the process. Their relationship was, respectively, $rs = -0.139073$ and $rs = 0.144985$.

Thus, the prognosis for the long-term

outcome of treatment for distributed cancer of the thoracic oesophagus depends on the stage of the disease, the location of the tumour and the presence of regional metastases. When the prehospital diagnosis of cancer localisation in the mid-thoracic oesophagus with a process length of more than 3 cm and the presence of regional metastases before the age of 65, it is necessary to plan a combined treatment (neoadjuvant radiation therapy + surgery). Radiation therapy for advanced cancer of the thoracic oesophagus is palliative in nature and should mainly be used in patients older than 70 years with comorbidities in the decompensation stage.

In conclusion, a radical treatment for cancer of the mid- and lower thoracic oesophagus is surgical, in which the median of cumulative survival is 19 months. Traditional radiation therapy should be applied in a limited way, as it is palliative in nature, the median survival rate does not exceed 9 months.

In the presence of regional metastases in cancer of the thoracic oesophagus, it is advisable to use the combined method (neoadjuvant radiation therapy + Lewis surgery with 2-zonal dissection), the median survival is 19 months.

The leading prognostic factor for cancer of the thoracic region is the presence of regional metastases, on which the choice of treatment depends. With an inadequate choice of treatment method in a long-term period, the frequency of relapses and progression of the tumour process increases. Especially high-risk mortality in the first 2 years after treatment, regardless of the method of treatment of cancer of the thoracic oesophagus.

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ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2019 Jan 15; 7(1):88-91.
https://doi.org/10.3889/oamjms.2019.079
eISSN: 1857-9655
Clinical Science

The Connection of the Level of Estradiol in Serum and Obesity with the Endometrial Bleeding in Postmenopausal Women

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Citation: Tofiloska V, Krstevska M, Daneva-Markova A, Jovanovska V. The Connection of the Level of Estradiol in Serum and Obesity with the Endometrial Bleeding in Postmenopausal Women. Open Access Maced J Med Sci. 2019 Jan 15; 7(1):88-91. https://doi.org/10.3889/oamjms.2019.079

Keywords: Postmenopausis, bleeding; Postmenopausal patients; Serum estradiol; BMI; Endometrial malignancy

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Received: 25-Dec-2018; **Revised:** 27-Dec-2019; **Accepted:** 29-Dec-2019; **Online first:** 13-Jan-2019

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

Abstract

BACKGROUND: Postmenopausis is a period that begins one year after the last menstrual period. Abnormal uterine bleeding could be of different origins.

AIM: This study aimed to determine the association of serum estrogen hormone levels and obesity with the occurrence of endometrial bleeding in post-menopausal women.

MATERIAL AND METHODS: Prospective clinical study involving 120 postmenopausal patients treated at the University Clinic for Gynecology and Obstetrics-Skopje, divided into two groups: control and study. The control group consisted of 40 postmenopausal patients without endometrial bleeding, hospitalised and operated due to urogenital pathology. The study group consisted of 80 patients with endometrial bleeding who were divided into three subgroups according to the thickness of the endometrium: from 5-8 mm, 8-11 mm and above 11 mm. In all subjects, estradiol and BMI was determined.

RESULTS: Estradiol levels were statistically higher in the study group compared to control while statistically significant difference among the three subgroups according to the thickness of the endometrium about the levels of estradiol in blood is not found. About BMI, the results showed that there was no statistical significance between the two examined groups.

CONCLUSION: Patients with endometrial bleeding have increased levels of estradiol and are at increased risk of endometrial cancer about controls, the likelihood of endometrial cancer significantly increases by 1,108 times.

Introduction

Over the last 30 years, there has been an increased incidence of endometrial malignancy in the world, and especially in highly developed countries. The incidence of postmenopausal women is the highest with a peak of 85% in the age group 55-63 [1], [2]. Postmenopausis is a period that starts one year after the last menstrual period. It is divided into early and late menopause. Late menopause, after 70 years, is called senile. Almost 5% of all the visits by a gynaecologist are due to postmenopausal bleeding [3]. During this period, abnormal uterine bleeding may range from polyps, endometrial atrophy, endometrial hyperplasia, endometrial cancer, submucosal fibroid, hormone therapy, uterine infections or cervix, use of certain medications such as blood thinners [4], etc. In most cases, endometrial bleeding is associated with endometrial polyps (6% of cases) [5] or endometrial atrophy (almost 50% of cases) [6]. Studies suggest that only 3.7%-17.9% of cases with postmenopausal bleeding are due to endometrial cancer [7].

Ultrasonography is widely used as a diagnostic method for evaluating irregular bleeding in postmenopausal women [8]. For the time being, there is no consensus on the limit value of endometrium thickness in which there is no need for sampling for analysis in cases of endometrial bleeding [8]. In postmenopausal ovaries, they have no more follicles, thus stopping the production of estradiol and progesterone. The value of oestradiol in postmenopausis ranges from 10 to 20 pg/mL. According to the results of many studies, oestradiol is

a significant predictor of endometrial malignancy and endometrial bleeding [8], [9]. According to Naomi's study and chronic endometrial malignancy, endometrial bleeding in patients in menopause is positively associated with an elevated estradiol level [9].

Obesity is also strongly associated with the development of endometrial bleeding [10]. Almost 57% of endometrial bleeds in the United States are due to overweight [11], [12]. The meta-analysis involving 26 studies, the American Cancer Research Institute, found that an increase in Body Mass Index (BMI) by five units increases the risk of endometrial bleeding by 50% [13]. The mechanism of action of obesity as a risk factor for endometrial bleeding in postmenopausal women is explained by the increased adiposity that increases the activity of aromatase, which leads to the conversion of androgenic hormones into estrogens and directly promotes the proliferation of endometrium and transcription of the pro-proliferative genes. Chronic inflammation associated with visceral adiposity is mediated by proinflammatory adipokines and leads to hyperinsulinemia, to an increase in insulin-like growth factor 1 (IGF1) and hyperglycaemia, which increases endometrial proliferation. At the same time, reduction of anti-inflammatory cytokines was observed. Inflammation and an increase in estrogen metabolites additionally contribute to DNA damage and genetic instability. Finally, stem cells can be recruited from the adipose tissue, where they contribute to supporting the tumour. The purpose of this study was to determine the eventual difference in the level of serum estrogen hormones, i.e. obesity in endometrial bleeding in postmenopausal women.

This study aimed to determine the association of serum estrogen hormone levels and obesity with the occurrence of endometrial bleeding in post-menopausal women.

Material and Methods

This is a prospective clinical study involving 120 postmenopausal patients treated at the University Clinic for Gynecology and Obstetrics-Skopje. Patients were divided into two groups: control and examination group. The control group included 40 postmenopausal patients without endometrial bleeding, hospitalised and operated due to urogenital pathology, and with an orderly histopathological finding of the material obtained with an explorative endometrial curettage. The study group included 80 postmenopausal patients with endometrial bleeding. According to the ultrasound verified thickness of the endometrium, they were divided into three subgroups: from 5 -8 mm, 8-11 mm and above 11 mm. Each patient was given a detailed history, weight and height were determined for BMI calculation and 5 mL of venous blood was taken to determine the serum estradiol level. This study excluded patients who received hormone replacement therapy, patients to whom fractional explorative curettage cannot be performed, patients with a personal history of malignant diseases, benign or malignant ovarian changes and patients who were operated of breast cancer under treatment with tamoxifen.

The concentration of oestradiol in serum was determined by the chemiluminescent method of the Immulite 2000 analyser in the biochemical laboratory at the University Clinic for Gynecology and Obstetrics Skopje. For the reference values of oestradiol in postmenopausis, values of 4 to 71.2 pg/mL were taken. Ultrasound analysis was performed with an ultrasonic device Voluson E8, and the measurement was done with a transvaginal probe whereby the patients were in the supine position at the gynaecological chair. The examination was performed on an empty bladder with a probe for each patient separately. All the analyses were made in a longitudinal section of the uterus, where both the cervix and the fundus of the uterus are displayed simultaneously, and the endometrial media line is also visualized.

Quite often in the study was voluntary and anonymous without any procedures or conditions that imply coercion and it was approved by the responsible bodies of the Medical Faculty at the Ss Cyril and the Methodius University of Skopje, Skopje.

Statistical analysis

All tests were performed with SPSS 20.0. Continuous variables were described using mean and standard deviation (SD) or median and interquartile range. Categorical variables were described using frequencies and percentages. Data were tested for normality by Shapiro-Wilk tests and graphically checked for symmetry. Differences between groups were assessed by Mann-Whitney U tests and Kruskal-Wallis ANOVA test. P values < 0.05 was set as the threshold for statistical significance.

Results

The average blood estradiol level among the examinees in the sample was 29.8 ± 14.1 pg/ml with a minimum value of 20 pg/mL and a maximum value of 99.5 pg/mL. In the control group, the mean estradiol level in the blood was 25.5 ± 12 pg/mL with a minimum value of 20 pg/ml and a maximum value of 80.9 pg/mL. In patients in the test group with endometrial bleeding, the mean blood estradiol concentration was 31.9 ± 14.7 pg/mL with a minimum

value of 20 pg/mL and a maximum value of 99.5 pg/mL and was significantly elevated relative to the control group, $p < 0.05$ (Mann-Whitney U Test: $Z = 3.9108$, $p = 0.00009$). In 50% of patients in the test and control group the blood estradiol value was greater than the corresponding IQR = 29 pg/mL (21.9-34.9) vs. IQR = 20 pg/mL, Table 1 (20-25 pg/mL).

Table 1: Concentration of serum estradiol (pg/mL) in the control and examination group

Group	Number n	Mean value	SD	Minimum	Maximum	Mediana IQR
Control	40	25.48	11.98	20	80.9	20 (20-25)
Examination	80	31.95	14.72*	20	99.5	29 (21.9-34.9)
Total value	120	29.79	14.15	20	99.5	26 (20-32.7)

According to the division of patients from the study group into three subgroups according to the thickness of the endometrium, an analysis of blood estradiol values was performed in each of these groups, Table 2. In the first subgroup, with a thickness of endometrium 5-8 mm, blood estradiol was 30 ± 11.5 pg/mL with a minimum value of 20 pg/mL and a maximum value of 66 pg/mL. According to the media analysis, 50% of patients in this subgroup had a blood estradiol value greater than IQR = 27.1 pg/mL (20.8-32.5 pg/mL). In the second subgroup with an endometrial thickness of 8 mm-11 mm, the mean value of oestradiol in the blood accounted for 33.3 ± 19.2 pg/mL with a minimum value of 20 pg/mL and a maximum value of 87 pg/mL. According to the analysis of the media, 50% of patients in this subgroup had a blood estradiol value greater than IQR = 28.8 pg/mL (21.5-36 pg/mL). In the third subgroup with a thickness of endometrium > 11mm, the mean blood estradiol concentration was 33.6 ± 15.7 pg/mL with a minimum value of 20 pg/mL and a maximum value of 99.5 pg/mL. According to the media analysis, 50% of patients in this subgroup had a blood estradiol level greater than IQR = 29.6 pg/mL (25.4-36.3 pg/mL). Statistical analysis of the results showed that there was no significant significance in blood concentrations of estradiol in patients with different endometrial thickness (Kruskal-Wallis ANOVA: $H = 1.815984$ $p = 0.4033$), Table 2.

Table 2: Blood oestradiol concentrations (pg/mL) in patients with different endometrial thickness

Subgroups Endometrial thickness (mm)	Number N	Mean value	SD	Minimum	Maximum	Mediana IQR
5 – 8	36	30.03	11.47	20	66	27.1 (20.8-32.5)
8 – 11	17	33.35	19.17	20	87	28.8 (21.5-36)
> 11	27	33.64	15.67	20	99.5	29.6 (25.4-36.3)
Total value	80	31.95	14.72	20	99.5	29.2 (21.9-34.9)

Within the research, the respondents in the whole sample were analysed according to the BMI value. The average BMI value in the entire sample was 29.2 ± 4.9 kg/m^2 with a minimum value of 14.9 kg/m^2 and a maximum value of 42.7 kg/m^2, Table 3. According to the IQR, 50% of patients in the whole sample had BMI with a height greater than 29.3 kg/m^2.

Table 3: Values for BMI (kg/m^2) in the control and examination group

Group	Number	Mean Value	Sd	Minimum	Maximum	Mediana IQR
Examination	80	29.46	5.42	14.9	42.7	29.7 (26-32.3)
Control	40	28.66	3.85	21.8	41.4	28.2 (26.1-30.3)
Total value	120	29.19	4.95	14.9	42.7	29.3 (26-32)

The statistical analysis of the BMI results showed that there is no statistical significance between the two groups of patients. Patients in the examined group had an average BMI of 29.5 ± 5.4 kg/m^2 with a minimum value of 14.9 kg/m^2 and a maximum of 42.7 kg/m^2. According to the median analysis (IQR), 50% of patients in this group had a BMI value greater than 29.7 kg/m^2. In the control group, the average BMI of the patients was 28.7 ± 3.8 kg/m^2 with a minimum value of 21.8 kg/m^2 and a maximum value of 41.4 kg/m^2. According to the median (IQR), fifty per cent of the subjects in the control group had a BMI value greater than 28.2 kg/m^2.

Discussion

Estradiol is a significant predictor of endometrial malignancy, especially in patients with endometrial bleeding. The results of serum estradiol levels in this study showed that according to the media analysis, 50% of patients in this subgroup had an estradiol level greater than IQR = 29.6 pg/mL (25.4-36.3 pg/mL), which can be assumed to be at increased risk of endometrial cancer. Studies of Naomi et al., suggest a positive association between endometrial malignancy in postmenopausal patients and the level of estradiol [9]. Under the influence of relatively high levels of estrogen stimulation, postmenopausal endometrium may develop mild architectural changes and turn into so-called impaired proliferative endometrium [14] or may show an increase in degrees of architectural and cytological atypia and have a form of atypical endometrial hyperplasia (endometrial intraepithelial neoplasia) [15] or endometrial intraepithelial carcinoma [16] from which endometrial cancer can develop.

The endometrial carcinoma shows a positive association with the increased body mass index, so in 39% of cases with endometrial cancer is in obese women [17]. Several mechanisms can promote endometrial cancer or progression in overweight or obese women. Primarily, this association can be explained by an increase in circulating estrogens arising from the aromatisation of androgenic precursors in adipose tissue, leading to endometrial cell proliferation [18]. The data in the consulted literature coincide with the results of this study. Thus, in the study of Widderpas et al., [19], it was found that the assembled patients with BMI of 30-33.9 kg/m^2 had

a three-fold risk of developing endometrial malignancy, which is consistent with the results in this study in which the statistical analysis showed that for each unit an increase in BMI, the probability of endometrial carcinoma increased by 1.484 times (p = 0.0001, 95% CI = 1.205-1.829).

In conclusion, concentration of serum estradiol in patients with bleeding (31.9 ± 14.7 pg/mL) was statistically significantly elevated relative to the control group, (25.5 ± 12 pg/mL), $p < 0.05$. Among the subgroups of patients with different endometrial thickness, statistical significance for the concentration of oestradiol in serum was not found. No statistical significance was found for the values of the body mass index between the control group (28.7 ± 3.8 kg/m^2) and the test group (29.5 ± 5.4 kg/m^2), but the values are in between obesity and obesity. If it is known that increased body mass, that is, obesity and increased concentrations of estrogen hormones are a risk factor for developing malignancy of the endometrium, then both groups of subjects are at increased risk of endometrial cancer.

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ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2019 Jan 15; 7(1):92-96.
https://doi.org/10.3889/oamjms.2019.038
eISSN: 1857-9655
Clinical Science

Predictors of Mortality in Pulmonary Haemorrhage during SLE: A Single Centre Study Over Eleven Years

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Citation: Masoodi I, Sirwal IA, Anwar SK, Zaidi A, Balbaid KA. Predictors of Mortality in Pulmonary Haemorrhage During SLE: A Single Centre Study Over Eleven Years. Open Access Maced J Med Sci. 2019 Jan 15; 7(1):92-96. https://doi.org/10.3889/oamjms.2019.038

Keywords: SLE; Nephritis; Neuropsychiatric manifestations; IVIG; Steroids; Mechanical ventilation; Pulmonary haemorrhages

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Received: 22-Oct-2018; **Revised:** 22-Dec-2018; **Accepted:** 10-Jan-2018; **Online first:** 14-Jan-2019

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

BACKGROUND: Pulmonary haemorrhage (PH) is a serious complication during Systemic Lupus Erythematosus (SLE).

AIM: The aim was to present data on 12 patients of SLE with classic symptoms and signs of PH admitted throughout eleven years.

METHODS: This retrospective study was carried out at King Abdul Aziz Specialist hospital in Taif-a tertiary care hospital in the western region of Saudi Arabia. The data was analysed from the case files of SLE patients who had episodes of PH throughout 11 years (January 2007 to December 2017).

RESULTS: Twelve patients (10 females and 2 males) were found to have diffuse pulmonary haemorrhage during their SLE in the study period. Of 12 patients with confirmed pulmonary haemorrhage (hemoptysis, hypoxemia, new infiltrates on chest radiography, fall in haemoglobin and hemorrhagic returns of bronchoalveolar lavage with hemosiderin-laden macrophages) 4 patients had PH as the first presentation of SLE and 8 patients developed this complication during the disease. All patients presented with shortness of breath and hemoptysis. The most common extra-pulmonary involvement in the study cohort was renal (83%), which ranged from clinical nephritis, nephrotic syndrome to acute renal failure. All patients were managed in intensive care of the hospital, and of 12 patients, 9 (75%) required mechanical ventilation. All patients were uniformly treated with pulse Methylprednisolone; 9 received Cyclophosphamide, 6 received IVIG, and 4 received Plasmapheresis. Only 3 patients (25%) survived despite maximum possible support during their mean hospital stay of 18 ± 5 days.

CONCLUSION: The requirement of mechanical ventilation and the association of renal and neuropsychiatric complications predicted mortality in patients with pulmonary haemorrhage.

Abstract

Introduction

With its chronic and relapsing course, SLE can involve many organ systems, and pulmonary haemorrhage (PH) remains the devastating complication of this disease. The frequency of PH ranges from 0.63 to 5.4% in various cohorts of SLE [1]. While in admitted patients PH ranges from 0.5 to 9% [2], [3] of hospital admissions, the frequency of this complication steeps to 5.7% in an intensive care setting. Further, various autopsy series in SLE patients have demonstrated PH up to 12.3% connoting clinical PH to be the tip of the iceberg [4], [5]. The frequency of PH is higher in women as SLE is more common in this gender and the mean or median progression of SLE at the time of PH varies from 6 months to 14.1 years. The usual presentation of PH is shortness of breath with or without hemoptysis. However, the absence of hemoptysis in SLE patients doesn't rule out PH in a given case. The presence of radiological evidence of infiltrates on CT scan with a corresponding drop in haemoglobin is the usual scenario among SLE cases with PH. The high-resolution CT scan is more sensitive than conventional radiography in detecting PH [6]. The characteristic features on imaging are diffuse bilateral alveolar infiltrates in most series and some researchers have reported alveolar-interstitial infiltrates as well. There is a paucity of data regarding the type of immune response that triggers PH in

patients with SLE. In an animal model of PH pristane-induced SLE in susceptible mice, the involvement of the innate immune response was shown to have played a key role. The severity or recovery from the insult of PH is dependent on adaptive immunity with significant participation of B cells. The haemorrhage has been shown to be preceded by infiltration of macrophages and neutrophils [7].

Regarding lung biopsy, some of the first studies by Myers and Katzenstein [8], demonstrated the small vessel vasculitis or microangitis in 4 patients with lupus. The above study highlights the characteristic expression of PH in SLE and immune complex deposits. Capillaritis may have immune complexes associated with SLE [9]. We reiterate that many patients with PH in SLE described in the literature are reported with “soft bleeding” or without capillaritis.

We at this moment present data on 12 patients of SLE with classic symptoms and signs of PH admitted throughout eleven years.

Methods

This was a retrospective study conducted to assess the predictors of mortality due to pulmonary haemorrhage during SLE at King Abdul Aziz Specialist, Taif, Saudi Arabia. All patients with PH fulfilled the criteria of Systemic Lupus International Collaborating Clinics (SLICC) group [10].

Patients with signs of alveolar haemorrhage like hemoptysis, hypoxemia, new infiltrate on chest radiography fall in haemoglobin concentration, and hemorrhagic returns of bronchoalveolar lavage with hemosiderin-laden macrophages were included.

The detailed history and thorough clinical examination were tabulated. Patients previously diagnosed to have SLE were studied regarding their disease manifestation and the treatment modalities before PH.

The laboratory investigations included complete blood counts, erythrocyte sedimentation rate (ESR) serum complement (C3, C4), autoimmune profile, urine analysis, coagulation, hepatic and renal functions were tabulated in the study cohort. Appropriate microbiological cultures data was tabulated as well.

The chest x-ray and CT scans were analysed, and patients with characteristic alveolar haemorrhages were included. Patients who were diagnosed with SLE previously, their records were reviewed.

Exclusion criteria: Pulmonary haemorrhage due to Pulmonary, renal syndromes Good pastures syndrome, Wegner’s Granulomatosis; Necrotising pneumonia;

Data were statistically described regarding frequencies (number of cases) and valid percentages for categorical variables. Mean, standard deviations, minimum and maximum were used to describe parametric numerical variable while median and inter-quartile range (IQR) were considered for non-parametric data. Comparison of categorical variables between the subgroups (cross-tabulation) was made using a Chi-square test. All statistical calculations were done using computer program IBM SPSS (Statistical Package for the Social Science; IBM Corp, Armonk, NY, USA) release 21 for Microsoft Windows.

Results

A total of 14 patients were found to have 15 episodes of pulmonary haemorrhage during the study period. The data on two patients were excluded as they had necrotising pneumonia. Finally, data on 12 SLE patients with PH was analysed.

The mean ± SD age of participants was 25.8 ± 8.3 years, and age ranged from 17-52 years. Of 12 patients, 8 patients 8/12 (66%) were known cases of SLE and 4 patients (33%) PH was the first presentation of SLE, as shown in Figure 1.

All the patients presented with hemoptysis (100%) in our series, 9 patients (75%) had shortness of breath, and 8 patients (66%) had coughed. Dyspnea at rest was mild, to begin with, and of 12 patients, the shortness of breath rapidly worsened necessitating mechanical ventilation in 9/12 (75%) patients. Only 2 patients (16%) had a fever and florid signs of sepsis. While as 7 patients (58%) had constitutional symptoms characteristic of SLE.

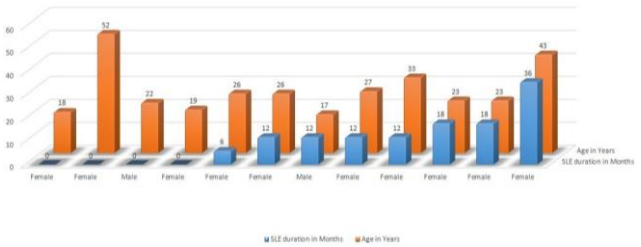

Figure 1: Clinical characteristics of SLE patients with Pulmonary haemorrhage

The characteristic rash of SLE was seen in 6 patients (50%) and musculoskeletal involvement was observed in 5 patients (40%) of patients in the study cohort. Five patients (40%) had serositis in the form of pericardial effusion. Of 12 patients, 3 (55%) had

central nervous system involvement of SLE in the form of seizures. The details are shown in Figure 2.

Severe disease activity according to SLE disease activity index was observed in 9, while moderate activity in 3 patients.

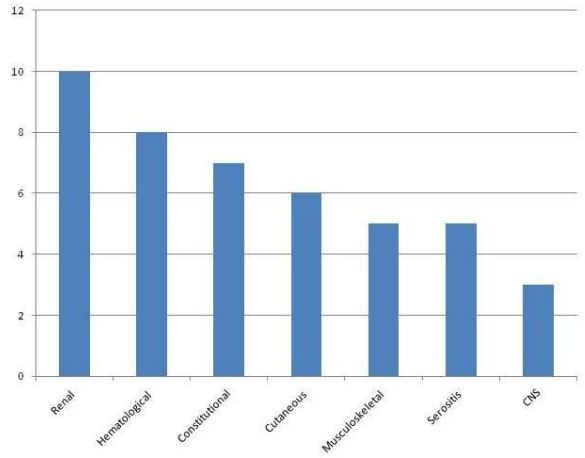

Figure 2: Extra Pulmonary involvement in PH patients

All patients in our study cohort were found to have severe anaemia, normal platelets and normal coagulation profiles as shown in Table 1. The chest radiographs and HRCT revealed diffuse alveolar infiltrates in all patients. Bronchoalveolar lavage in 5 patients revealed uniformly hemorrhagic fluid with hemosiderin-laden macrophages. In three patients BAL grew Acinetobacter on culture, Table 1.

Table 1: Investigative profile of SLE patients with pulmonary haemorrhage

No	Age	Hb	WBC	Platelets	INR	Crea te	24 Hr UP	C3 /C4	ANA dsDNA	ECHO	BAL
1	18/F	7.9	3.8	145	1.2	1.5	1.2	Low	Positive	Normal	Sterile
2	52/F	6.9	3.8	132	1.1	6	1.8	Low	Positive	Moderate MS	Sterile
3	22/M	7.9	3.9	116	1.2	1.5	1.5gm	Low	Positive	Global hypokinesia	Sterile
4	19/F	6.8	7.3	150	1.1	2.9	3.9	Low	Positive	Pericardial Effusion	Sterile
5	26/F	7.9	6.1	248	0.9	1.2	3.2	Low	Positive	Pericardial Effusion	Sterile
6	26/F	5.3	2.4	204	1.4	2.2	8gm	Low	Positive	Marantic Endocarditis	Acineto bacter
7	17/M	6.5	8.2	136	1.3	3.7	3.7gm	Low	Positive	Normal	Sterile
8	27/F	6.2	2.3	154	1.4	3.2	6.6gm	Low	Positive	Pericardial Effusion	Acineto bacter
9	33/F	7.9	4.5.	155	1.1	1.9	6.9gm	Low	Positive	Pericardial Effusion	Sterile
10	23/F	7	7.4	273	1.3	5.4	2-4gm	Low	Positive	Pericardial Effusion	Sterile
11	23/F	7.5	7.1	258	1.7	1.9	2.6gm	Low	Positive	Normal	Acineto bacter
12	43/F	6.9	6.9	153	1.1	7	3gm	Low	Positive	Normal	Sterile

Echocardiography was normal in 4/12 (34%) while as pericardial effusion was seen in 5 patients, one patient had marantic endocarditis, and the other had global hypokinesia. The pericardial effusion improved in 3 without drainage. Moderate Mitral stenosis was seen in one of the patients. The details are shown in Table 1.

The patients with PH were managed in intensive care of the hospital. All patients received pulse Methylprednisolone, IVIG had been given to 6 (50%), and 4 (33%) had received plasmapheresis. Of 12 patients with PH 9 patients succumbed despite maximum available supportive treatment. Thus the mortality rate was 75% (9/12) in this study. The cause of death was respiratory failure compounded by polymicrobial sepsis in 3 patients and Varicella pneumonia after Immunosuppression therapy was observed in one patient who succumbed.

Three patients who survived episodes of PH didn't require mechanical ventilation and had no sepsis. They were treated with pulse steroids and IVIG while as plasmapheresis was given to one as shown in Table 2. However, all the three had active disease, anaemia and renal derangements but none of the three patients had neuropsychiatric manifestations.

Table 2: Management & outcome of SLE patients with pulmonary haemorrhage

No	Age	BT	M.V	Steroids	IVIG	PF	Outcome
1	18/F	1unit	NO	YES	YES	NO	SURVIVED
2	52/F	2units	YES	YES	NO	NO	DIED
3	22/M	0	YES	YES	YES	NO	DIED
4	19/F	2units	YES	YES	NO	NO	DIED
5	26/F	1	NO	YES	YES	NO	SURVIVED
6	26/F	2units	YES	YES	YES	YES	DIED
7	17/M	1unit	YES	YES	NO	NO	DIED
8	27/F	0	YES	YES	NO	NO	DIED
9	33/F	2units	YES	YES	YES	YES	DIED
10	23/F	0	NO	YES	NO	YES	SURVIVED
11	23/F	2	YES	YES	NO	NO	DIED
12	43/F	2	YES	YES	YES	YES	DIED

Abbreviation used BT = Blood transfusion; M.V = Mechanical ventilation; IVIG = Intranvenous immunoglobulins; PF = Plasmapheresis.

All the three patients who survived are on our follow up for the last two years now. Two patients are in remission, and one patient is on maintenance hemodialysis. Details are shown in Table 3.

Table 3: Clinical and investigative profile of patients who survived

No	Age	SLE duration	Hb	WBC	Platelets	INR	Create	24 Hr UP	C3 &C4	ANA dsDNA	ECHO	Treatment +No MV
1	18/F	First	7.9	3.8	145	1.2	1.5	1.2	Low	Positive	Normal	S+IVIG
2	26/F	6 M	7.9	6.1	248	0.9	1.2	3.2	Low	Positive	Pericardial Effusion	S+IVIG+
3	23/F	18M	7	7.4	273	1.3	5.4	2.4	Low	Positive	Pericardial Effusion	S+IVIG+PF

Hb = gm/dl; S-STEROIDS; IVIG = Intravenous immunoglobulin; PF = Plasmapheresis; MV = Mechanical ventilation; Seizures: None; Sepsis: None; Blood transfusion: None; Patients 1 and 2 are in remission while as patient number 3 is on maintenance Hemodialysis.

Discussion

The classical triad of hemoptysis, abrupt fall in haemoglobin level and new lung infiltrate on CT scan chest were observed in all the patients in this study. However, overt hemoptysis may not be present in all SLE patients presenting with PH as reported by Zamora et al., [9], which means thereby that a physician needs to have a high degree of clinical suspicion in a given case of SLE presenting with shortness of breath and radiological changes. Even at high altitudes PH should not be confused with high altitude pulmonary oedema as reported by Lis et al., [11]. PH is one of the rare but catastrophic complications in the course of SLE, and the mortality

continues to be high as was observed in this study. We observed that patients requiring mechanical ventilation had a poor outcome in line with other workers [1], [9]. Mechanical ventilation (MV) per se predisposes patients to many complications, and one of the life-threatening complications of MV is Ventilator-associated pneumonia (VAP). The risk of VAP is highest immediately after intubation and steadily increases after that. Even though broad-spectrum antibiotics had been given to all patients but keeping in view concomitant immunosuppressant treatment all patients who received MV succumbed.

Contrary to these three patients who didn't receive MV, despite their active disease survived after PH episodes were aggressively managed. PH was the first presentation of SLE in four patients in our study and 3 of them had seizures in addition to PH. Their shortness of breath progressively deteriorated and required mechanical ventilation meaning thereby that PH can present irrespective of the duration of SLE as described in the literature [12], [13]. Even pulmonary haemorrhage in SLE can present without extra pulmonic manifestation of SLE as reported by Bajantari et al., [14].

Patients with the associated neuropsychiatric manifestation of SLE had a poor outcome in this study. The data on 21 Korean patients by Kwok et al., [15], revealed that SLE patients with neuropsychiatric manifestation were at an increased risk for developing PH. Our results are in coherence with their study. Renal impairment was associated with the majority of patients with PH in this study. Similar results were demonstrated by Chang et al., [16].

The profile of autoantibodies in our study including anti-DNA, lupus anticoagulant, anticardiolipin, anti-Sm, anti-Ro and anti-RNP were similar in patients with PH compared with SLE patients without PH. Hence these may not serve as biomarkers to predict PH in SLE. None of our patients had thrombocytopenia or deranged coagulation. However, all our patients had hypocomplementemia, and overwhelming sepsis contributed towards mortality in 3 patients. In a study data on 50 patients with pulmonary haemorrhage in SLE the bivariate analysis, factors associated with mortality were high Acute Physiology and Chronic Health Evaluation II scores, the requirement of mechanical ventilation, infections, renal failure and thrombocytopenia [17].

All patients in our study cohort received pulse Methylprednisolone which has been shown to provide a survival advantage compared to prednisone 1 mg/kg [1]. In our study 4 patients (33%) were given plasmapheresis and one of them survived, but it is difficult to assess its utility alone. Intravenous immunoglobulin was given to 6patients (50%) in our study and of these, a 26-year-old female patient survived as shown in Table 2, and 3. This is not sufficient to prove the efficacy of IVIG but in a retrospective study by Shen et al., [17], the data on 29 SLE patients with PH, authors advocated early aggressive management with high-dose steroids, intravenous immunoglobulin and cyclophosphamide.

Infections have been reported by different series as an important factor associated with PH [18], [19]. In our study bronchoalveolar lavage (BAL) in three patients grew Acinetobacter species and BAL was sterile in rest of the patients. It is difficult to separate whether the infections occurred at the time of diagnosis of PH or after this complication. In a study by Rojas-Serrano et al., [18] data on 14 events of PH in SLE, evaluated during the first 48h with bronchoscopy and bronchoalveolar lavage, showed the presence of infection in 57%; of the patients leading to the observation that an infection could be a precipitating or contributing factor. Thus it is prudent to mention that all efforts must be utilised to identify and treat infection in a given case of PH.

Despite aggressive treatment the outcome in our study was poor as only 3 patients (25%) survived which is, however higher than reported by Marino et al., [19]. Various experimental attempts to treat this devastating complication have been attempted recently in which Umbilical cord-derived mesenchymal stem cell transplantation (UC-MSCT) has been shown to be effective in the treatment of four SLE patients with PH. Authors concluded that UC-MSCT could be applied as a salvage strategy for SLE patients with PH [20]. However, the data is too small, and the availability of this technique is not universal. In another study data on 21 patients from China, Bronchoalveolar lavage in combination with high-dose pulse therapy of methylprednisolone was found to be superior to methylprednisolone alone. The authors observed a statistically significant difference between the two groups. Broncho alveolar lavage alleviated hypoxemia and dyspnea in patients with PH [21].

To conclude, PH is a devastating complication of SLE, and despite aggressive treatment mortality remains high. Our study showed that the requirement of mechanical ventilation during the disease and association of neuropsychiatric manifestations and renal impairment predicted mortality in patients with PH. Further studies may be carried out to confirm the association.

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ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2019 Jan 15; 7(1):97-102.
https://doi.org/10.3889/oamjms.2019.012
eISSN: 1857-9655
Clinical Science

Role of Soluble Transferrin Receptor and Transferrin Receptor-Ferritin Index to Detect Iron Deficiency Anemia in Regular Hemodialysis Patients

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Citation: Suega K, Kandarini Y, Tubung J. Role of Soluble Transferrin Receptor and Transferrin Receptor-Ferritin Index to Detect Iron Deficiency Anemia in Regular Hemodialysis Patients. Open Access Maced J Med Sci. 2019 Jan 15; 7(1):97-102. https://doi.org/10.3889/oamjms.2019.012

Keywords: Regular hemodialysis; Iron deficiency anaemia; sTfR; TfR-F index

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Received: 16-Oct-2018; **Revised:** 13-Nov-2018; **Accepted:** 15-Nov-2018; **Online first:** 14-Jan-2019

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

Abstract

BACKGROUND: Several iron indicators can be used to detect iron deficiency anaemia (IDA) where confounding comorbidities occurred such as patients with regular hemodialysis.

AIM: This study was aimed to determine the diagnostic value of serum transferrin receptor (sTfR) and transferrin receptor-transferrin index (TfR-F index) and to combine these two markers in detecting IDA in regular hemodialysis anaemic patients.

METHODS: There were 70 patients recruited consecutively. IDA was diagnosed based on TS < 20% and ferritin level < 200 ng/L and functional iron deficiency when TS < 20% and ferritin > 200 ng/L. TfR-F index calculated as sTfR/log ferritin.

RESULTS: Correlation of ferritin to iron level was changed when its correlation adjusted by confounding inflammation (CRP level > 10). The correlation strength of ferritin to iron serum before adjusted was r = 0.37 with p = 0.02 but became r = 0.65 with p = 0.023 after adjusted to CRP > 10. In inflammation (CRP > 10), ferritin mild-moderately correlated with iron but became moderately strong when there was no inflammation (CRP < 10). AUC for sTfR was 0.77 with p = 0.028 (95% CI 0.55-0.99), and for TfR-F index has larger AUC 0.85% with p = 0.004 (95%CI 0.69-1.00), hence TfR-F index more superior than sTfR. sTfR and sTfR-F index were not correlated with CRP with p > 0.05, and sTfR and TfR-F index mean level was different between IDA and ACD patients although not statistically significant.

CONCLUSION: When sTfR and the TfR-F index used in combination to detect IDA, we found the largest AUC on ROC 0.98 (95% CI 0.94-1.00).

Introduction

Anaemia is a well-known problem in chronic kidney disease (CKD) especially end-stage renal disease who need regular hemodialysis (RH) leading to higher morbidity and mortality rate. Anaemia affects 49-55% of patient with CKD and more prevalent once diseases become more advanced. Moreover, in the population-based survey, anaemia is an essential indicator of iron deficiency [1], [2], [3]. Although many conditions contributing to anaemia in CKD patients such as the diminished production of erythropoietin stimulating agent (ESA), blood loss due to bleeding disorders and frequent laboratory test, impaired of iron absorption and iron retention within reticuloendothelial [4], every anaemia should be defined of its original causes to be well managed. ESA according to KDOQI is one of many important treatment options for anaemia in CKD patients, and to achieve a maximal response, iron status should be determined. Unresponsiveness to ESA treatment is defined when iron availability in time for erythropoiesis deficient, therefore iron management is an essential element for anaemia in CKD patients. There were two major iron disorders in this group of patients' absolute iron deficiency and functional iron deficiency. These two different iron deficiency (ID) might be hard to

distinguish since definitive tools for each of those conditions is lacking [5], [6].

Several iron indicators can be used to detect iron deficiency anaemia (IDA) in a setting where confounding comorbidities co-occur such as patients with RH. The most available iron indicators for IDA in the complicated area are transferrin saturation (TS) and ferritin level [4], [7]. But these two indicators have been known affected by inflammation where hemodialysis itself, an inflammation condition through uremic toxin, underlying diseases, hemodialysis process, making their interpretation hindered by physiologic factors and cause failure to detect ID status [8], [9]. The best diagnostic tool to identify IDA in CKD is still iron stained bone marrow aspiration (BMA) but because BMA is invasive, could not be used as a standard of care in daily practice. Therefore more convenient non-invasive and reliable enough method to detect iron status is needed. Recently two markers emerged, e.g., soluble transferrin receptor (sTfR) and the ratio of sTfR/log ferritin (TfR-F index) as a new promising indicator that can differentiate IDA with others especially anaemia of chronic disease (ACD). These two markers not entirely influenced by concurrent chronic disease as well as inflammation [4], [10], [11], [12].

sTfR is a monomer glycoprotein that detached from transmembrane TfR protein after truncated and lost their first 100 amino acid then released into the blood became sTfR. While TfR-F index is calculated from rationing sTfR over logarithm of ferritin, there was a close linear relationship between TfR-F index and iron store. Their value may be negative in the condition where iron is in a deficit state to maintain normal haemoglobin level [13]. The Clinical role of sTfR in identifying IDA patients has been studied in numbers of researches. Majority of these studies support the value of sTfR to detect ID and be able to differentiate IDA from ACD [14], [15], [16], [17]. However, Fussaro et al. showed sTfR was not much better than TS or ferritin to detect ID in patients with CKD [18]. Data from thalassemia population revealed sTfR is a diagnostic tool with moderate accuracy to detect IDA patient, as well as in sickle cell anaemia sTfR level reflecting more to erythropoietic activity than to ID [19], [20]. Punnonen et al. presented that TfR-F index has higher sensitivity and specificity to distinguish IDA from ACD and this ratio has corroborated by several other studies [4], [21], [22]. However a recent meta-analysis by Infusino et al. claimed that sTfR has better clinical performance than TfR-F index in identifying IDA [23].

This study aim was to determine the diagnostic value of sTfR and TfR-F index and to combine these two markers in detecting IDA in RH anaemic patients.

Material and Methods

This observational cross-sectional study was performed to determine the diagnostic value of sTfR and TfR-F index to detect IDA in RH anaemic patients at Sanglah Hospital Bali. There were 70 patients recruited consecutively and agreed to sign an informed consent approved by the Institutional Review Board of Sanglah hospital by the ethical principles of the Declaration of Helsinki. IDA was diagnosis based on TS < 20% and ferritin level < 200 ng/L and functional ID when TS < 20% but ferritin > 200 ng/L [24], [25], [26]. Medical history, physical examination, conventional haematology parameters including CBC, Iron serum, Total iron binding capacity (TIBC), ferritin serum, haemoglobin level, CRP, sTfR were studied. sTfR was measured using Biovender Human ELISA kit on RD 1940 11100. TfR-F index calculated as sTfR/log ferritin [4], [21].

Table 1: Characteristics of study subjects

Characteristics	Number (mean or %)
Gender	
Male	40 (57.1)
Female	30 (42.9)
Age (year), median (min-max)	51 (23-60)
BMI (kg/m^2), mean ± SD	21.35 ± 2.14
Hemoglobin (gram/dl), mean ± SD	7.77 ± 1.24
MCV (fl), mean ± SD	88.45 ± 6.07
MCHC (%), mean ± SD	30.41±2.01
MCH (pg), mean ± SD	27.22±2.58
RDW (%), mean ± SD	14.65±2.09
SI (μg/dl), median (min-max)	58.67 (11.52-316.80)
TIBC (μg/dl), median (min-max)	175.00 (91.00-701.00)
Ferritin (ng/ml), median (min-max)	795.65 (24.35-3944.00)
Transferrin saturation (%), median (min-max)	34.67 (4.93-99.62)
CRP (mg/L), median (min-max)	24.82 (1.00-92.09)
sTfR (μg/ml), median (min-max)	0.61 (0.16-4.23)
IDA, n (%)	6 (8.57)
ACD, n (%)	18 (25.7)
TfR-F index, median (min-max)	0.28 (0.05-3.05)

BMI: Body Mass Index; MCV: Mean Corpuscular Volume; MCHC: Mean Corpuscular Hemoglobin Concentration; MCH: Mean Corpuscular Hemoglobin; RDW: Red Cell Distribution Width; SI: Serum Iron; TIBC: Total Iron Binding Capacity; CRP: C-Reactive Protein; sTfR: Soluble Transferrin Receptor; IDA: Iron Deficiency Anemia; ACD: Anemia of Chronic Disease; TfR-F index: TfR/log ferritin ratio.

Statistical analysis was performed using SPSS software for windows with p-value < 0.5 indicating statistical significance. ROC (receiver operating curve) was performed, and the AUC (area under the curve) was calculated to assess the power of sTfR and TfR-F index to detect IDA in RH anaemic patients. The AUC is a measure of test accuracy, with the largest area under curve indicating the better test. The optimal cut-off value of sTfR and TfR-F index were determined using ROC curve for sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), the accuracy of the test. The closer the curve to the upper left corner of the plot the more accurate the test was. In this study, the cut-off value was selected to the point where sensitivity slightly overwhelming specificity by the goal of sTfR and TfR-F index to screen more IDA patients. Multiple regression analysis was performed to compare the AUC area of sTfR, TFR index and when sTfR and TfR-F index were combined. Person's correlation analysis was also performed to evaluate the possible

connections of each parameter especially main iron indicators to inflammation marker.

Results

Out of 70 patients enrolled in this study, 40 (57.1%) were male, and 30 (40.2%) were female. A total of 6 patients (8.57%) and 18 patients (25.7%) were with IDA and ACD, respectively. Characteristics of the study subjects are presented in Table 1.

Correlation of serum ferritin with serum iron is depicted in Table 2. It could be seen that the strength was altered due to the existence of inflammation (CRP).

Table 2: Partial correlation between serum ferritin and iron

	CRP < 10 (No Inflammation)		CRP < 10 (With Inflammation)	
	Correlation coef	p	Correlation coef	p
Ferritin	0.648	0.23	0.321	0.016

CRP: C-Reactive Protein.

In Table 3, no correlation observed between new indicators chosen with inflammation (CRP), although these new iron indicators (STFR and TFR index) differed between IDA and Non-IDA (Table 4). However, the difference was not significant ($p > 0.05$).

Table 3: Correlation of STFR and TFR index with CRP

Variable	Correlation coef	p
sTfR	-0.129	0.287
TfR-F index	-0.76	0.531

sTfR: Soluble Transferrin Receptor; TfR-F index: TfR/log ferritin ratio.

Our study revealed that AUC (area under curve) for sTfR was 0.77 with $p = 0.028$ (95% CI 0.55-0.99). The cut-off value, at its maximum sensitivity of 83.3% and specificity of 67.2%, was 0.71. The TfR-F index has larger AUC, which is 0.85, with $p = 0.004$ (95% CI 0.69-1.00).

Table 4: Mean difference between IDA with Non-IDA

	IDA (n = 6)	Non-IDA (n = 64)	p
sTFR	-0.0946	0.2368	0.474
TfR-F index	-0.0124	0.6772	0.141

sTfR: Soluble Transferrin Receptor; TfR-F index: TfR/log ferritin ratio.

The cut-off value, at its best sensitivity of 83.3% and specificity of 81.2%, was 0.33. The TfR-F index was superior compared to sTfR, as seen in Figure 1A. When sTfR and TfR-F index were used in combination to determine the existence of IDA in regular hemodialysis patients (Figure 1B), it was found that they carry the largest AUC, which is 0.98 (95% CI 0.94-1.00).

A) B)

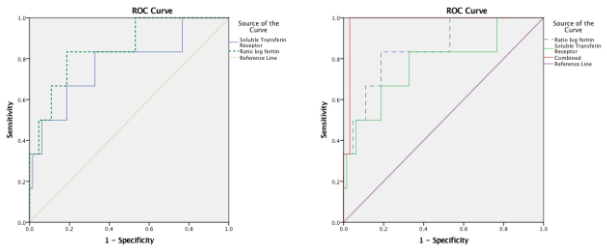

Figure 1: A) The receiver operating curve (ROC) of sTfR and TfR-F index to detect IDA in RH patients. B) The receiver operating curve (ROC) of sTfR and TfR-F index combination to detect IDA in RH patients

Discussion

CKD is a steady, gradual progressive chronic disease where kidney functions finally diminished, and this group of patients will mostly require RH with a higher mortality rate when compared to the general population [27], [28]. Data from 5 European countries in DOPP study found that the prevalence of anaemia in RH patients was 49% (by the year 1989-1999) – 55% (by the year 2000) [29]. Among three main type of anaemia in CKD population which are decreased ESA production due to kidney disorders, anaemia of chronic disorder and IDA, IDA in CKD population in term of clinical practice posed substantial challenges [30]. There was numerous and multifactorial process contributed including occult bleeding, chronic bleeding, defect in iron absorption because of inflammation, frequent laboratory testing, massive ESA treatment with supraphysiologic erythropoiesis. The annual blood loss estimated to be 1.5-3 gram [1], [30]. Besides absolute ID where iron storage is severely reduced or absent in bone marrow, however patient on RH also have the functional ID that partly related to ESA treatment and ACD due to inflammatory state of its underlying pathologic [31]. This functional ID markedly defines by sufficient iron store on body tissue but lack of iron availability for erythropoietic processed [5], [6], [32].

Our present study found that 6 (8.6%) patient with IDA using TS < 20% and ferritin < 200 ng/L, and 18 (25.7%) with functional ID based on KDOQI, Pernefri, and Bahrainwalla et al., [3], [24], [26]. It is generally believed that ID should clinically determine whether an absolute iron deficiency or functional iron deficiency is. However, this separation is often practically impossible to delineate. One clue to hold that help determined functional ID related to inflammation is retrospectively by observing ESA treatment responses of intravenous iron with or without concomitant raise of ESA dose and decreased ferritin level [32]. It is well known that TS and ferritin

are not proper iron indicators in the setting of inflammation because of the confounding effect of immune response to inflammation could compromising the true role of TS and ferritin. Ferritin is a positive acute phase protein where its level increased begin early during inflammation reach its peak in a week. From an experimental longitudinal study on serum ferritin, reported that ferritin level reaches its maximal after 3 days and gradually returned to the normal level in the next 10 days [1]. The confounding effect of inflammation can mislead conclusion whether over or underestimate of ID prevalence. According to BRIDA (Biomarkers Reflecting Inflammation and Nutrition Determinants of Anemia) project to assess the effect of inflammation on ferritin level to estimate ID, one should consider regression correction factors. The study performed on 29765 pre-school children and 25731 women of reproductive age using CRP and a1-acid glycoprotein (AGP) as a marker of inflammation reported that regression correction changes the estimated prevalence of ID in pre-school by median + 25 percentage points when ferritin serum was used [33]. BRIDA project suggested the need to determine marker of inflammation when assessing iron indicators status even at the population level [34], e.g., CRP for acute inflammation (rapid onset within hours) or AGP (late rising in 24 hours and lasted 4-5 days) for chronic inflammation [35]. The precise underlying pathophysiologic how inflammation influence and change iron indicators are yet to be defined. Inflammation and iron status (nutrition in a broad sense) are well connected in a way bidirectionally such that nutrition disorders can compromise immune function. Also, inflammation through immune response released acute phase protein ferritin and also hepcidin in order to withhold iron that certain microbe growth desperately depends on [1]. Disorder of iron metabolism happened because acute phase protein ferritin, transferrin, hepcidin have their ability to disturb the distribution of iron in every cell of the human body. Whether acute inflammation resulted from infection or injury, or chronic inflammation causes metabolic disturbance through releasing cytokines, and this can affect the regulation and production of the acute hepatic protein that contributed to the disorder of iron metabolism. Besides, several studies support the notion that during inflammation iron absorption on gastrointestinal also is compromised [33], [36].

In this study, we found that the correlation of ferritin to iron level was changed when its correlation adjusted by confounding inflammation (CRP level > 10). The correlation strength of ferritin to iron serum before adjusted was r = +0.37 with p = 0.02 but became r = +0.65 with p = 0.023 after adjusted to CRP > 10. In the setting of inflammation (CRP > 10), ferritin mild-moderately correlated to iron but became moderately strong when there was no inflammation (CRP < 10) where ferritin level truly represents tissue iron storage. In the future, knowledge about inflammation biomarkers should fill the gaps whether specific causes of inflammation (e.g., infection, injury, arthritis, malignancy, obesity, autoimmune diseases) also have their influence on each and specific iron indicators status. For example, the liver disease where ferritin is produced may directly cause a higher level of ferritin without followed by an increased level of inflammation biomarkers [33].

Using sTfR and TfR-F index to identify IDA in RH anaemic patient, we found that our results also corroborated several other studies that confirmed sTFR and TfR-F index have a significant role to detect IDA in the setting of confounding inflammation such as regular hemodialysis. We found AUC for sTfR was 0.77 with p = 0.028 95% CI 0.55-0.99, and for TfR-F index has larger AUC 0.85% with p = 0.004 95%CI 0.69-1.00. A prospective multicenter study to differentiate IDA and ACD conducted by Skikne et al., using sTfR and TfR-F index, reported that sTfR has AUC 0.74% with p < 0.0001 95%CI 0.66-0.83, and TfR-F index has larger AUC of 0.87% with p < 0.0001 hence more superior than sTfR in detecting IDA in the setting of inflammation. Bone marrow iron stained as golden standard was not used, but established opinion and practice for diagnosis and classification of anaemia were followed. Another study also found sTfR and TfR-F index value are useful parameters in assessing iron status in CKD patients. However, they were best in complementing to existing indices of serum ferritin and TS. TfR-F index also showed more superior than sTfR in distinguishing IDA and ACD [37]. The study using bone marrow iron staining as the golden standard for IDA which only pure no stained iron was considered IDA, meanwhile +1 to +6 iron staining considered non-IDA [4]. Study on inflammatory bowel disease and regular hemodialysis on ESA treatment also reported that TfR-F index is more accurate at distinguishing between IDA and ACD [26], [28], [38], [39]. The superiority of TfR-F index over sTfR was not so surprising since this index was derived from two elements that reciprocally associated (sTfR increased, and ferritin decreased) affected by ID. Moreover, especially in RH patients, ID was partly related to inflammation status that can increase ferritin level [4], [37].

TfR-F index has been found to have close, linear relationships with stored iron expressed as per kg body weight. This finding resulted from the experimental study that performs repeated phlebotomy of 14 healthy subjects where sTfR and ferritin were measured consecutively in serial [22]. A longitudinal study of 129 anaemic hospital patients observed that TfR-F index increased in IDA but not in ACD patient [21]. Peterson et al. reported that TfR-F index was decreased in ACD, but increased in IDA and patient with mixed IDA and ACD [40]. These studies again supported TfR-F index was a useful tool to detect IDA in the complicated area [41]. However, a recent meta-analysis by Infusino et al. showed that

the TfR-F index was no better than sTfR in detecting IDA in the presence of confounding condition [23].

sTfR is a soluble fragment of membrane-bound TfR that truncated from nearly all cells mostly from erythroblast and reticulocyte, has become easier to perform in the past 10 years and can be measured quantitatively. It is sensitive to represent iron availability during the erythropoietic process in bone marrow and other tissue as well as represent tissue iron status. sTfR increased in absolute ID and during stimulated erythropoietic either post ESA treatment or other condition such as thalassemia, sickle cell anaemia, hemolytic anaemia. In the situation where marrow activity is depressed due to hypoplasia, chemotherapy-induced marrow depressed, sTfR concentration decreased [10], [17], sTfR level can range from 8 times below normal to 20 times above the normal level [1]. A study in Pakistan reported sTfR in RH patients could differentiate between iron replete and iron deplete [42]. sTfR also represent iron availability during erythropoiesis activity supported by a study of Yin et al., through GEE model, sTfR was found significantly associated with the time point when hemodialysis was performed, meaning iron availability in time for undergoing erythropoietic process making sTfR an important marker of erythropoietic [43].

Our study also found out sTfR, and the TfR-F index was not correlated with CRP with $p > 0.05$, and sTfR and TfR-F index mean level was different between the patient with ADB and ACD although not statistically significant due to low power (small samples size). When sTfR and TfR-F index use in combination to detect IDA, we found the largest AUC on ROC 0.98 95%Ci 0.94-1.00. These findings also other studies claimed that sTfR less influenced by inflammation and can be used to determine IDA in the situation where inflammation and infection co-exist. The limitation of our study was not using iron stained bone marrow as a golden standard to determine IDA patient due to inconvenience and invasiveness. STFR was measured by Biovender Human sTfR ELISA kit on RD 194011100, not the one that WHO recommended [44]. There is no international reference standard exist for sTfR assay. It is impossible to compare single threshold value that would be accurate for all commercial kits and chemical device [14]. Since every available commercial kit is method-dependent, and this difference may cause by the disparity of TFR preparation used as standard and raise antibodies [14], [16], [45].

In conclusion, this study conclusion was sTfR, and TfR-F index proved to be important tools to determine IDA in RH anaemic patients and TFR index has superior accuracy than sTfR. When sTfR and TfR-F index used in combination, their diagnostic value reaches the best.

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ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2019 Jan 15; 7(1):103-106.
https://doi.org/10.3889/oamjms.2019.029
eISSN: 1857-9655
Clinical Science

The Correlation between Malondialdehyde and Nerve Growth Factor Serum Level with Diabetic Peripheral Neuropathy Score

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Citation: Decroli E, Manaf A, Syahbuddin S, Syafrita Y, Dillasamola D. The Correlation between Malondialdehyde and Nerve Growth Factor Serum Level with Diabetic Peripheral Neuropathy Score. Open Access Maced J Med Sci. 2019 Jan 15; 7(1):103-106. https://doi.org/10.3889/oamjms.2019.029

Keywords: Malondialdehyde; Nerve growth factor; Diabetic peripheral neuropathy score

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Received: 24-Aug-2018; **Revised:** 02-Dec-2018; **Accepted:** 03-Dec-2018; **Online first:** 14-Jan-2019

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

Abstract

AIM: This study was conducted to identify malondialdehyde (MDA) serum level, nerve growth factor (NGF) serum level, diabetic peripheral neuropathy score and the correlation between MDA and NGF serum level with diabetic peripheral neuropathy score.

METHODS: A cross-sectional study was conducted to observe diabetic patients in the internal medicine department in Dr M. Djamil Hospital, Padang, Indonesia. The MDA serum level was measured using Beuge method with thiobarbituric acid. The NGF serum level was analysed using ELISA method. Diabetic peripheral neuropathy score was defined when history score in Michigan Neuropathy Screening Instrument (MNSI) ≥ 7 and physical assessment score in MNSI > 2.

RESULTS: Thirty subjects with diabetes has diabetic peripheral neuropathy score 3.53 (± 0.91), MDA serum level 2.16 (± 2.89) nmol/ml, and NGF serum level 10.56 (± 2.89) pg/dl. There were significant correlations between the MDA serum level and the diabetic peripheral neuropathy score ($r = 0.364$, $p = 0.048$), and between the NGF serum level with the diabetic peripheral neuropathy score ($r = -0.59$, $p = 0.001$).

CONCLUSION: There are high MDA serum level and low NGF serum level in patients with diabetic peripheral neuropathy. Low NGF serum level plays a bigger role than high MDA serum level in diabetic peripheral neuropathy.

Introduction

Diabetes mellitus is a chronic disease that may cause a long term health problem and multiple quality of life depriving complications. One of the diabetic complications is diabetic peripheral neuropathy that can be assessed by Minnesota Neuropathy Screening Instrument (MNSI). The MNSI includes two separate assessments, a 15-item self-administered questionnaire and a lower extremity examination. Diabetic peripheral neuropathy is based on the existence of chronic hyperglycemia. Chronic hyperglycemia is associated with oxidative stress, endothelium damages, and it causes microvascular and hemorheological disturbances [1], [2].

Oxidative stress can be determined by several ways, such as MDA. MDA is one of recommended marker for oxidative stress. MDA, a product of the lipid peroxidation, has been accepted as one of the reliable biological markers for oxidative stress, based on BOS (Biomarker Oxidative Stress) Study in 2002 [3], [4], [5]. MDA has been documented as a primary biomarker of free radical-mediated lipid damage and oxidative stress. Increased MDA level in serum and may other tissues have been reported in diabetic patients. MDA may impact peripheral nerve among diabetic patients [6], [7].

NGF is an important protein to maintain life and survival of the neurons, and it works by increasing cell regeneration and decreasing the degeneration. NGF decline level is associated with the disturbances

in Schwann cells regeneration because NGF produced by the neurons and the Schwann cells. NGF decline level induces peripheral nerve lesions in diabetic patients. The forms of lesions are axonal atrophy, demyelination, and reduced number of nerve fibres [8], [9]. Pittinger and Vinik (2003) found that a decrease of NGF serum level in diabetes correlates with the clinical symptoms of neuropathy [10]. The clinical symptoms of diabetic neuropathy can be assessed with diabetic peripheral neuropathy score by Minnesota Neuropathy Screening Instruments (MNSI) [11].

The objective of this study is to identify MDA serum level, NGF serum level, diabetic peripheral neuropathy score and the correlation between MDA and NGF serum level with diabetic peripheral neuropathy score.

Methods

This was a cross-sectional study, conducted in the internal medicine department in Dr M. Djamil Hospital, Padang, West Sumatera, Indonesia. This study was conducted from January 2017 until December 2017.

Inclusion criteria in this study are type 2 diabetes mellitus (T2DM) patients with peripheral neuropathy, aged between 18 to 59 years old that has agreed to participate and signed the informed consent form.

Exclusion criteria in this study are patients with chronic renal disease, liver cirrhosis, stroke, Alzheimer's disease, allergic disease, rheumatoid arthritis, psoriatic arthritis, systemic lupus erythematosus, systemic sclerosis and other autoimmune disorder, leprosy, anemia, thrombocytosis, patients currently using antituberculosis drugs, cytostatic drug, steroid, alcohol, and patients with foot ulcer that is difficult to examine.

The diabetic peripheral neuropathy score was calculated by history score in MNSI score ‘7’ or higher and physical assessment score in MNSI more than two. The history score in MNSI is a self-fulfilled questionnaire. Physical assessment in MNSI was the appearance of feet, ulceration, ankle reflexes, vibration perception at great toe, and monofilament [6]. The appearance of feet was scored 1 when there was deformities, dry skin, callus, infection, fissure.

Ulceration was score 1 when present, and scored 0 if absent.

The ankle reflexes, also known as the Achilles reflex, examined by tapped the Achilles tendon while the foot is dorsiflexed. When there was no reflex, they would get score 1, when the reflex which appeared only after assisted by Jendrassic manoeuvre, they would get score 0.5.

Vibration perception at great toe was examined by placing the fork on the projection of distal interphalangeal joint of the hallux. Subjects would tell the examiner whether they felt the vibration when it disappeared with closed eyes. When subjects did not report any vibration, the score was zero. When the subjects felt the vibration less than 10 seconds, they would get score 0.5.

The monofilament testing was done on ten points in each foot. The monofilament was perpendicular and pressed for two seconds only, while the subjects closed their eyes. All points would be tested for three times. Score zero would be given for eight right answers or more, and a half point for one to seven right answers.

MDA and NGF serum level were measured by laboratory methods. About 3 mL blood samples were taken from the cubital vein using an anticoagulant-free container to examine MDA and NGF serum levels. MDA serum level was measured by using Beuge method with thiobarbituric acid, while NGF serum level was measured by using the ELISA method for human nerve growth factor-*β*.

The numerical data would be presented as mean and standard deviation. Data were analysed using Statistical Package for Social Sciences (SPSS) 22.0. The normality test was performed only for the numerical data using the Kolmogorov-Smirnov test. Correlation between MDA and NGF serum levels with peripheral diabetic neuropathy score were examined using the Spearman correlation test. The coefficient correlation was calculated and the level of significance for the correlation was counted. The level of statistical significance was 5%.

Results

Patient’s age in this study ranged from 41 to 59 years old. The range of the MDA serum level in this study was between 1.6 nmol/mL to 3.01 nmol/ml while the NGF serum level ranged from 6.8 pg/dl up to 15.7 pg/dl. The peripheral diabetic neuropathy score in this study is 3.53 (± 0.91)

Table 1: Characteristics of the subjects

Variable	n (%)	mean (SD)
Age (years)		53.9 (5.7)
Sex		
Male	13 (43)	
Female	17 (57)	
Body Mass Index (Kg/m^2)		24.6 (3.7)
HbA1c (%)		8.8 (2.0)
Neuropathy score		3.53 (0.91)
MDA (nmol/mL)		2.16 (0.28)
NGF (pg/dL)		10.56 (2.89)

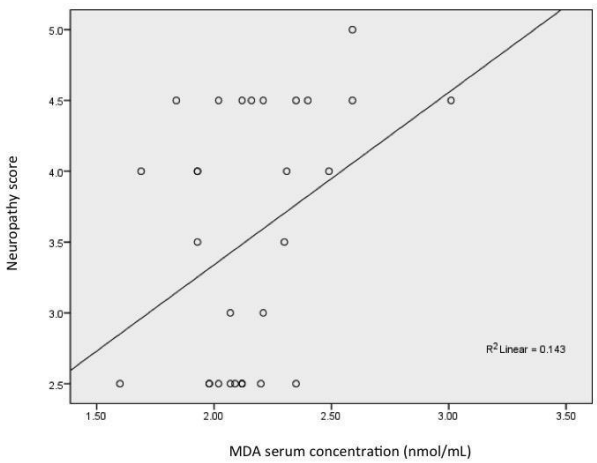

Figure 1: Correlation between the MDA serum level and the diabetic peripheral neuropathy score (r = 0.364 and p = 0.048)

There was a positive correlation (r = 0.364, p = 0.048) proved between the MDA serum level with diabetic peripheral neuropathy score (Figure 1). The correlation between NGF serum level and diabetic peripheral neuropathy score was r = -0.59 and p = 0.001 (Figure 2).

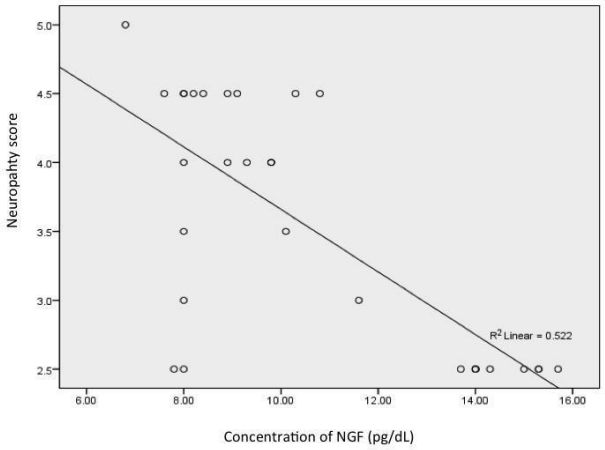

Figure 2: Correlation between NGF serum level and diabetic peripheral neuropathy score (r = -0.59 and p = 0.001)

Discussion

The mean MDA serum level of this study was higher than normal. Maitreyess (2011) and Martin-Gallan (2003) has also found that MDA serum level on T2DM with complication was higher than the no complication group [12], [13]. Bhutia (2011) and Jalees (2017) found that the MDA serum level was significantly elevated in patients with diabetes compared to those in normal subjects [14], [15]. Zavar-Reza (2014) observed significant increased MDA serum level in T2DM patients with complications [16]. Increased MDA serum level can be explained by increasing oxidative stress in diabetic patients. Increased level of oxidative stress associated with free radical-mediated lipid peroxidation. MDA serum level increased because of free oxygen radicals in diabetes that cause peroxidative breakdown of phospholipids.

In this study, the mean serum level of NGF was lower than normal. Mahmoud et al. in 2009 found a significant lower NGF serum level among patients with peripheral neuropathy [17]. Obrosova et al., (2001) had shown that NGF serum level in the diabetic patient was lower than the control [18]. Tosaki et al., (2008) reported that the Schwann cell planted on high-glucose culture media had a low concentration of NGF [19]. Yilmaz et al., (2013) obtained the result of lower NGF production by the Schwann cell of diabetic subjects [20]. NGF decline level is associated with the disturbances in Schwann cells regeneration because NGF produced by the neurons and the Schwann cells. The oxidative stress that occurs in diabetic patients will diminish the nutrient and oxygen supply for the nerve cells, resulting in interfering the life of the nerve and the Schwann cells and increasing the cell degeneration [2], [21].

In this study, we found a positive correlation between MDA serum level with diabetic peripheral neuropathy score. Aziza (2014) found that an increase of MDA serum level in diabetic neuropathy rats [22]. Martinez-Hevaz (2017) reported that altered in MDA values is associated with polyneuropathy in diabetic patients [23].

We found a significant negative correlation between NGF serum level with diabetic peripheral neuropathy score. Yasuda et al., (2003) discovered a positive correlation between decreasing NGF serum level and the neuron regeneration. NGF regulates intraneural homeostasis during development by providing neurotrophic and regulating intracellular pathways to promote neuronal sprouting [11]. Li (2017) showed that NGF has a protective effect in diabetic peripheral neuropathy [24].

In conclusion, there is a high MDA serum level and low NGF serum level in patients with diabetic peripheral neuropathy. Low NGF serum level play a bigger role than high MDA serum level in diabetic peripheral neuropathy.

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ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2019 Jan 15; 7(1):107-113.
https://doi.org/10.3889/oamjms.2019.078
eISSN: 1857-9655
Clinical Science

Predictors of Renal Dysfunction in Adults with Childhood Vesicoureteral Reflux after Long-Term Follow-Up

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Citation: Bundovska-Kocev S, Kuzmanovska D, Selim G, Georgievska-Ismail Lj. Predictors of Renal Dysfunction in Adults with Childhood Vesicoureteral Reflux After Long-Term Follow-Up. Open Access Maced J Med Sci. 2019 Jan 15; 7(1):107-113. https://doi.org/10.3889/oamjms.2019.078

Keywords: Childhood vesicoureteral reflux; Renal dysfunction; Chronic renal failure; Predictive risk factors

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Received: 04-Dec-2018; **Revised:** 02-Jan-2019; **Accepted:** 03-Jan-2019; **Online first:** 14-Jan-2019

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

Abstract

BACKGROUND: Triad of childhood vesicoureteral reflux (VUR), urinary infection (UTI) and renal scarring might initiate potentially serious consequences that lead to renal dysfunction manifested at the second or third decade of life.

AIM: To identify the risk factors predictive for renal dysfunction in adults with primary VUR after long-term follow-up.

METHODS: We evaluated the records of 101 children (94.1% female, 5.9% male) at a median age of 5.2 ± 2.3 years (1-12 years), suffering from UTI and VUR. The patients were interviewed after mean 21 years from the first episodes of VUR (8 to 32 years). Renal function was determined from the estimated glomerular filtration rate (eGFR).

RESULTS: Renal scarring was detected in 68.3% out of 82 patients and bilateral one in 7.3% patients. Linear regression analysis revealed that presence of proteinuria (B = -33.7, p=0.0001), the greater number of years from VUR diagnosis (B = -1.6, p = 0.002) and renal scarring (B = -14.8, p = 0.005) appeared as independent predictors of reduced global eGFRcreat. The same variables plus microalbuminuria (B = -1.0, p = 0.012) appeared as independent predictors of reduced global eGFRcreat-cys. Bilateral scarring (OR=25.5, p = 0.003) appeared as independent predictor of greater risk for CKD assessed using eGFRcreat while greater number of years from VUR diagnosis (OR = 1.7, p = 0.092), microalbuminuria (OR = 1.3, p = 0.047) and again bilateral scarring (OR = 31.3, p = 0.040) appeared as predictors of risk for CKD assessed using eGFRcreat-cys.

CONCLUSION: Identification of those with an increased risk of progression to CKD should be the goal in all patients with childhood VUR. Their systematic follow-up should be till adulthood and older age.

Introduction

Primary vesicoureteral reflux (VUR) is a congenital urinary tract anomaly manifested by either a unilateral or bilateral reflux of urine from the bladder to the kidney which is diagnosed mostly after an episode of urinary tract infection (UTI) [1], [2]. The incidence of VUR is hard to establish but approximately affects 1-2% of children [3], and is much higher among children with UTI (15-70%, depending on age) [4]. The association between VUR and UTI with potentially serious consequences that ultimately lead to reflux nephropathy and renal dysfunction and/or failure in children is well established, from both clinical and experimental studies [5], [6], [7], [8]. Also, the association between VUR and the renal scarring has been confirmed in numerous published studies, and predictive risk factors for its occurrence have been established [8], [9], [10], [11], [12], [13], [14]. Furthermore, despite many advances have been made over the past decades in understanding the childhood VUR, its relations to UTI and prognosis as well as its choice of treatment, there have been still conflicting reports on the risk factors for worsening renal function in adult patients who were treated for childhood VUR.

Thus, the present study aimed to evaluate the renal function and to identify the predictors of its dysfunction in a cohort of patients with childhood VUR who were assessed after long-term follow-up.

Methods

We evaluated the records of 101 children of both sexes, aged between one and 12 years, suffering from UTI and primary VUR. According to the availability at that time, VUR was diagnosed with direct radionuclide cystography (RNC) at the Institute of pathophysiology and nuclear medicine in the presence of pediatric nephrologist and the specialised nurse from University Children' Hospital. Patients with VUR associated with posterior urethral valves, ectopic ureterocele, neurogenic bladder, and other obstructive uropathies below bladder were excluded from the study.

The following clinical variables were obtained from patient record: gender, the age of diagnosis of primary VUR (years), grade of primary VUR, unilateral or bilateral primary VUR, history of UTI, treatment modality (medical, surgical or endoscopic). VUR was graded according to International Reflux Study Committee into five grades [1], [15], [16]. Renal scarring was detected with technetium-99 m labelled dimercaptosuccinic acid (DMSA) renal scintigraphy, which was performed within 6 months of UTI [17].

The patients were interviewed after 20,75 ± 6,46 years from the first episodes of VUR (minimum 8 to maximum 32 years). At the office visit, clinical history was taken. On physical examination weight and height as well as blood pressure were measured (systolic and diastolic). Also, blood and urine samples were collected for further analysis (concentration of creatinine and cystatin C in serum, albumin in urine and proteinuria using a dipstick) and ultrasonographic examination (measurements) of kidneys (Siemens Acusson S3000) was done. Creatinine clearance (CrCl) was calculated according to the Cockcroft-Gault Equation [18]. According to the recommendation of Kidney Disease Improving Global Outcomes (KDIGO) [19] renal function was determined from the estimated glomerular filtration rate (eGFR) using the CKD-EPI Creatinine Equation (2009) for eGFRcreat and 2012 CKD-EPI creatinine-cystatin C equation for eGFRcreat-cyst. In accordance with this recommendation [19] eGFR was categorized into 5 categories as follows: G1-normal or high (≥ 90 ml/min/1.73 m^2), G2-mildly decreased (60-89 ml/min/1.73 m^2), G3a-mildly to moderately decreased (45-59 ml/min/1.73 m^2), G3b-moderately to severely decreased (30-44 ml/min/1.73 m^2), G4-severely decreased (15-29 ml/min/1.73 m^2) and G5-kidney failure (< 15 ml/min/1.73 m^2). Thus patients were stratified by eGFR into groups. Chronic kidney disease (CKD) was confirmed if eGFRcreat and/or eGFRcreat-cyst was < 60 ml/min/1.73 m^2 [19].

Statistical analysis

Categorical parameters were summarised as percentages and continuous parameters as mean ± SD. Comparisons between groups were performed using Student-t-test and ANOVA analysis of variance with Bonferroni post-hoc analysis for continuous parameters and Pearson's chi-square test for categorical parameters. Assessment of correlation of various echorenographic parameters was done using Pearson's correlation analysis. Multiple linear and logistic regression analyses were performed to determine independent predictors of reduced eGFR and/or CKD presence, respectively. The area under the receiver operating characteristics (ROC) curve (AUC) was performed to quantify the value of independent predictors in discrimination of patients with and without CKD. All data analysis was performed using SPSS version 25.0 (IBM SPSS, Inc., Chicago, Illinois) and p-value ≤ 0.05 was considered significant.

Results

The baseline characteristics of patients are summarised in Table 1. A total of 101 patients diagnosed with primary VUR were recruited, of which 95/94.1% were female and 6/5,9% male. The median age at VUR diagnosis was 5.2 ± 2.3 years. VUR was diagnosed at one year in 2 patients, at two years in 10 patients, at 3 years in 9 patients, at 4 years in 19 patients, at 5 years in 12 patients, at 6 years in 18 patients, at 7 years in 16 patients, at 8 years in 9 patients, at 10 years in 2 patients, at 11 years in 3 patients and at 12 years in one patient. In all patients fever and UTI was present before VUR diagnosis was established. VUR was diagnosed at right kidney in 63 patients and left kidney in 74 patients. Among patients with unilateral VUR, 48/76.2% had low-grade VUR (i.e. I–III) at right kidney and 61/82.4% at left kidney, while high-grade VUR (i.e. IV–V) was present in 15/23.8% patients at right kidney and 13/17.6% patients at left kidney. Among those with bilateral VUR, 37 had a low-grade VUR, and 49 had a high-grade VUR. Most patients (94.0%) were administrated prophylactic antibiotics, and the rest of all (7.0%) have STING (subureteral Teflon injection) procedure. In one patient nephrectomy of the right kidney was done.

Of the 101 patients, 82 had a record of undergoing a DMSA within the time of VUR diagnosis. Considering both the right and left kidney, renal scarring was detected in 56/68.3% out of 82 patients

and bilateral one in 6/7.3% patients (Table 1).

Table 1: Baseline characteristics of patients

Parameters	Numbers (%)	
Gender (n/%)		
Female	95/94.1	
Male	6/5.9	
Age at onset of VUR (years)		
Mean	5.2 ± 2.3	
Range	1 to 12	
1-2 years (n/%)	12/11.9	
2-5 years (n/%)	40/39.6	
> 5 years (n/%)	49/48.5	
UTI (n/%)	101/100	
Treatment		
Antibiotics	94/94.0	
STING	7/7.0	
Nephrectomy	1/0.9	
VUR grade	Right kidney (n = 63)	Left kidney (n = 74)
	2.89 ± 0.80	2.70 ± 0.75
Grade II (n/%)	23/36.5	35/47,3
Grade III (n/%)	25/39.7	26/35,1
Grade IV (n/%)	14/22.2	13/17,6
Grade V (n/%)	1/1.6	-
Bilateral VUR* (n/%)	37/37	
Renal scaring** (n/%)	56/68.3	
Bilateral scaring**	6/7.3	

* For 100 patients; ** For 82 patients; VUR = vesicoureteral reflux; STING = subureteral teflon injection

After mean 20.8 ± 6.5 years from the first episode of VUR (8 to 32 years), laboratory analysis (serum, urine and ultrasound) were done. Patients who were 26.1 ± 4.9 of age (17 to 36 years) were divided according to the value of eGFRcreat into 3 categories of renal dysfunction and compared regarding characteristics considered of importance for renal function. Due to the small number of patients, 3Ga and 3Gb were considered as one group. Out of all, 67/66.3% were with normal renal function (G1), 27/26.7% were with mildly decreased (G2), and 7/6.9% were with moderately decreased (G3) renal function. The results of the comparison are shown in Table 2.

A comparison of the characteristics among patients stratified according to the eGFRcreat (Table 2) revealed that those with moderately impaired renal function in comparison to those with normal function were older, diagnosed with VUR at a younger age with significantly more years passed by after VUR was diagnosed, had a more serious grade of VUR (especially for left kidney) along with more frequently bilateral one. Of note was that renal scarrings were significantly more frequently present in the same patients' group while bilateral one was significantly absent in patients with normal renal function in comparison to those with some grade of dysfunction. Blood pressure measurements didn't show any difference between the groups. Furthermore, patients with mildly and moderately impaired renal function compared to those that had normal function showed a significantly lower value of CrCl. Those with moderately impaired renal function also showed ultrasonographical measured the significantly smaller size of both kidneys, a higher percentage of proteinuria as well as the higher value of albuminuria in comparison to those with normal function (Table 2). When the same comparison was made according to the value of eGFRcreat-cys, we obtained almost identical results.

Table 2: Comparison of clinical and laboratory finding in adult patients who were diagnosed with childhood VUR divided according to the categories of eGFRcreat

Characteristics	eGFRcreat (ml/min/1.73 m^2)			p
	G1 = ≥ 90 (n = 67)	G2 = 60-89 (n = 27)	G3 = 30-59 (n = 7)	
Age (years)	24.8 ± 5.0	28.5 ± 3.5	29.0 ± 4.3	0.001
Gender (%)				
male	6/9.0	0	0	P = 0.197
female	61/91.0	27/100	7/100	
Age of VUR diagnosis (years)	5.1 ± 2.4	5.8 ± 2.2	4.5 ± 1.4	0.293
Time from the first episode of VUR (years)	19.6 ± 4.5	22.6 ± 4.0	24.4 ± 3.6	0.001
VUR grade/right kidney	(n = 40)	(n = 17)	(n = 6)	0.324
	2.7 ± 0.7	3.1 ± 0.9	3.0 ± 0.8	
VUR grade/left kidney	(n = 48)	(n = 21)	(n = 5)	0.008
	2.5 ± 0.6	2.9 ± 0.8	3.4 ± 0.8	
VUR bilateral				
no	44/66.7	16/59.3	3/42.9	0.415
yes	22/33.3	11/40.7	4/57.1	
Treatment(%)				0.173
antibiotics	64/95.5	23/85.2	7/100	
STING	3/ 4.5	4/14.8	0	
Scarring (%) (n=82)				0.051
no	22/40.7	3/14.3	1/14.3	
yes	32/59.3	18/85.7	6/85.7	
Bilateral scarring (%) (n=82)				0.001
no	51/94.4	21/100	4/57.1	
yes	1/50.0	0	3/42.9	
BPs (mmHg)	115.9 ± 9.4	116.6 ± 8.1	113.8 ± 11.6	0.772
BPd (mmHg)	76.9 ± 6.0	77.7 ± 4.5	77.8 ± 4.0	0.765
Right kidney (mm)	84.5 ± 14.4	91.3 ± 11.9	76.4 ± 16.3	0.022
Left kidney (mm)	84.0 ± 16.2	89.4 ± 13.0	72.1 ± 14.2	0.027
CrCl (ml/min)	118.9 ± 26.9	77.9 ± 12.6	55.2 ± 14.4	0.0001
Proteinuria (n/%)				0.0001
no	64/95.5	23/85.2	0	
yes	3/4.5	4/14.8	7/100	
Albuminuria (mg/L)	11.2 ± 4.2	13.6 ± 6.2	14.4 ± 1.5	0.038

eGFRcreat = glomerular filtration rate according serum creatinine concentration; VUR = vesicoureteral reflux; STING = subureteral teflon injection; BPs = blood pressure in systole; BPd = blood pressure in diastole; CrCl = creatinin clearence.

In addition, correlation of either eGFRcreat or eGFRcreat-cys with parameters that were related revealed significant relation between lower eGFRcreat or eGFRcreat-cys and longer time since VUR diagnosis (r = -0.450, p = 0.0001; r = -0.445, p = 0.0001; respectively), higher grade of primary unilateral VUR (r = -0.352, p = 0.002; r = -0.324, p = 0.005; respectively), presence of unilateral renal scarring (r = -0.244, p = 0.027; r = -0.294, p = 0.007; respectively) and bilateral ones (r = -0.307, p = 0.005; r = -0.329, p = 0.003; respectively) as well as presence of proteinuria (r = -0.486, p = 0.0001; r = -0.463, p = 0.0001; respectively) and greater level of albuminuria (r = -0.251, p = 0.012; r = -0.307, p = 0.002; respectively).

Predictive variables of reduced eGFR

To determine the independent predictors of reduced eGFR among patients with childhood VUR, we performed multiple stepwise linear regression analysis with covariates that showed a significant relation to it. The results demonstrated that the presence of proteinuria, the greater number of years since from VUR diagnosis and the presence of renal scarring appeared as independent predictors of reduced global eGFR assessed according to serum creatinine value (eGFRcreat) (Model 1, Table 3, Figure 1). Microalbuminuria (B = −0.159, p = 0.117), bilateral renal scarring (−0.088, p = 0.382) and VUR grade of left kidney (B = −0.082, p = 0.434) were

excluded as non-predictive variables in the regression analysis. When we used eGFR assessed according to serum creatinine and cystatin C values (eGFRcreat-cys) the results demonstrated that the presence of proteinuria, value of microalbuminuria, the greater number of years since VUR diagnosis and the presence of renal scarring appeared as independent predictors of reduced global eGFRcreat-cys (Model 2, Table 3, Figure 1).

Table 3: Multiple linear or logistic regression models of eGFR as the dependent variable and its independent predictors

Linear regression	B	Beta	Sig.	95%CI
Model 1				
Proteinuria	-33.783	-0.481	0.0001	(-47.387)-(-20.178)
Years VUR dgn	-1.651	-0.307	0.002	(-2.675) – (-0.628)
Renal scarring	-14.835	-0.274	0.005	(-24.973)-(-4.697)
Model 2				
Proteinuria	-33.031	0.522	0.0001	(-44.540)-(-21.521)
Years VUR dgn	-1.177	-0.243	0.005	(-1.991)-(-0.364)
Microalbuminuria	-1.027	-0.230	0.012	(-1.821)-(-0.233)
Renal scarring	-9.888	-0.203	0.018	(-18.032)-(-1.744)
Logistic regression	Exp(B)	Wald	Sig.	95%CI
Model 3				
Bilateral scarring	25.500	8.842	0.003	3.016-215.594
Model 4				
Years VUR dgn	1.769	3.998	0.092	0.911-3.436
Microalbuminuria	1.333	3.946	0.047	1.004-1.771
Bilateral scarring	31.304	4.233	0.040	1.177-832.313

Model 1: linear regression analysis with eGFRcreat as dependent variable; Model 2: linear regression analysis with eGFRcreat-cys as dependent variable; Model 3: logistic regression analysis with eGFRcreat with and without value of < 60 ml/min/1.73 m^2 as dependent variable. Model 4: logistic regression analysis with eGFRcreat-cys with and without value of < 60 ml/min/1.73 m^2 as dependent variable; CI = confidence interval; VUR = vesicoureteral reflux; Dgn = diagnosis.

Again bilateral renal scarring (−0.086, *p* = 0.330) and VUR grade of left kidney (*B* = −0.030, *p* = 0.742) were excluded as non-predictive variables in the regression analysis. To confirm the role of the same variables in the prediction of the presence of CKD (< 60 ml/min/1.73 m^2) we performed a logistic regression analysis using almost the same variables (proteinuria was excluded as an indisputable significant predictor) as independent predictors.

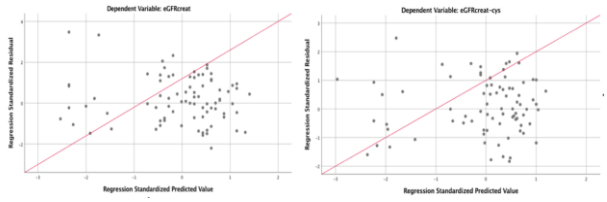

Figure 1: Scatter plot of standardised residual vs standardised predicted value with a regression line for eGFRcreat (left) and eGFRcreat-cys (right)

The results showed that only presence of bilateral scarring appeared as an independent predictor of risk for CKD (Model 3, Table 3) assessed using eGFRcreat. Years of VUR diagnosis (2,206, p = 0.137), VUR grade of left kidney (0.780, p = 0.337), microalbuminuria (0.106, p = 0.745) and renal scarring (0.161, p = 0.281) were excluded as non-predictive variables in the regression analysis. When we used eGFRcreat-cys, the greater number of years since VUR diagnosis, the value of microalbuminuria and presence of bilateral scarring appeared as predictors of risk for CKD (Model 4, Table 3). VUR grade of left kidney (2.798, p = 0.094) and renal scarring (0.578, p = 0.447) were excluded as non-predictive variables in the regression analysis.

In order to confirm the role of independent predictors in discrimination of patients with and without CKD assessed using eGFRcreat-cys, ROC analysis (Figure 2) revealed that addition of microalbuminuria to bilateral scarring (AUC=0.667, 95% CI: 0.435-0.899, p = 0.123) and greater years since diagnosis VUR (AUC = 0.789, 95% CI: 0.660-0.919, p = 0.007) significantly improved the AUC (AUC = 0.809, 95% CI: 0.657–0.962, p = 0.004). Also, we determined 21.5 years since VUR diagnosis as a cut-off value with the highest sensitivity of 87.5% and a specificity of 57.5% for determining the existence of CKD according to eGFRcreat- cys.

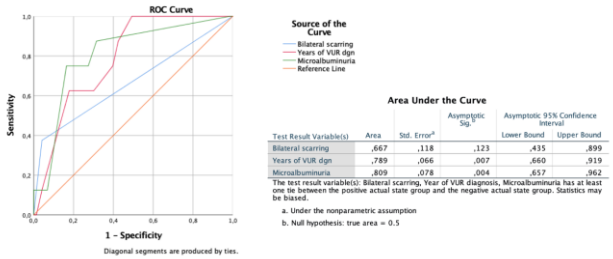

Figure 2: ROC curve for the presence of chronic kidney disease assessed using eGFRcreat-cys

Discussion

In the present study, a total of 101 patients were diagnosed with primary VUR at a median age of 5 years. Among patients with unilateral VUR, low-grade VUR (i.e. I–III) was present in over 70% of patients while high-grade VUR (i.e. IV–V) was present in 23.8% patients at right kidney and 17.6% patients at left kidney. Considering both the right and left kidney, renal scarring was detected in 56/68.3% out of 82 patients and bilateral one in 6/7.3% patients.

As we already stressed an association between childhood VUR and the renal scarring had been confirmed in numerous published studies [8], [9], [10], [11], [12], [13], [14]. In the meta-analysis of Shaikh et al., [12] the prevalence of renal scarring was 2.6 times (95% CI: 1.7–3.9) higher among children with VUR than among children without VUR (41% vs 17%; *P* < 001). Prospective clinical studies showed that the risk of renal scarring after acute DMSA abnormalities detected at acute febrile UTI is significantly greater in patients with high-grade VUR, affecting up to 89% with grade IV–V VUR [8], [20], [21]. In the meta-analysis of 27 clinical studies, Faust et al., [11] demonstrated an increased risk of acquired renal scarring in children with VUR *vs* without VUR

(OR 2.8 and 3.7, respectively).

Hence, the prevalence of renal scarring has been reported to be in the range from 15-62%. Bailey et al., [22] showed that after a mean of 24 years of follow-up out of 21 patients only 4/19% had normal kidneys, although the study included only those with gross childhood VUR. Also, Olbing et al., [23] in a prospective trial of 223 patients with severe VUR who were followed-up for 10 years showed unscarred kidneys on urography only in 38% of these children. Smellie et al., [24] found scars in 44% of their patients with severe VUR that were a follow-up for 10-41 years. Also, Vasama-Lahdes et al., in the study of 127 patients treated for non-obstructive VUR of any grade with the mean age of 41 years documented presence of unilateral scarring in 35% and bilateral one in 24% of subjects assessed by ultrasound.

Furthermore, Swerkersson et al., [8] in their retrospective analysis of 303 children younger than 2 years with a culture verified UTI and VUR in 22% of them, reported 26% permanent renal damage according to DMSA scintigraphy. Recently, among 958 patients studied by Madani et al., [25] DMSA scan showed renal damage in 41.2% of patients. Almost all published data found a strong association between severity of VUR and renal scarring. However, our study showed a higher percentage of renal scarring which was in line with the study of Abeysekara et al., [26] and Macedo et al., [27] who detected renal scarring among patients with primary VUR in 55.3% and 55.2% of them, respectively. The higher percentages probably should be explained by ineffective treatment of UTI and VUR in that period when patients were diagnosed along with the fact that patients might have had multiple febrile UTIs before their first cystography. Furthermore, the higher percentage of renal scarring was also found when DMSA scan was used instead of ultrasound for detection.

Given that renal scarring was recognised as a predictive factor for an increased risk of renal dysfunction that may not be present until the second or third decade of life, it was of great importance to conduct our study to confirm such findings [1]. Thus, in our study of patients with unilateral or bilateral childhood primary VUR it was found that patients with moderately impaired renal function in comparison to those with normal function stratified according to the eGFRcreat or eGFRcreat-cys were older, diagnosed with VUR at a younger age with significantly more years passed by after VUR was diagnosed, had a more serious grade of VUR (especially for left kidney) along with more frequently bilateral one. Also, renal scarring was more frequently present in patients with renal dysfunction while bilateral one was significantly absent in patients with normal renal function in comparison to those with some grade of dysfunction. As for predictors of renal dysfunction regression analysis revealed that presence of proteinuria, the greater number of years since VUR diagnosis and the presence of renal scarring appeared as independent predictors of reduced global eGFRcreat while the same variables plus microalbuminuria appeared as independent predictors of reduced global eGFRcreat-cys. Furthermore, presence of bilateral scarring appeared as an independent predictor of greater risk for CKD assessed using eGFRcreat while a greater number of years since VUR diagnosis, the value of microalbuminuria and again the presence of bilateral scarring appeared as predictors of risk for CKD assessed using eGFRcreat-cys.

Our results were in general in line with those from literature. Several studies have focused on risk factors for developing renal dysfunction and/or CKD in patients with childhood VUR. Ardissino et al., [28] in the epidemiological study conducted in Italy (i.e. ItalKid Project), documented that VUR was found to be the single leading cause of CKD in children, accounting for 25.8% of cases. When the population was subdivided according to the creatinine clearance (Ccr) levels, patients with VUR and Ccr < 25 mL/min/1.73 m^2 had an overall risk of 68% for progressing to end-stage renal disease (ESRD) by the age of 20. In the study of El-Khatib et al., [29] 147 patients with reflux nephropathy and/or primary VUR were followed for two years or more (range 2-19 years) and deterioration in renal function was documented in 37% of them; the identified risk factors were the presence of proteinuria, an elevated plasma creatinine concentration, bilateral scarring, male sex and the presence of hypertension. Nakashima et al., [30], followed 95 patients who had a renal scar or grade III or higher VUR and found that 35% demonstrated renal function deterioration; the identified risk factors were the presence of bilateral scarring, proteinuria > 300 mg per day, diastolic hypertension and low eGFR. In addition, Vasama-Lahdes et al., [9] in 147 (55%) of 267 patients treated for childhood VUR found that eGFR was normal only in 33% of patients and those with bilateral scarring (3%) were significantly more likely to have reduced eGFR, while approximately 7% of patients with VUR progress to ESRD. Caione et al., [31] followed-up for 1-16 years 50 patients with bilateral VUR and found CKD in 54% of them with significant risk for its development in those with bilateral high-grade VUR and serum creatinine levels > 6.0 mg/L in the first year of life. However, Silva et al., [32] determined that age at diagnosis > 24 months, VUR grade V, bilateral renal damage, and a delay in the diagnosis of VUR of > 12 months after UTI were independent predictors of CKD. In addition, North American Pediatric Renal Trials and Collaborative Studies (NAPRTCS) Registry found that in only 8.5% of patients VUR was the cause of CKD whereas Novak et al., [33] on data from the NAPRTCS suggested that older age, higher CKD stage, and history of UTI are significant risk factors for CKD progression in children with VUR. Furthermore, Chen at al., [34] recruited total of 173 patients with primary VUR and found that older age of VUR diagnosis (≥ 5 years vs < 1 year), higher grade of

VUR and higher number of UTI were risk factors for renal scarring, whereas a younger age of VUR diagnosis, renal scarring and acute pyelonephritis were risk factors for developing CKD stage two or higher.

Study limitations: The main limitation of our study was a relatively small number of patients. Also, the number of repetitive UTI during a follow-up period was not documented. We didn't analyse either biochemical parameters of renal function in the childhood or the aetiology of infection.

In conclusion, in adult patients who were treated for childhood VUR, the presence of proteinuria, the greater number of years since VUR diagnosis and renal scarring appeared as independent predictors of reduced global eGFRcreat along with microalbuminuria for reduced eGFRcreat-cys. Bilateral scarring appeared as an independent predictor of greater risk for CKD assessed either using eGFRcreat or eGFRcreat-cys.

A better understanding of the risk factors for renal scarring, and deteriorating renal function can be useful in tailoring the management and therapeutic approach for VUR. Additionally, identification of those with an increased risk of progression to CKD should be the goal in all patients with childhood VUR. Their systematic follow-up should be till adulthood and further to an older age.

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ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2019 Jan 15; 7(1):114-116.
https://doi.org/10.3889/oamjms.2019.042
eISSN: 1857-9655
Case Report

Drug-Induced Melanoma: Irbesartan Induced Cutaneous Melanoma! First Description in the World Literature!

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Citation: Tchernev G, Temelkova I. Drug-Induced Melanoma: Irbesartan Induced Cutaneous Melanoma! First Description in the World Literature! Open Access Maced J Med Sci. 2019 Jan 15; 7(1):114-116. https://doi.org/10.3889/oamjms.2019.042

Keywords: Losartan; Valsartan; Melanoma; Surgery procarcinogenic effect

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Received: 03-Dec-2018; **Revised:** 22-Dec-2018; **Accepted:** 23-Dec-2018; **Online first:** 09-Jan-2019

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

Abstract

BACKGROUND: Melanoma appears to be a malignant disease, whose development can be potentiated by different drug groups. More and more data are in favour of the claim that commonly used antihypertensive drugs also contain the risk of developing melanoma. The most evidence is that angiotensin receptor blockers may be carcinogenic. Two representatives from this group, valsartan and irbesartan, produced by certain pharmaceutical companies are being withdrawn from the market due to finding content of NDMA and NDEA, which are believed to be potent carcinogens. Another representative of this group, losartan, according to in vitro data, potentiates cell adhesion and invasion of human melanoma cells.

CASE REPORT: We present a 45-year-old man with arterial hypertension. For year and a half/two years, the patient is on systemic therapy with Aspirin and Irbesartan/Hydrochlorothiazide. The patient also reported about the presence of a pigmented lesion in the abdominal area, which occurred 5-6 years ago, before the onset of cardiac therapy. According to him, there was a change in the colour and size of the lesion within the framework of cardiac therapy (from 1.5-2 years). Innovative one step melanoma surgery was performed, and the lesion was radically removed with a 1 cm operational safety margin in all directions within one operative session. The subsequent histological verification found the presence of thin melanoma.

CONCLUSION: Drug-induced melanoma turned out to be a problem of significant importance. The group of angiotensin receptor blockers should be investigated more thoroughly and in detail on the probability of potentiating carcinogenesis. We describe an interesting case showing the progression of pigment lesion to melanoma as a possible result of irbesartan therapy, i.e. we share a theory that differs from that of drug-induced de novo melanomas. It should not be overlooked the fact that another widely used drug-Aspirin, is also likely to potentiate the development of melanoma. Furthermore, the case is indicative of the use of one step melanoma surgery in a melanoma patient with a thickness less than 1 mm.

Introduction

The number of studies, according to which different groups of antihypertensive drugs are likely to potentiate the development of melanoma, increases significantly [1], [2], [3]. According to the literature, angiotensin receptor blockers (ARBs), which are widely used as antihypertensive drugs, carry an increased risk of developing cancer [4]. The latest data suggest that the occurrence of cutaneous cancer, including cutaneous melanoma, is also associated with this group of drugs [2], [5]. Experimental in vitro data show that losartan (ARB) stimulates cell adhesion and invasion of human melanoma cells [3].

Case report

We present a 45-year-old man in good general condition and concomitant arterial hypertension, controlled through medication with Aspirin 100 mg (0-0-1) and Irbesartan/ Hydrochlorothiazide 150 mg/12.5 mg (1-0-0). The

patient was hospitalised at the clinic on the occasion of worsening, for several months, of psoriasis vulgaris, diagnosed from 20 years. During the dermatological examination, despite the psoriatic skin changes, in the area of regio abdominalis dextra, was visualised as finding a hyperpigmented macula with uneven pigmentation and uneven edges (Figure 1a and 1b). According to the patient, the pigmented lesion appeared 5-6 years ago. Medication therapy with Aspirin and Irbesartan/Hydrochlorothiazide dates from 1.5-2 years. According to anamnestic data, within the framework of cardiac therapy, the patient observed a change in the colour and size of the lesion. Clinically and dermatoscopically, the pigmented macula met the requirements for a malignant melanocytic lesion. Also, clinical and dermatoscopic data spoke in favour of melanoma less than 1 mm thick. Based on these data, one step melanoma surgery (OSMS) was performed. The lesion was removed by elliptical excision, under local anaesthesia, with a surgical field of 1 cm in all directions (Figure 1b and 1c). The surgical defect was closed by a single interrupted sutures (Figure 1d). Histological examination confirmed the diagnosis: superficial malignant melanoma, Clark II level, Breslow's thickness below 1 mm, no ulceration, low mitotic activity, well-expressed lymphocytic stromal reaction, clear resection lines. The staging was performed according to which it was found to be melanoma Stage I (T1aN0M0).

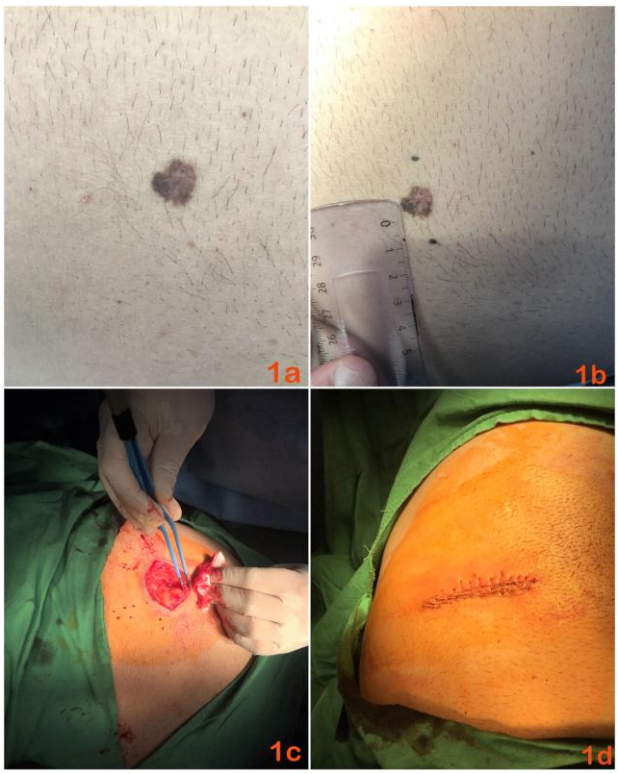

Figure 1: a) Clinical picture of primary cutaneous melanoma located in regio abdominalis dextra with uneven pigmentation; b) Outlining the 1 cm operational security boundaries in all directions, preoperative finding; c) Intraoperative picture of the lesion removed by elliptical excision; d) Postoperative clinical picture of surgical defect closed by single interrupted sutures

Discussion

Antihypertensive drugs are the most common and widely used drugs among the population; however, according to very new data, they may be associated with an increased risk of melanoma occurrence [1]. As possibly potentiating the development of this type and another type of cutaneous tumours, different groups of drugs for the treatment of arterial hypertension are mentioned [1], [2], [5]. Studies of the carcinogenic effect of angiotensin receptor blockers (ARBs) predominate [2], [3], [4], [5], [5]. It is believed that this group of antihypertensive drugs generally carries a risk of developing cancer [4], but the individual risk of different agents is not yet known [4]. The prevailing number of data, however, is in the direction of claiming that ARBs are highly associated with melanoma development [2], [3], [5]. In vitro, experimental data show that losartan inhibits NHE1 (Na + /H + exchanger isoform 1) activity and migration of human melanoma cells (MV3), but at the same time stimulates MV3 cell adhesion and invasion [3].

Some events in 2018 lead to important analyses and conclusions. In June 2018, US manufacturer Prinston Pharmaceuticals Inc. stopped producing products containing valsartan because it detected traces of N-nitrosodimethylamine (NDMA) in the active pharmaceutical ingredient of valsartan (API) provided by a Chinese manufacturer (Zhejiang Huahai Pharmaceutical Co) [6]. NDMA is defined as a chemical substance in the group of potent carcinogens and, according to the US Department of Health and Human Services, exposure to high doses of NDMA may cause liver damage [7], [8]. This is confirmed by animal studies that indicate that NDMA can cause tumours in the liver, kidneys and the respiratory tract, making it a likely human carcinogen [8]. Subsequently, the withdrawal of valsartan expands, following the detection of NDMA in drugs manufactured by a second Chinese manufacturer (Zhejiang Tianyu Pharmaceuticals of Taizhou) and by a manufacturer in India (Hetero Labs Limited, called Camber Pharmaceuticals), followed by voluntary withdrawal of valsartan products from several companies (Major Pharmaceuticals, Solco Healthcare and Teva Pharmaceuticals Industries, as well as valsartan/hydrochlorothiazide from Solco and Teva) [9], [10].

In September 2018, information about a second potential carcinogen in the product valsartan-N-nitrosodiethylamine (NDEA) was published [11].

In November 2018, SciGen Pharmaceuticals voluntarily withdrew ARB-irbesartan due to NDEA content and according to the US Food and Drug Administration (FDA): „This is the first non-valsartan drug product the agency has found to contain the NDEA impurity" (Aurobindo Pharma Ltd manufactures

the active pharmaceutical ingredient (API) for ScieGen's irbesartan products) [12].

The case we have described supports the thesis that, in the presence of pigment lesion, irbesartan probably promotes carcinogenesis in the direction of cutaneous melanoma. This statement differs from that shared in other articles, according to which valsartan are a possible inducer of de novo melanomas, i.e., without the presence of precursor lesions.

Also, the FDA shares the likelihood of cancer being dose-dependent (daily intake of the highest dose of valsartan (320 mg) throughout 4 years, 1/8000 patients are likely to develop cancer) [13].

According to the EMA (European Medicines Agency) (as a result of an electronic correspondence), there have been 9 reported cases of melanoma in patients receiving valsartan. None of them has been formalised and suggestions remain as to whether products are contaminated with NDMA/NDEA, or it is possible that the carcinogenic effect comes directly from the generic substance of valsartan as well as from the presence of a potential another carcinogen [3], [11]. The same open question remains about the carcinogenic effect of irbesartan.

According to a study of 2015, angiotensin II therapy potentiates melanogenesis (increases melanin content and increases tyrosinase activity) through angiotensin type 1 receptors, and losartan eliminates this effect [13]. This, however, indicates that ARBs directly affect melanogenesis and the carcinogenic effect may be related to another, unknown pathogenetic chain.

In conclusion, the case presented is an interesting example due to 1) the possibility of Irbesartan induced cutaneous melanoma in the presence of precursor pigmented lesion and 2) the perfect therapeutic result due to perform one step melanoma surgery.

The content of probable carcinogens, in representatives of the group of angiotensin receptor blockers, suggests the need for further studies on the individual carcinogenic risk for particular drugs. Interestingly, one of the most widely used drugs, i.e. the antihypertensive drugs and acetylsalicylic acid, are pointed out as probably triggering the development of melanoma.

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ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2019 Jan 15; 7(1):117-120.
https://doi.org/10.3889/oamjms.2019.031
eISSN: 1857-9655
Case Report

Trigonocephaly: Case Report, Review of Literature and a Technical Note

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Citation: Sumkovski R, Kocevski I, Micunovic M. Trigonocephaly: Case Report, Review of Literature and a Technical Note. Open Access Maced J Med Sci. 2019 Jan 15; 7(1):117-120. https://doi.org/10.3889/oamjms.2019.031

Keywords: Trigonocephaly; Metopic suture; Craniosynostosis; Open-cranial vault reconstruction

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Received: 08-Nov-2018; **Revised:** 17-Dec-2018; **Accepted:** 18-Dec-2018; **Online first:** 10-Jan-2019

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

Abstract

BACKGROUND: Premature fusion of the metopic suture results in a type of craniosynostosis known as trigonocephaly. The treatment of trigonocephaly is surgical and is likely to remain so. Surgical methods and techniques for correction of craniosynostosis-related skull deformities have evolved, and a single best procedure is yet to be presented.

CASE REPORT: Here we present a technical remark in a case of open cranial vault reconstruction.

CONCLUSION: Although the literature, in general, prefers barrel stave (radial) frontal bone osteotomies, a technique with longitudinal frontal bone osteotomies were performed, without fixation of the bony flaps, frontal bone or supraorbital arch, with a quite satisfactory result.

Introduction

Trigonocephaly is one of the many types of craniosynostosis. Craniosynostosis represents the premature fusion of calvarial sutures. The incidence of craniosynostosis is estimated at approximately 1 in 2000-2500 live births [1], [2], [3]. It has traditionally been classified as syndromic and non-syndromic based on phenotypic features. The syndromic type of craniosynostosis, which represents fewer than 5% of all cases of craniosynostosis, is usually associated with a positive family history of craniosynostosis, fusion of multiple sutures and other abnormalities (skeletal most often), whereas the non-syndromic type, which is the more common variant, often has single-suture synostosis without other abnormalities. It is generally assumed that non-syndromic craniosynostosis (single suture synostosis) have no genetic abnormalities, but recent advances have shown that almost all craniosynostosis have genetic background [4]. Single suture synostosis is more commonly observed. There is a slight male predominance with a ratio of 2:1. [2], [3]. The metopic synostosis, which this case is about, has a frequency of about 4-10%, just behind the sagittal suture synostosis, accounting for 53-60% and coronal synostosis accounting for 17-29%. Accounting for less than 2% the lambdoid suture synostosis is the least common. The case we are presenting is about a 6-month-old infant, previously diagnosed with trigonocephaly. Presented here is the case with a special note regarding the technical management (operative technique), as the frontal bone was managed differently from the operative techniques proposed in most of the literature where barrel stave (radial) osteotomies are mostly used. Also, the bone flaps were left loose, as no fixating plates or other fixating techniques were used.

Case Report

This case report describes a case of trigonocephaly in a 6-month-old infant, diagnosed at the age of two months. No previous treatment is undertaken. The cranial perimeter is 37 cm, weight is 8.5 kg. The infant is asymptomatic, despite the visible head deformity.

A preoperative CT scan was obtained, demonstrating metopic suture synostosis, noted as a mid-forehead ridge, hypotelorism, flattening of the frontal bones, anterior displacement of the coronal sutures, compensatory bulging of the parieto-occipital region and temporal narrowing (Figure 1, Figure 2).

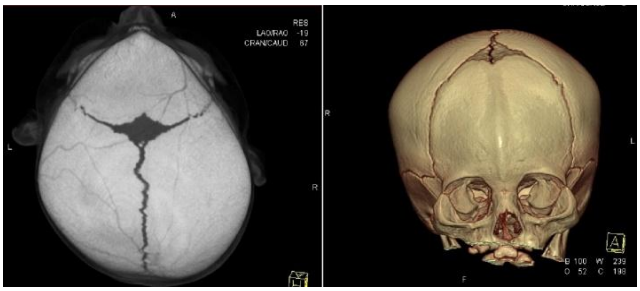

Figure 1: 3D reconstructed CT scan. Note the metopic suture synostosis, seen as a mid-forehead ridge, hypotelorism, flattening of the frontal bones, anterior displacement of the coronal sutures, compensatory bulging of the parieto-occipital region and temporal narrowing

After intubation and introduction into general anaesthesia, the patient, 6 month old male infant, in this case, was placed in supine position with the head stabilised on a horseshoe headrest. Next, the operative field was prepped with antiseptic betadine solution and sterile single-use drapes were used for isolation.

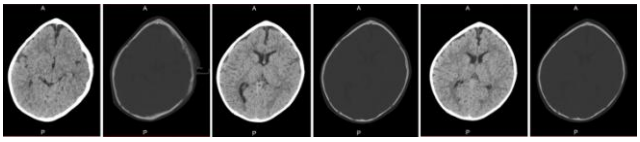

Figure 2: Axial cross section head CT scan: note the triangular forehead, prominent midline sagittal ridge and shortening of the anterior cranial fossa, with compensatory bulging of the parieto-occipital region and temporal narrowing

A bicoronal sinusoid (zigzag) incision was made; subperiosteal skin flap was created and elevated. It was secured in place by three fish hooks. After the exposition of the bone structure of the scalp, frontal and parietal bones were identified, midline frontal bone ridge (metopic suture), and also anterior fontanel. The skin flap was elevated just below the glabella and the nasofrontal suture enough to expose the supraorbital ridge. Next, using an Olivecrona dissector, the dura was easily separated from the tabula interna of the midline of the frontal and parietal bones at the level of the anterior fontanel, just above the superior sagittal sinus. Kerrison rongeur was used to perform suturectomy at the level of the coronal suture, starting from major fontanel. Using high-speed pneumatic saw (Medtronic Midas Rex system) the initial osteotomy was extended to the level of the sphenofrontal and zygomaticofrontal suture, then turning toward midline just above the supraorbital arch (about 1 cm above) to form a frontal bone flap. The bone flap was left fixed at the midline level just above the nasion. Kerrison rongeur was used to safely complete the frontal bone craniotomy in one peace, to avoid injury to the dura or the anterior part of the superior sagittal sinus. Next, using an Olivecrona dissector the periorbita was dissected from the orbital bone, the point is the protection of the periorbital tissues from the drill and exposition of the bony supraorbital arch for additional drilling. The same was performed using a high-speed drill (Medtronic Midas Rex pneumatic high-speed drill), used for drilling of the ridge at the level of nasofrontal suture, superomedial and superior orbital wall, extending to the previously addressed zygomaticofrontal suture. Intracranial drilling was performed just anterior to the cribriform plate and the crista gali. The bony supraorbital arch was removed in one peace, thus safely opening the orbits and exposing the periorbita (Figure 3).

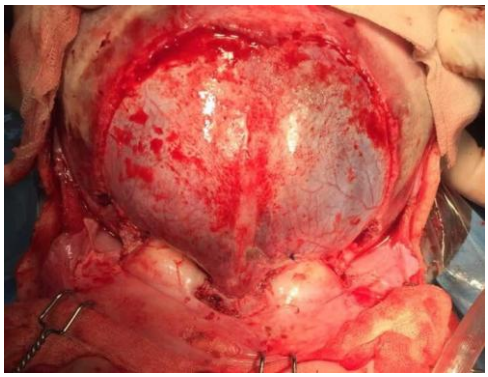

Figure 3: Intraoperative look just after frontal bone craniotomy, supraorbital arch osteotomy. Exposition of the dura underneath and the orbital tissues

The frontal bone, initially removed in one peace, was cut down the middle longitudinally through the metopic suture using a high speed saw, to the level just above the glabella, leaving the glabella as a separate peace, thus creating two halves of the prematurely fused frontal bone, plus the glabella. Each half was additionally cut down the middle (longitudinally), again using high speed saw, thus creating four free bone flaps from the frontal bone. Following, the previously removed supraorbital ridge was addressed.

Two vertical osteotomies were made from each side of the glabella, creating three different parts of the supraorbital ridge. Next, two barrel stave (radial) osteotomies approximately 1 cm wide were cut into the parietal and temporal bone from each side

to facilitate reshaping to match the widened frontal bones, using a Kerrison rongeur. In the next step, the bones were arranged — first, the bony supraorbital ridge, next to the bony glabella and then the four frontal bone flaps. The bone fragments were left loose; they were not fixed in place, as no fixating plates, or other fixating techniques, were used (Figure 4).

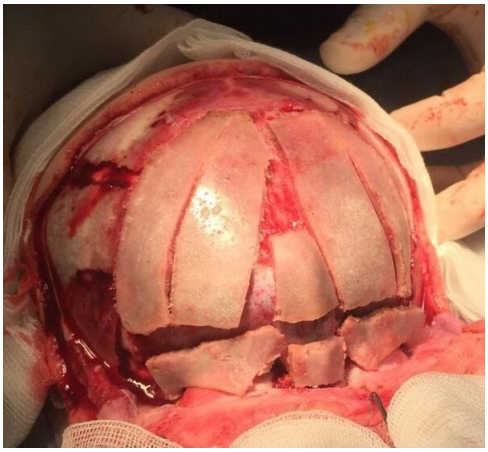

Figure 4: Arrangement of the bone flaps. The bone fragments were left loose, as no fixating plates or other fixating techniques, were used

The previously elevated periosteum, undissected from the skin flap was sutured to the rest of the periosteum using rapidly resorbable polyfilament suturing material, thus covering all of the free bone flaps/bone fragments. The skin was sutured in single vertical mattress sutures using a monofilament nonresorbable suturing material. No epicranial/subperiosteal drainage was used.

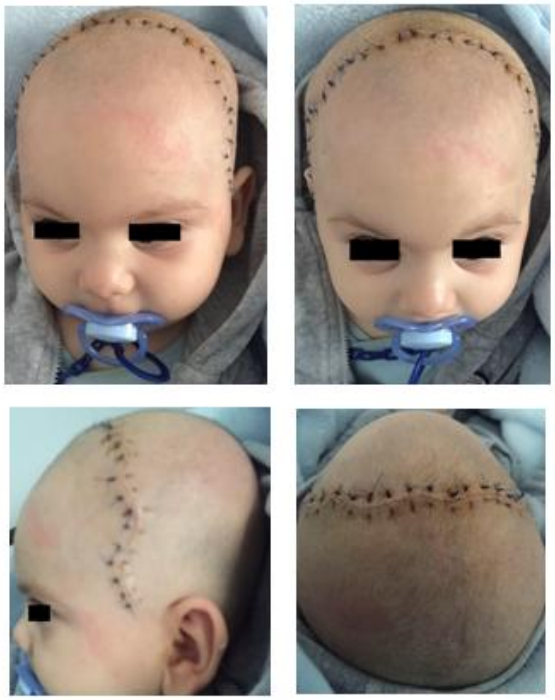

Figure 5: Follow up at 10 days postop, just before suture removal

The operating time was 3 hours 45 minutes, and the estimated blood loss was less than 50 ml. The procedure was well tolerated by the patient, and there were no postoperative complications. There was periocular and facial oedema, most severely expressed on the 2nd postoperative day. The patient was discharged on the 3rd postoperative day. At follow up visit at ten days from the operation, the incision was well healed, the facial and mid-forehead oedema was in resolution, and no other complications were noted.

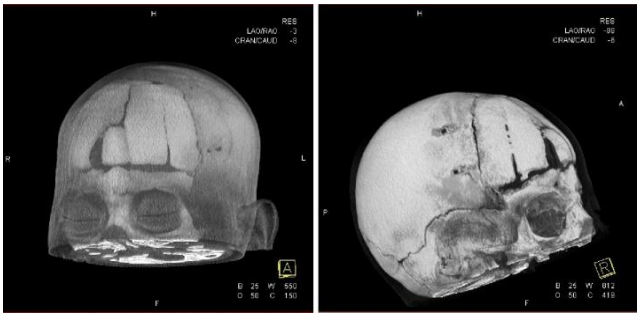

Figure 6: Post-operative 3D reconstructed head CT at 3 months follow up visit

A postoperative CT scan at three months follow up was also obtained demonstrating a satisfactory anterior cranial base decompression with no displacement of the bone flaps (Figure 6, Figure 7).

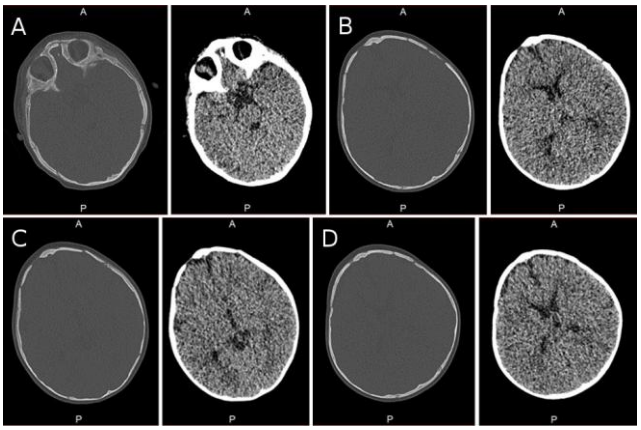

Figure 7: Postoperative head CT scan at 3 months follow up. Note the anterior cranial base decompression

Discussion

Trigonocephaly is the result of premature fusion of the metopic suture, resulting from restricted lateral growth of the frontal bones, leading to characteristic appearance of “keel forehead”, posterior displacement of the superolateral orbital rims, hypotelorism, flattening of the frontal bones, anterior displacement of the coronal sutures, compensatory

bulging of the parieto-occipital region and temporal narrowing. This results in a triangular forehead, prominent midline sagittal ridge and shortening of the anterior cranial fossa [5]. Normal closure of the metopic suture is expected to occur by nine-month, although normal closure can occur as early as 3 months of age, and yet not all children with prematurely closed metopic suture develop trigonocephaly [6]. Interestingly, trigonocephaly has the highest rate of associated cognitive impairment among the single suture synostosis [7]. The diagnosis can be made based on clinical appearance, although radiographic imaging is often used to rule out an associated intracranial anomaly. Skull x-rays or craniogram is often obtained when a diagnosis is suspected, with minimal clinical value, compared with detailed physical examination of an experienced clinician. Three-dimensional computed tomography (CT) provides a comprehensive view of the suture as well as the overall head shape. However, if a diagnosis of synostosis is suspected and concern for brain pathology exists the use of ultrasound (US), or magnetic resonance (MR) imaging should be considered as a safer alternative to CT. The treatment of trigonocephaly and craniosynostosis remains surgical and is likely to remain so. In general, the two main indications for surgical treatment of any craniosynostosis including trigonocephaly include correction of the skull deformity for aesthetic and psychosocial purposes and ensuring there is adequate space for normal brain growth [8].

The aesthetic deformity associated with craniosynostosis alone is considered sufficient to justify the treatment, the point being the social and psychological impact on the affected children. Increased intracranial pressure (ICP) is an absolute indication for surgical repair. Surgical methods and also techniques for correction of craniosynostosis-related skull deformities have evolved. However, available data have yet to demonstrate a single best procedure for the treatment of synostosis. At the moment the surgical techniques available allow performing an open cranial vault reconstruction or minimally invasive reconstruction using endoscopic techniques. The timing of the operation should also be considered, as the procedures in infants tend to be less invasive. Open cranial vault remodelling procedures are often delayed until 6 to 12 month, mainly because the observation of an increased incidence of revision surgery in patients operated on before the age of 6 months. The endoscopic techniques are generally best performed by 3 to 6 month of age. In our patient, an otherwise a healthy 6-month-old infant, the open technique for cranial vault reconstruction was used. A technical remark in the management in this specific case is the osteotomies performed on the frontal bone and also the remodelling of the supraorbital arch, which, traditionally, is not often addressed. Although the literature, in general, prefers barrel stave (radial) frontal bone osteotomies, another technique was used in this case. As previously noted, longitudinal frontal bone osteotomies were performed, and the result is quite satisfactory. Also, none of the bone flaps was fixed, frontal bone or supraorbital arch. The postoperative head CT scan at three months postop demonstrates an acceptable anterior cranial base decompression. However, this type of cranial vault reconstruction, as reasoned by the author, may only be used in trigonocephaly, as the approach itself only allows for anterior cranial base decompression. Other types of craniosynostosis require a different exposition, perhaps through the same skin incision, and also different sutures to be addressed.

In conclusion, this modified approach for anterior cranial base decompression in metopic suture synostosis is technically simple and appears to be associated with no greater morbidity than the traditional approach. Potential advantages include reduced blood loss; less postoperative pain decreased the length of stay. Surgical indications are identical as for any other approach. However, due to the satisfying outcome, this technique may become a surgical option of choice in patient's metopic suture synostosis.

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Open Access Macedonian Journal of Medical Sciences. 2019 Jan 15; 7(1):121-123.
https://doi.org/10.3889/oamjms.2019.043
eISSN: 1857-9655
Case Report

Irbesartan Induced Cutaneous Melanoma! Second Case in the Medical Literature!

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Citation: Tchernev G, Temelkova I. Irbesartan Induced Cutaneous Melanoma! Second Case in the Medical Literature! Open Access Maced J Med Sci. 2019 Jan 15; 7(1):121-123. https://doi.org/10.3889/oamjms.2019.043

Keywords: Irbesartan; ARB; Drug-induced melanoma; Surgery; Survival benefit

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Received: 08-Dec-2018; **Revised:** 17-Dec-2018; **Accepted:** 23-Dec-2018; **Online first:** 09-Jan-2019

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

Abstract

BACKGROUND: Drug-induced melanoma is a topic, concept or "reality" becoming more and more popular as the list of drugs considered as potential inducers of cutaneous melanoma is constantly growing. Interesting and current at the moment is the question/dilemma of "Irbesartan induced melanomas" and "Valsartan induced melanomas"! The following questions are without answers: 1) the general risk which angiotensin receptor blockers contain for potentiating the carcinogenesis and cancer development (as a whole); 2) available officialized data for withdrawal from the market of products with valsartan and irbesartan due to detected potential carcinogens-NDMA/NDEA, and 3) the missing official information on the most likely forms of cancer potentiated by these drugs. That is precisely why many questions remain open, and the inevitable assumption arises for the key, although according to some conspiratorial role of so-called "pharmaceutical giants" in the concept of drug-induced malignancies.

CASE REPORT: We present a 72-year-old man with arterial hypertension in connection with which he is taking Irbesartan 300 mg (1-0-0), Amlodipine 5 mg (0-0-1) and Moxonidine 0.2 mg (0-0-1). The patient reported the presence of pigment lesion in the head area, which dates from many years and 3 years ago it was at the size of the nail plate on the index finger. Irbesartan therapy dates from 1.5-2 years, and according to the patient 1.5-2 years after the start of irbesartan therapy, the lesion grew sixfold, accompanied by sensitivity and discomfort in the area. Clinically and dermatoscopically the lesion had data on superficial spreading cutaneous melanoma. Tumour thickness ≤ 1 mm was measured preoperatively by ultrasound. The so-called one-step melanoma surgery (OSMS) was performed, and the lesion was removed by elliptical excision with an operative surgical margin of 1 cm in all directions within one operative session. The subsequent histological study (and screening staging) found that it was a superficial spreading melanoma stage IA (T1bN0M0).

CONCLUSION: Possible, but unlikely, in our opinion, is that the intake of angiotensin receptor blockers (in particular irbesartan), and the progression of benign precursor lesions to malignant do not have a direct relationship. The growing number of data in the literature for drug-induced melanoma and massive withdrawal of products with valsartan and irbesartan due to the content of probable carcinogens speaks, however in favour of the opposite, namely that it is more likely to speak about established dependence than of a sporadic association. Drug-induced melanoma-rather a reality than a myth.

Introduction

In September 2018, NEJM-Journal Watch/New England Journal of Medicine shared information for revealed content of two potential carcinogens-NDMA and NDEA in the drug valsartan [1], [2]. It can be said that spreading drugs containing one or more carcinogens... becomes modern [1], [2]. Or just a trend? Normal and should not impress us!? [1], [2], [3], [4], [5], [6], [7]. Interesting is also the fact that high-grade NEJMs formalise information for carcinogens in medications. However, it does not tell what type of cancer is observed at, during or after their use [1], [2]. We specifically refer to the group of angiotensin receptor blockers [1], [2].

Case report

We present a 72-year-old man with arterial hypertension controlled through medication with Irbesartan 300 mg (1-0-0), Amlodipine 5 mg (0-0-1) and Moxonidine 0.2 mg (0-0-1). The treatment with Irbesartan dates from 1.5-2 years. The patient was hospitalised for surgical removal of a pigment lesion in

the head area. According to the patient's data, the lesion dates from several years, and 3 years ago it was at the size of a nail plate on the index finger. One and a half to two years after the start of irbesartan therapy, the patient observed a sixfold increase in the size of the lesion, sensitivity and discomfort, which is the reason for his presentation at the hospital of the Ministry of Interior and the attendant surgical removal. During the dermatological examination in the area of regio parietalis dextra, a melanocytic formation with irregular shape, dark brown to black colour, uneven distribution of pigment and presence of regression zones, clinically and dermatoscopically suspected for superficial spreading melanoma was visualised (Figure a and b). Tumour thickness ≤ 1 mm was measured preoperatively by ultrasound. The so-called one-step melanoma surgery was performed, and the lesion was removed by elliptical excision with an operative safety margin of 1 cm in all directions within one operative session (Figure c and d). Due to the impossibility of closing the defect in its middle part, the decision was made to be performed advancement flap, and the skin flap was mobilised from the proximal part of the skull in the distal direction (Figure d). The wound edges were adapted and sewn with single interrupted sutures. The subsequent histological study found that it was a superficial spreading melanoma, Clark II level, Breslow's thickness of 1 mm, without ulceration. The staging was performed which established melanoma stage IA (T1bN0M0). The postoperatively established tumor thickness of 1 mm does not require re-excision, i.e. the required safety margin was respected within one surgical intervention. Due to this fact, the patient was left for clinical observation and a perfect cosmetic result was observed (Figure e).

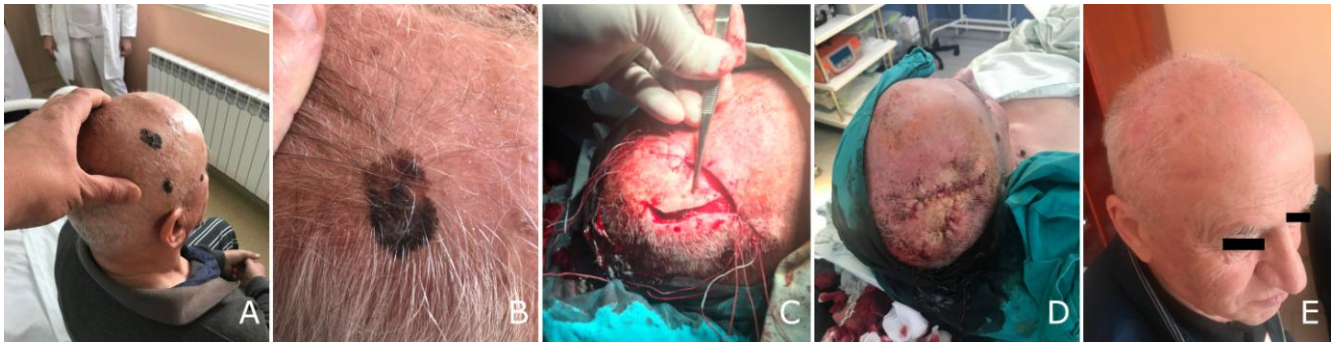

Figure 1: A), B) Clinical view of a melanocytic formation with irregular shape in the right parietal region, presence of regression zones, clinically and dermatoscopically suspected for melanoma; C) Elliptical excision of the lesion with 1 cm operational safety margin in all directions and performing of modificated advancement flap; D) Postoperative clinical status of surgical defect closed by single interrupted sutures; E) Clinical view of perfect cosmetic outcome

Discussion

The latest and newest literature data speak in favour of the concept that the definition of drug-induced melanoma exists and becomes significance [8], [9], [10], [11] increasingly. Numerous data in the literature show that drugs for the treatment of schizophrenia and Parkinson's, for example, by various mechanisms affect melanogenesis and are capable of leading to a possible imbalance, ensuring respectively on the one hand 1) protection from melanoma development or 2) eventual progression to the last [11], [12], [13], [14], [15]. Interestingly, however, these are drugs for which there is no published or reported evidence of carcinogenic content [11], [12], [13], [14], [15]. But the latter are not stopped from production and application? Antihypertensive angiotensin receptor blockers are associated with an increased risk of not only melanomas development, but also other skin tumors [10]. With the case we presented, we ask two important questions: 1) are the so-called "pharmaceutical giants", with their produced and distributed drugs, in the root of potentiating carcinogenesis and the development of de novo malignant lesions? and 2) are, the so called "Pharmaceutical companies", able to distribute the same drugs that, within existing precursor formations (normal or dysplastic nevi), potentiate their progression to malignant?

The mass withdrawal of products with valsartan and subsequently irbesartan (due to found content of potential carcinogens-NDMA and NDEA) by various drug companies, puts a number of "troubling questions", namely: First, "Based on what grounds or data companies have decided to check their products for carcinogens?" and secondly, "Do these" giants "have data for development or existing risk for the development of tumor formations (or already existing ones) within a possible reception of the indicated medications (currently unofficial)?" [1], [2], [3], [4], [5], [6], [7]. Only the answer to these questions can explain the rapid withdrawal of different products from different pharmaceutical companies. The rationale in the media and medical space at the moment for "potential carcinogenic risk" is not enough!

The following question remains open: 1) whether, for medications that currently have unclear

pro-carcinogenic action (???), there are data that the incidence of melanoma is increased? And (2) why drugs with a proven carcinogenic effect are withdrawn without explanation at the moment what forms of cancer are found within their application [1], [2], [3], [4], [5], [6], [7]. The facts are the least disturbing. And such data surely exists! This indirectly speaks that pharmaceutical companies in all likelihood have databases for the frequency of a particular type of cancer that occurred within or after the application of a particular drug? However, such data should be formalised at the moment. Namely-that such facts (as shared by us) could hardly be confessed by companies that, for example, pay 370 million dollars to the US court and keep the direction of their domestic policy only for "inner consumption" or far from the media noise [16].

The other contradictory issue, statement, or point of view should be the following: Is it possible for certain pharmaceutical companies to distribute products with carcinogenic substances, while at the same time the company/is in question offer/s a wide range of medications for the treatment of melanomas and other advanced cancers? Although conspiratorial, the answer is categorical: Yes! Companies that withdraw certain medications or for which there is evidence of association with cutaneous melanomas, at the same time are engaged in the treatment of the latter! And are among the top 10 pharmaceutical companies! One superficial analysis of the data shows that this is a reality. And it can be verified in all media.

Interestingly, the case described is the second formalised by us regarding the relationship between irbesartan and melanoma. We presented a patient with a precursor melanocytic lesion, initially identified as a melanocytic nevus, which changes its size six times and progressed to melanoma, the latter occurring 1.5-2 years after systemic intake of irbesartan. A sporadic association may be available, but we think it is less likely.

In conclusion, the probability, in the presence of precursor lesions, the intake of irbesartan to trigger their development towards melanoma, is completely real. This is confirmed by the presented by us (so far) two different patients with arterial hypertension, controlled with irbesartan, manufactured by various pharmaceutical companies. This indirectly speaks in favour of the notion that the primary substance, not possibly NDMA/NDEA contamination, is the basis for potentiation of carcinogenesis? The possible, but highly unlikely hypothesis that the melanoma development in adults is simply associated with the intake of angiotensin receptor blockers is not excluded.

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ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2019 Jan 15; 7(1):124-126.
https://doi.org/10.3889/oamjms.2019.025
eISSN: 1857-9655
Case Report

Retrieval of Intravascular Fractured Fragment of Tunnelled Double Lumen Catheter in Hemodialysis Patient

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Citation: Semadi IN, Koerniawan HS, Irawan H. Retrieval of Intravascular Fractured Fragment of Tunnelled Double Lumen Catheter in Hemodialysis Patient. Open Access Maced J Med Sci. 2019 Jan 15; 7(1):124-126. https://doi.org/10.3889/oamjms.2019.025

Keywords: Double lumen catheter; Intravascular fractured fragment; Retrieval; Surgical venous cut-down

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Received: 28-Oct-2018; **Revised:** 04-Dec-2018; **Accepted:** 05-Dec-2018; **Online first:** 11-Jan-2019

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

Abstract

BACKGROUND: Intravascular fractured fragment of double lumen catheter with embolisation is a serious and rare complication. Another serious complication includes infection, thrombosis, arrhythmias, and pulmonary embolism. We report a successful surgical venous cut-down technique in the retrieval of an intravascular fractured fragment of tunnelled double lumen catheter in a hemodialysis patient.

CASE REPORT: A 51-year-old female underwent hemodialysis through a tunnelled double lumen catheter and had her arterio-venous graft matured. During retrieval of tunnelled double lumen catheter procedure, the distal part of the catheter was fractured and slipped into the internal jugular vein. Chest radiograph revealed intravascular double lumen catheter extending from the distal part of the right internal jugular vein to right atrium. The procedure of foreign body retrieval was done the next day under general anaesthesia and C-Arm guidance using right internal jugular venous cut-down approach. A right-angle clamp was used to retrieve the fragment without any post-procedure complications.

CONCLUSION: Intravascular fractured fragment of double lumen catheter is a dangerous situation as are all the intravascular foreign bodies. The choices of the technique for retrieval of the fractured fragment are varied. It depends on the type and site of a fractured fragment as well as the surgeon experiences.

Introduction

Central venous catheter (CVC) is used for the administration of drugs, hemodialysis, hemodynamic measurement, as well as cardiac pacing. The complication of foreign bodies is closely associated with the development of the intravascular catheter technique in 1945 by Meyers. In some case especially in trauma, CVC can be used using large bore to deliver fluid resuscitation. In the United States, more than 5 million of the central venous catheter are inserted every year, and the device drop behind is due to loss of wire or fractured fragment due to inexperiences [1]. Various studies have been performed and suggest that one of the causes of CVC fracture is huge traction force needed to retrieve CVC due to fibrin sheath formation around the CVC [1]. Complications of CVC are a fractured catheter, thrombosis, or even vein perforation (though it is rare). The danger of intravascular foreign bodies was formally recognised by the Federal Drug Administration in a 2008 public health announcement [2]. The authors report a case of an intravascular fractured fragment of tunnelled double lumen catheter in a hemodialysis patient. It was successful retrieval under C-Arm guidance using surgical venous cut-down of the right internal jugular vein.

Case Report

A 51-year-old female was admitted due to complaints of weakness as well as planning to have brachytherapy for her cervical cancer treatment. She was diagnosed with cervical cancer stage III-b with chronic kidney diseases caused by obstructive nephropathy because of the malignancy infiltration of primary cervical cancer. After underwent arterio-venous graft 10 weeks before, a tunnelled double lumen catheter for hemodialysis was accidentally fractured during its retrieval from the skin. The patient was asymptomatic. However, chest radiograph demonstrated of a long radio-opaque double lumen hemodialysis catheter fragment from the end of right internal jugular vein at the junction to the superior vena cava and lodge in this site (Figure 1). The distal tip was at the right atrium.

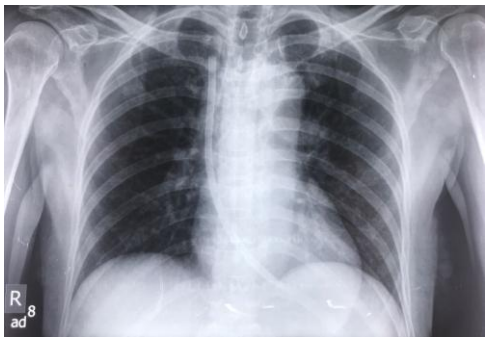

Figure 1: Chest radiograph before surgery

The patient then was planned for retrieval of the catheter. The patient and her husband, as well as her daughter, were discussed, and a small open trans-jugular retrieval technique via the right internal jugular vein was chosen. Informed consent was obtained from her daughter as well as verbal consent from her husband (due to distance and location of the husband).

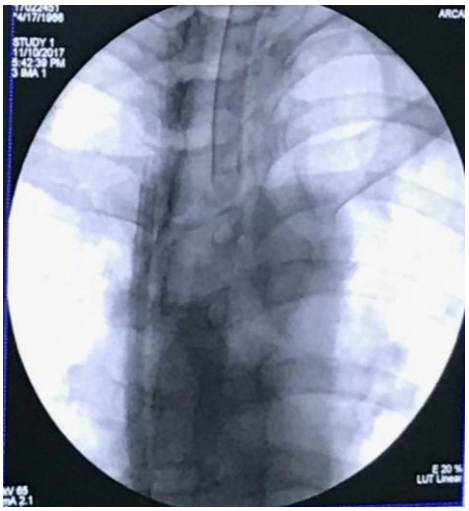

Figure 2: Radiology image shows the location of a fractured fragment of double lumen catheter in the clavicular level of the right internal jugular vein

The procedure was performed under general anaesthesia and C-Arm guidance. Intravenous antibiotic (cefazolin 2 gram) was administered during the procedure and first 24 hours after surgery. About 7 cm Golf stick incision was made at the right Sedillot's triangle. The incision was done until the internal jugular vein was exposed. Purse string was made around prior incision with 4-0 non-absorbable monofilament suture. Under C-arm guidance (Figure 2), a mixter right angle clamp was inserted to retrieve the distal tip of dislodging double lumen catheter. The fractured fragment of the catheter can be retrieved smoothly (Figure 3). The purse sting then tightened. The extravasation from vein was evaluated. The total procedure time was 35 minutes and C-arm time was 5 minutes.

After the procedure, the patient was monitored, electrocardiogram and examination were normal. There were not any major or minor complications such as arrhythmia, bleeding, vascular or cardiac perforation or local hematoma were observed during or after the procedure.

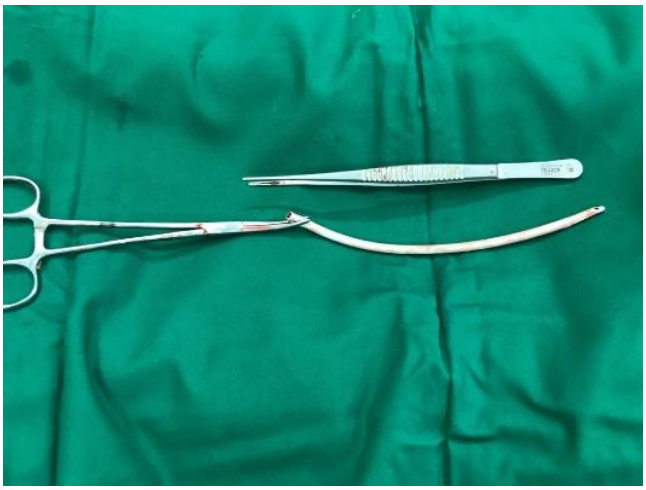

Figure 3: Foreign body of a fractured fragment of double lumen catheter after retrieval

Discussion

We report a case of a patient with a fractured fragment of double lumen catheter for hemodialysis in the right internal jugular vein, which was successfully retrieved using open jugular approach retrieval technique. The main complication of any vein catheter insertion was bloodstream infection and venous thrombosis, and mechanical (such as arterial puncture, nerve injury, air embolism, and hemothorax or pneumothorax between 5% and 19%) [3], [4]. Fractured fragment of the catheter with or without embolisation was a serious but rare complication in a patient [3], [4], [5]. There a few cases of cardiac

tamponade following a fractured fragment of a catheter. Although it was seldom occurred, a fractured intravascular catheter might cause thrombosis in the right atrium [2], [6]. The complication also included cardiac perforation [7].

Improper procedure handling was the most common cause of fractured fragment of a catheter [3]. The previous study suggested that the silicone catheter could be a risk factor for fractured and migration of the catheter [7]. Some mechanisms played a role in the pathogenesis of catheter embolism. According to the literature, the pinch-off syndrome was a frequent cause of catheter injury with an incident 1.1%. The port separation of a catheter from chamber or cuff or disconnection was another mechanism of catheter embolism with incident estimated to be in the range from 0.2 to 1.7% [5]. Incorrect locking of the catheter connection has also been suspected as a cause [5].

Significant complications had formerly reported include fractured of a catheter in the central vein, metal guide wires, pacemaker electrodes port-A fragment, and vascular stents. Retrieval of a fractured fragment of the catheter from the central vein as quickly as possible was needed because the complications might be potentially life-threatening. Complication such as sepsis, Pulmonary embolism, and abscess formation was reported [7], [8], [9]. If a fractured fragment of the catheter was free floating intravascular, a percutaneous retrieval technique is the method of choice. Percutaneous intravascular foreign body retrieval was a safe and effective method of retrieval embolized fragments from venous access devices even in children [7].

If the fractured fragment was remained at the point of skin entry or stay in the vein that can be accessed superficially, a surgical cut-down technique might be preferred as a first approach [6]. The decision of retrieval of the intravascular fractured fragment was often based on the shape, size, and location. The smooth-edged intracardiac foreign body can be treated non-operatively. Sometimes the retained shaped-edged foreign body can remain asymptomatic for a long time, and suggest complete fixation of the metallic foreign body to the cavity wall and further retrieval may not be needed [10], [11]. However, when an intravascular foreign body is identified, endovascular retrieval should be attempted due to its high success rate and minimal morbidity [1].

In our case, a chest radiograph revealed that the catheter fragment stays in the right internal jugular vein near the junction with superior vena cava. We decided to choose the venous cut-down technique. The catheter fragment was able to be retrieved easily under C-Arm guidance successfully without any complications.

In conclusion, an intravascular fractured fragment of double lumen catheter is a dangerous situation as are all intravascular foreign bodies. They should be managed at the earliest by knowing the exact site of the foreign body using chest radiograph. Intravascular catheter fragment with or without embolisation might cause fatal complication (though it was rare), and they should be retrieved in most instance. There were many methods to retrieve an intravascular fractured fragment of the catheter, including venous cut-down, percutaneous trans-catheter retrieval with loop snare, or even thoracotomy or sternotomy. The choices of the technique for retrieval of the fractured fragment are varied. It depends on the type and site of a fractured fragment as well as the surgeon experiences.

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ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2019 Jan 15; 7(1):127-133.
https://doi.org/10.3889/oamjms.2019.021
eISSN: 1857-9655
Dental Science

Three-Dimensional Prospective Evaluation of Piezocision-Assisted and Conventional Rapid Maxillary Expansion: A Controlled Clinical Trial

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Citation: Abdul-Aziz AI, Refai WM. Three-Dimensional Prospective Evaluation of Piezocision-Assisted and Conventional Rapid Maxillary Expansion: A Controlled Clinical Trial. Open Access Maced J Med Sci. 2019 Jan 15; 7(1):127-133. https://doi.org/10.3889/oamjms.2019.021

Keywords: Piezocision; Rapid maxillary expansion; Skeletal; Dental; CBCT; Controlled study

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Received: 21-Oct-2018; **Revised:** 29-Nov-2018; **Accepted:** 30-Nov-2018; **Online first:** 05-Jan-2019

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

Abstract

BACKGROUND: Piezocision-assisted orthodontics (PAO) is considered one of the modern techniques aiming at reducing the treatment time and overcoming some limitations of orthodontic treatment. The use of piezocision as an adjunct in the treatment of posterior crossbite is limited, so additional research in this area is required.

AIM: To three-dimensionally compare the skeletal and dental effects produced by piezocision-assisted rapid maxillary expansion (PARME) and conventional rapid maxillary expansion (RME) using cone beam computed tomography (CBCT).

MATERIALS AND METHODS: This prospective controlled study comprised 14 consecutive non-syndromic patients with posterior crossbite. In 7 patients (mean age = 16.1 ± 0.3 years), PARME was used to correct the crossbite; whereas in the remaining 7 (mean age = 15.9 ± 0.5 years), RME was done. Cone beam computed tomography (CBCT) scans were performed before expansion (T1) and 3 months later after expansion (T2) to compare the skeletal and dental effects produced by the two expansion techniques. Transverse skeletal, dentolinear, and dentoangular variables at the level of maxillary first and second premolars and maxillary first molars were measured and compared within and between groups using the appropriate statistical test.

RESULTS: For the transverse skeletal variables, PARME showed a non-significant increase; whereas, RME showed a significant increase. Regarding the dentolinear measurements, a significant increase in coronal widths and an insignificant increase in apical widths was seen in PARME, whereas, the RME showed a non-significant increase for both coronal and apical widths. Non-significant decreases (protrusion of teeth) in the dentoangular measurements were seen in both groups. Between-group comparisons showed a non-significant difference except for the dentolinear coronal widths.

CONCLUSION: PARME is effective in treating posterior crossbite. Because of the more dental expansion produced by PARME as compared to the conventional RME, PARME should be limited only to mild or moderate not severe forms of palatal constriction. The available evidence regarding the effectiveness of corticotomy- and/or piezocision-assisted maxillary expansion for correction of posterior crossbite is limited and inadequate.

Introduction

Maxillary transverse deficiency (MTD) is a type of malocclusion commonly seen in daily orthodontic practice. Narrow maxilla and palatal vault; crossbite, unilateral or bilateral; and dental crowding are clinical signs that could be the result of maxillary deficiency [1]. The aetiology of MTD can be due to both genetic and environmental factors: soft tissue influences, cleft palate, and habits [2].

MTD can be treated by expanding the maxilla through several approaches including slow maxillary expansion (SME), rapid maxillary expansion (RME), and surgically assisted rapid palatal expansion (SARPE); however, the choice of the suitable approach is dependent upon the amount of expansion needed [3], the skeletal age of the patient [4], and the presence of vertical or sagittal problem in addition to the transverse discrepancy [5].

Widening the dental arch by opening the midpalatal suture is the goal of RME. The concept of RME was given in 1860 by E. H. Angell, and because of the efforts done by Hass, RME has become a routine [6] [7], [8], [9]. The ideal age for expansion

with RME is before 13 to 15 years of age. The more the age increases, the more the midpalatal suture becomes tightly interdigitated; however, in most individuals, it is possible to obtain considerable amounts in maxillary width up to age 15 to 18 [10], [11].

The use of corticotomy in orthodontics was first reported in orthodontics by Köle in 1959 [12] and then followed by Converse and Horowitz in 1969 [13]. Also, maxillary expansion accompanied by corticotomy was reported by Lines in 1975 [14]. In 2001, the concept was reintroduced by Wilcko et al., and was named “accelerated osteogenic orthodontics” (AOO); also, it is called “periodontally accelerated osteogenic orthodontics” (PAOO) [15].

In 2006, Park introduced the corticision technique to eliminate the need for flaps using a blade and a surgical hammer to make incisions through the gingiva [16]. On the other hand, Vercellotti reported a technique using a piezosurgical micro-saw in 2007 [17]. In this technique, the elevation of a flap before the corticotomy was maintained, and only vestibular incisions were performed. To overcome the disadvantages and combine the advantages of the previous corticotomy techniques, the Piezocision was introduced [18].

According to a previous systematic review [19], no studies were published on the effects of corticotomy on the transverse expansion; also, only, few case reports [20], [21], [22] addressed the corticotomy- or Piezocision-assisted maxillary expansion (PAME). These case reports had some shortcomings including the absence of the control group and the small sample size.

Therefore, the aim of this controlled clinical trial was to three-dimensionally compare the skeletal and dental effects produced by piezocision-assisted rapid maxillary expansion (PARME) and conventional rapid maxillary expansion (RME) using cone beam computed tomography (CBCT).

Material and Methods

We designed this study as a two-group controlled study that included fourteen consecutive non-syndromic patients (8 males, 6 females) who were prospectively included at the Department of Orthodontics and Dentofacial Orthopedics of the Faculty of Dentistry, Minia University, Egypt. Sex and age distributions for the two groups are shown in Table 1. Inclusion criteria were a maxillary transverse deficiency (MTD) with posterior crossbite, no severe gingival inflammation or active periodontal disease and free from any systemic disease. Exclusion criteria were a history of any previous orthodontic or orthopaedic treatment, the presence of congenital or developmental deformity, or absence of more than four teeth in the posterior maxillary arch. The study protocol was approved by the Ethical Committee of the Faculty of Dentistry, Minia University, Minia, Egypt. All patients and/or parents consented to the treatment procedures.

Table 1: Sex and age distribution of the groups

	PARME (n = 7)	RME (n = 7)	P value
Gender			
Male	3 (43%)	5 (71%)	0.592
Female	4 (57%)	2 (29%)	
Age (years)			
Range	15.3 – 16.9	15.8 – 16.6	
Mean ± SD	16.1 ± 0.3	15.9 ± 0.5	0.329

SD = standard deviation.

The piezocision surgery was performed by the first author (A.I.) under local anaesthesia without any flaps or sutures. Gingival vertical incisions were made interproximally only on the buccal aspect of the alveolar bone distal to canines, first premolars, second premolars, and first molars below the interdental papilla and kept as much as possible in the attached gingiva using a number 15 blade. These incisions must cross the periosteum allowing the blade to come into contact with the alveolar bone. Ultrasonic instrumentation (US2 piezoelectric tip, ULTRASURGERY, Woodpecker, Guilin, Guangxi, China) was then used to perform corticotomy cuts through the gingival micro-incisions to a depth of 3 mm (Figure 1). Antibiotics, nonsteroidal anti-inflammatory drugs and mouthwashes containing chlorhexidine were prescribed for the patients after the surgery. The patients were instructed to avoid the surgical sites while brushing during the first postoperative week to allow harmonious gingival healing.

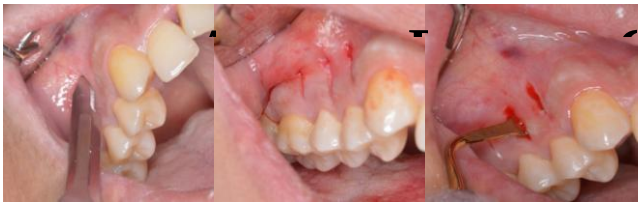

Figure 1: Piezocision surgery; A) scalpel blade No. 15 before making the vertical gingival incisions; B) after making the vertical gingival incisions; C) US2 piezoelectric tip inserted 3 mm into bone

Immediately after the surgery, a bonded tooth-borne hyrax (Leone, Sesto Fiorentino, Firenze, Italy) was cemented, and the activation of the appliance was started the day after the surgery (Figure 2). The expander was activated at a rate of 0.5 mm/day (two quarter turns daily) for eleven days to achieve an expansion of 5.5 mm to standardise the amount of expansion. The patients were reassured about the appearance of the midline diastema with the expansion. The patients were observed after two days from the beginning of the hyrax activation then every 3 days until completion of the expansion to assess the following: subject’s compliance in keeping proper oral

hygiene, compliance in activation of the expander, and observation of proper seating and sealing of the appliance. Once the activation of the appliance was completed, the Hyrax was left to act as a retainer for three months. After completion of the activation, the patients were seen after one week and then every month to ensure proper plaque control and reinforce necessary hygiene techniques. The 5.5 mm expansion was enough to achieve over correction of the posterior crossbite in all patients.

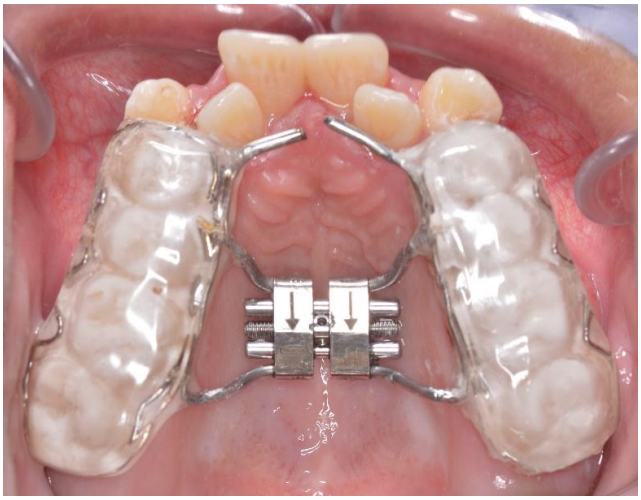

Figure 2: Cemented bonded Hyrax appliance before activation

As with PARME, the same protocol of Hyrax activation was followed in RME (0.5 mm/day for eleven days to achieve 5.5 mm expansion), but without performing the piezocision surgery. After activation, the expander was retained in place for three months before the CBCT scans can be taken. The 5.5 mm of expansion was sufficient to achieve overcorrection of the posterior crossbite in all patients.

The CBCT scans were taken before the expansion (T1) and after expansion (T2) (three months from completion of expansion) with the expander removed. Both before and after CBCT images were taken with a Scanora 3D machine (Soredex, Tuusula, Finland).

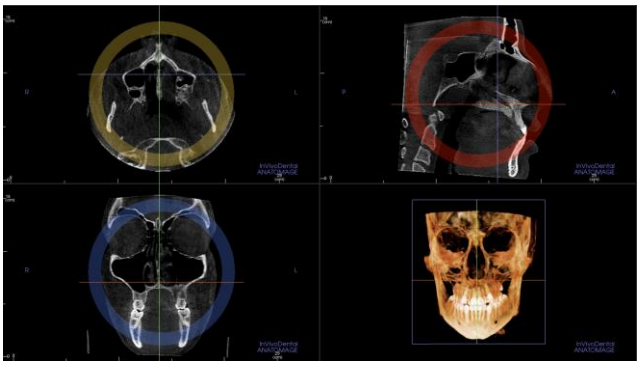

Figure 3: Reorientation of the patient position in all 3 axes (Axial, sagittal and coronal)

Intra-oral splint with a thickness of 2 mm fabricated for each patient was worn before the CBCT scan to allow for measurements in the axial plane. Image reconstruction was performed with Anatomage software (Invivo version 5.2; Anatomage Dental, San Jose, Calif) to obtain the 3D data.

Before 3D analysis, reorientation of the patient views was performed in all 3 axes (axial, coronal, and sagittal) (Figure 3). Landmark identification for all measurements was made by the first author (A.I.). With the help of the “slice locator” which allowed each point to be seen in the three planes, fine adjustment of the position of the points was performed (Figure 4). Once the points were digitised, the measurements were recorded by the software and then compared to assess intra-observer agreement.

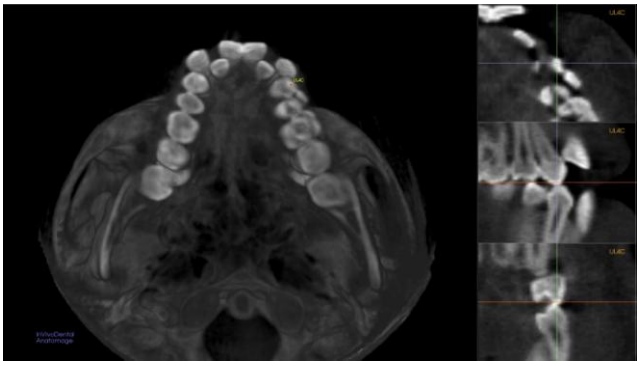

Figure 4: Fine adjustment of the position of the point on the slice locator in the axial, coronal and sagittal views

The 3D analysis was obtained by identifying the 3D reference landmarks, lines, and planes to evaluate transverse skeletal (Figure 5A), dentolinear (Figure 5B), and dentoangular measurements in relation to the maxillary plane (Figure 5C).

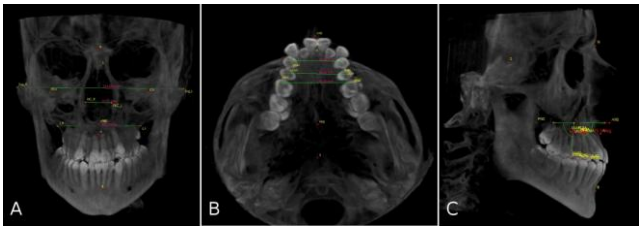

Figure 5: A) Skeletal transverse measurements: facial width (Zyg_R - Zyg_L), nasal width (NC_R - NC_L), and Maxillary width (J_R - J_L); B) Dentolinear measurements: coronal first premolar (UR4-UL4 coronal), coronal second premolar (UR5-UL5 coronal), coronal molar (UR6-UL6 coronal), apical first premolar (UR4-UL4 apical), apical second premolar (UR5-UL5 apical), and apical molar widths (UR6-UL6 apical); C) Dentoangular measurements: external buccopalatal inclination angle of the maxillary right and left first premolars (UR4 BP incl, UL4 BP incl), the maxillary right and left second premolars (UR5 BP incl, UL5 BP incl), and the maxillary right and left first permanent molars (UR6 BP incl, UL6 BP incl) in relation to the maxillary plane. The arrow indicates the external buccopalatal inclination angle of the upper right first premolar

Statistical analysis was performed using SPSS for Windows, version 22 (SPSS Inc., Chicago, Illinois, USA). Shapiro-Wilk test was used to

determine whether the variables were normally distributed or not, while Levene's test was used to assess the homogeneity of variances. Data were shown as mean ± standard deviation (SD). While the differences between pre- and post-treatment measurements for normally distributed parameters were analysed by the paired-samples t-test, Wilcoxon signed-rank test was used for not normally distributed parameters (U5 apical width, UR4 inclination, and UL5 inclination). The mean differences between the two groups for normally distributed parameters were compared by the independent t-test, and Mann–Whitney test was used for not normally distributed data. A *P* value less than 0.05 was considered statistically significant. To calculate the error of measurements, the measurements were repeated 2 weeks later by the same clinician. Cronbach's alpha was calculated for evaluation of intra-observer reliability.

Results

No difference was observed between the two groups regarding the baseline characteristics (age and gender) (Table 1). No dropouts were reported in the two groups. Loosening of the appliance occurred in two patients in the RME group and one patient in the PARME group.

Table 2: Descriptive statistics and dentoskeletal changes for PARME and RME

	PARME			RME		
	T1 Mean ± SD	T2 Mean ± SD	*P* value	T1 Mean ± SD	T2 Mean ± SD	P value
Skeletal linear measurements (mm)						
Facial width	116.72 ± 4.75	117.91 ± 5.44	0.107	114.18 ± 1.36	115.84 ± 1.44	0.001*
Nasal width	22.56 ± 2.86	23.70 ± 2.90	0.127	23.36 ± 3.31	25.54 ± 3.60	0.032*
Maxillary width	60.48 ± 1.08	63.15 ± 2.34	0.173	60.96 ± 8.57	64.77 ± 8.01	0.018*
Dentolinear measurements (mm)						
UR6-UL6 coronal	45.46 ± 5.24	51.41 ± 5.49	0.002*	46.17 ± 4.06	47.68 ± 3.71	0.159
UR6-UL6 apical	45.21 ± 6.97	47.15 ± 7.54	0.058	45.25 ± 3.25	45.93 ± 2.74	0.377
UR5-UL5 coronal	41.26 ± 4.41	46.92 ± 4.23	0.012*	40.99 ± 4.53	42.76 ± 4.14	0.096
UR5-UL5 apical	36.19 ± 5.88	38.57 ± 6.88	0.158	38.17 ± 5.45	36.58 ± 2.31	1.000
UR4-UL4 coronal	38.01 ± 4.45	43.69 ± 4.16	0.009*	36.93 ± 3.11	38.71 ± 3.84	0.096
UR4-UL4 apical	35.39 ± 4.49	37.60 ± 5.51	0.075	33.75 ± 3.69	33.43 ± 6.38	0.860
Dentoangular measurements (°)						
UR6 BP incl	81.07 ± 3.76	76.06 ± 7.28	0.135	80.96 ± 6.40	79.64 ± 5.57	0.321
UL6 BP incl	82.03 ± 2.42	78.66 ± 2.75	0.023*	81.72 ± 1.60	80.97 ± 0.38	0.497
UR5 BP incl	83.88 ± 3.26	78.96 ± 7.31	0.174	78.73 ± 3.96	77.96 ± 4.48	0.158
UL5 BP incl	78.38 ± 1.26	73.91 ± 3.26	0.061	78.89 ± 4.14	76.92 ± 3.42	0.109
UR4 BP incl	78.49 ± 3.66	73.00 ± 2.03	0.039*	78.26 ± 2.91	74.61 ± 2.04	0.067
UL4 BP incl	83.65 ± 3.68	79.58 ± 2.93	0.353	81.28 ± 6.01	79.45 ± 4.10	0.242

U = Maxillary; R = right; L = left; 6 = first molar; 5 = second premolar; 4 = first premolar; BP = buccopalatal; incl = inclination; *Significant at *P* < 0.05.

We started the expansion again after returning the loosened Hyrax to the pre-activation state and after performing a second piezocision surgery in PARME group to assure the effect of the regional acceleratory phenomenon (RAP). Cronbach's alphas were found to be between 0.765 and 0.987; thus, there was a good to very good intra-observer agreement regarding all measurements.

For the skeletal linear measurements, PARME had non-significant increases, but RME demonstrated statistically significant increases for all transverse skeletal variables (Table 2). In comparison, no statistically significant differences were found (Table 3).

Regarding the dentolinear measurements, the intercoronal widths increased significantly in PARME, while the interapical widths increased insignificantly. In RME, non-significant changes were detected for all dentolinear widths (Table 2). Comparing the two groups, PARME had a greater significant increase for the intercoronal widths and a greater non-significant increase for the interapical widths than RME (Table 3).

Table 3: Comparison of skeletal and dental mean changes between the PARME and RME groups

	PARME		RME		
	Mean	SD	Mean	SD	P value
Skeletal linear measurements (mm)					
Facial width	1.19	0.73	1.65	0.09	0.388
Nasal width	1.14	0.78	2.18	0.69	0.156
Maxillary width	2.67	2.22	3.81	0.91	0.459
Dentolinear measurements (mm)					
UR6-UL6 coronal	5.95	0.50	1.51	1.19	0.004*
UR6-UL6 apical	1.95	0.85	0.68	1.04	0.177
UR5-UL5 coronal	5.67	1.07	1.77	1.03	0.011*
UR5-UL5 apical	2.38	1.87	-1.59	4.32	0.200
UR4-UL4 coronal	5.69	0.96	1.78	1.03	0.009*
UR4-UL4 apical	2.21	1.12	-0.31	2.71	0.209
Dentoangular measurements (°)					
UR6 BP incl	-5.02	3.57	-1.32	1.75	0.182
UL6 BP incl	-3.37	0.90	-0.75	1.58	0.067
UR5 BP incl	-4.91	4.11	-0.77	0.61	0.159
UL5 BP incl	-4.46	2.01	-1.97	3.05	0.400
UR4 BP incl	-5.49	1.94	-3.65	1.73	0.400
UL4 BP incl	-4.07	5.88	-1.83	1.93	0.564

U = Maxillary; R = right; L = left; 6 = first molar; 5 = second premolar; 4 = first premolar; BP = buccopalatal; incl = inclination; *Significant at *P* < 0.05.

Concerning the dentoangular measurements, no statistically significant decrease was seen for the external buccopalatal inclination angle of all teeth in both groups except for the upper left maxillary first molar and upper right first premolar in PARME which showed a significant decrease (Table 2). After comparing the two groups, no statistical significance was detected (Table 3).

Discussion

To the best of our knowledge, this is the first prospective controlled clinical study to address the effects of piezocision on rapid maxillary expansion. All previous studies on this topic were case reports [20], [21], [22].

Although one of these articles [21] reported that the expansion done was rapid, the activation was performed to achieve 1 mm of expansion per week which is considered slow expansion. In addition to the rate of expansion, this report was different from our study in that a fixed appliance was worn during the expansion, banded not bonded expander was used,

and buccal flaps were done to perform the corticotomy on the buccal aspect.

The other two case reports [22] described the correction of a unilateral cross bite by corticotomy-assisted rapid maxillary expansion. One report [20] used fixed orthodontic appliance and quad-helix with the expansion assisted with piezocision on the buccal side, while in the other report, [22] fixed orthodontic appliance with heavy labial arch wire in one case and quad-helix in the second case were used to achieve the expansion which was assisted with corticotomies performed on the buccal and palatal sides after reflection of flaps. Therefore, all previous case reports used slow, not rapid maxillary expansion.

Because piezocision-assisted expansion requires periodontal surgery, it is considered an invasive procedure when compared to the conventional expansion. On the other hand, when piezocision is compared with conventional corticotomy, it is believed to produce less patient discomfort and trauma with the same clinical outcome [18], [23].

With the piezocision surgery, bone remodelling increases at the surgical site which is proportional to the surgical trauma. The activity of osteoblasts and osteoclasts increases which results in a decrease in bone density and an increase in bone turnover. This process facilitates the tooth movement and is called “regional acceleratory phenomenon” (RAP) [24]. The duration of the RAP can last for about four months based on the results two studies, one in humans [25] and the other in dogs [26].

Although the four-months duration is sufficient to perform the expansion in our study, we were very careful to gain the full advantage of the RAP. Therefore, the expansion was started immediately on the day following the surgery.

It can be difficult for the patients to brush around the teeth and the Hyrax; also, the gingival index can change because of the periodontal incisions of the piezocision. Because of this, plaque accumulation can increase around the expander with an increased tendency to develop periodontal problems. Therefore, the patients were informed and educated about oral hygiene.

To standardise the amount of expansion in the two groups, the Hyrax was activated by opening it twice daily (0.5 mm/day) for eleven days to achieve 5.5 mm expansion which is enough to achieve over correction in all patients.

During taking the CBCT scans, the patients were instructed to wear 2 mm thickness intra-oral splint to separate the two jaws and allow for measurements to be made in the axial plane.

In the present study, the period between the two CBCT scans was 3 months which is not enough for the growth changes to be combined with treatment effects; therefore, the growth was not a confounding factor.

For the skeletal linear (transverse) changes, RME showed significant increases in the mean facial, nasal, and maxillary widths. On the other hand, PARME showed a non-significant increase with no significant difference between the two groups. These changes might be attributed to lateral rotation of the two maxillary halves around the estimated centre of rotation located in the area of the frontonasal suture and the rear mid-palatal suture as a result of the lateral displacement and the stress distribution that occurred along the sutures of the circummaxillary structures [27], [28]. Also, these results may support the theory that maxillary expansion increases the airflow and improves nasal breathing [6], [29].

Regarding the facial width, our results were in agreement with Perillo et al., 2014 [30] for PARME and in disagreement for RME. Concerning the nasal and maxillary widths, Chung and Font, 2004 [31]; Gungor et al., 2012 [32]; Gopalakrishnan and Sridhar, 2017 [33]; Baratieri et al., 2014 [34]; Altug, Karasu, and Aytac, 2006 [35]; Perillo et al., 2014 [30]; Corekci and Goyenc., 2013 [36]; and Cordasco et al., 2012 [37] showed results consistent to ours for RME and inconsistent for PARME.

Regarding the dental transverse changes, PARME showed a significant increase in the coronal widths, and RME showed a non-significant increase for all teeth. By comparing the two groups, there was a significant difference. These results indicated that PARME produced more dental expansion than did RME.

The increased dental expansion in PARME as compared to RME conforms to the biological mechanism of RAP, which is characterised by transient bone demineralisation and increased bone metabolism.

The results of Baratieri et al., 2014 [34]; Perillo et al., 2014 [30]; Grassia et al., 2015 [38]; Corekci and Goyenc, 2013 [36]; Weissheimer et al., 2011 [39]; and Gunyuz, Germec-Cakan, and Tozlu, 2015 [40] agreed with our results for PARME but disagreed for RME.

On the other hand, our study showed a non-significant increase in the apical widths of studied teeth in the two groups. Also, there was no significant change between the two groups.

The insignificant increase in the apical width of most teeth found in our study was in disagreement with the significant increase found in Weissheimer et al., 2011 [39] and Gunyuz, Germec-Cakan, and Tozlu, 2015 [40].

The increase in the coronal widths was more than the increase in the apical widths in the two groups. These results demonstrated the controlled tipping of those teeth and the pyramidal nature of the

expansion with the base of the pyramid located at the oral side of the bone [41].

Concerning the buccolingual inclination of the expanded teeth, the two groups showed a statistically non-significant decrease (of the external angle) of the expanded teeth. This decrease could be attributed to buccal tipping of the expanded teeth, bending of the alveolar bone [42] and outward rotational movement of the two maxillary halves [10].

The insignificant increase in Bucco-palatal inclination found in this study agreed with that of Gunyuz, Germec-Cakan, and Tozlu, 2015 [40], but disagreed with the significant increase reported in Baratieri et al., 2014 [34]; Weissheimer et al., 2011 [39]; Kilic, Kiki, and Oktay, 2008 [43]; and Christie, Boucher, and Chung, 2010 [44].

In conclusion, PARME is effective in treating posterior crossbite. PARME produced significantly more coronal dental expansion than did RME. Non-significant difference was found for the skeletal transverse, dentolinear apical, and dentoangular variables between the two groups. Because of the more dental expansion produced by PARME as compared to the conventional RME, PARME should be limited only to mild or moderate not severe forms of palatal constriction. The available evidence regarding the effectiveness of corticotomy- and/or piezocision-assisted maxillary expansion for correction of posterior crossbite is limited and inadequate.

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ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2019 Jan 15; 7(1):134-138.
https://doi.org/10.3889/oamjms.2019.013
eISSN: 1857-9655
Dental Science

Analysis of Calcium Levels in Groundwater and Dental Caries in the Coastal Population of an Archipelago Country

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Citation: Yani RWE, Palupi R, Bramantoro T, Setijanto D. Analysis of Calcium Levels in Groundwater and Dental Caries in the Coastal Population of an Archipelago Country. Open Access Maced J Med Sci. 2019 Jan 15; 7(1):134-138. https://doi.org/10.3889/oamjms.2019.013

Keywords: Groundwater; Calcium; Dental caries

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Received: 13-Sep-2018; **Revised:** 06-Nov-2018; **Accepted:** 07-Nov-2018; **Online first:** 14-Jan-2019

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

Abstract

BACKGROUND: The coastal region is the largest region in Indonesia as a country of the archipelago. Characteristics of groundwater content in coastal areas are very influential on dental health, especially dental caries. The main elements contained in 1-1000 mg/litre groundwater are calcium, magnesium, sodium, potassium, chlorine, bicarbonate, and sulfate groups. Calcium is an essential ingredient for living organisms that play a role in the formation of bone and tooth along with permeability of cell walls.

AIM: This study aimed to analyse the relationship between calcium in groundwater with dental caries.

METHOD: Analytical observational study with cross-sectional approach was implemented was coastal communities in Watu Ulo Jember Regency in February 2018 (3,686 inhabitants), with sample criteria of the minimum age of 12 years and consumed groundwater as drinking water at least 2 years by purposive side. The variables in this study were calcium levels as the independent variable and dental caries as the dependent variable. Calcium was measured using the spectrophotometric method. Caries measurements were performed using the DMF-T index. Data were presented descriptively in the table and analysed by Spearman Correlation test to analyze the relationship between groundwater calcium with dental caries.

RESULTS: Average groundwater calcium content was 126.75 mg/litre (high category), and average dental caries was 2.2 (low category). Spearman correlation analysis showed $p = 0.029$ ($p < 0.000$), which means there was a correlation between groundwater calcium level with dental caries.

CONCLUSION: There is a positive relationship between the calcium content of groundwater with dental caries.

Introduction

Indonesia as an archipelago country extended geographically in such a way that major part of it is coastally located. About 60% of Indonesia's population is in 50 km range from the coastline, and more than 42 cities and 181 districts are in coastal areas [1]. Watu Ulo Jember Regency, East Java can represent the condition of coastal areas in Indonesia. Coastal areas have special characteristics that occur due to the interaction between processes found on land and in the oceans. These characteristics can have an effect on dental caries in the population living in the area, one of which is the groundwater content in coastal areas.

Data of Susenas (2017) stated that the majority of drinking water sources of Indonesian society is obtained from groundwater, bottled water and protected wells. Quantity, continuity, and affordability are the reasons for the use of groundwater as drinking water for the majority of Indonesians [2]. Groundwater is formed in the recharge area and flows into its surrounding area through the space between the constituent rocks. Groundwater quality from one place to another varies, depending on the type of rock, and where the groundwater location is pervasive, flowing, accumulating, as well as environmental conditions. Groundwater quality is determined by physical

properties, chemical content, and bacteriology [3].

The main elements that dissolved ions in water and contained in 1-1000 mg/litre groundwater are calcium, magnesium, sodium, potassium, chlorine, bicarbonate and sulfate groups. Calcium levels in freshwater are usually less than 15 mg/litre, in waters around the carbonate rocks are between 30-100 mg/litre and in ocean waters are around 400 mg/litre [4].

Calcium is an essential ingredient for living organisms that play a role in bone and teeth formation, along with permeability of cell walls [5]. Calcium is an important constituent of enamel and dentine structures in teeth bound in apatite crystals to form calcium hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) [6]. Calcium hydroxyapatite is the most stable phase of calcium phosphate compounds in physiological pH, temperature, and liquid compared to other phases [7]. Ca ion in calcium hydroxyapatite can be dissolved and displaced so that the process of demineralisation leading to dental caries. Dental caries is a disease of hard tooth tissue characterised by the destruction of enamel and dentine caused by the activity of bacterial metabolism in plaque, resulting in changes in enamel and dentine. Based on the Basic Health Research in 2013, the prevalence of dental caries in Indonesia in 2013 reached 53.2% [8], according to another research conducted in 2014 in coastal population, the prevalence of dental caries is 46.11%, which lower than the nationwide score [9].

To compensate for the demineralisation process in caries, it is necessary to have a calcium ion intake freely to assist the remineralisation process [4]. Based on the description, this study aimed to analyse the relationship between groundwater calcium content with dental caries.

Method

This was an observational analytic study with the cross-sectional method. The research approach was implemented coastal communities in Watu Ulo Jember Regency in February 2018 (3,686 inhabitants), with sample criteria of the minimum age of 12 years and consumed groundwater as drinking water at least 2 years by purposive side with the assumption that calcium in water had been absorbed by the body. Sampling was done by purposive sampling [10]. The variables in this study were calcium levels as the independent variable and dental caries as the dependent variable. Method of taking calcium in this study was done by taking groundwater from the well with a bucket then inserting the empty bottle into a bucket and opening bottle cap, filling water until full then closing and labelling bottle. The bottle was covered with plastic and put in a cool box and sent to Perum Jasa Tirta I laboratory in Malang, East Java to test the level of calcium by spectrophotometric method [11]. The caries data retrieval method was done with Decay, Missing, Filling-Teeth (DMF-T) index. The caries examination was done by drying the tooth surface, examined by the probe and dental mouth mirror. Caries cannot be filled but excavated; the tooth cavity can be filled with the restoring material. (decay), extracted teeth or indication of retraction due to caries (missing), and tooth filling because caries (filling). Then DMF-T component was summed up [12]. The results were presented in the form of frequency distribution tables and continued by Spearman correlation test to analyse the relationship of groundwater calcium content with dental caries. This research had been approved by the Health Ethics Committee of Faculty of Dentistry, Universitas Jember (No. 087/UN25.8/KEPK/DL/2018) on February 8, 2018.

Results

The results regarding the sex in this study can be seen in Table 1.

Table 1: Data of Respondents Characteristics by sex

Sex	Total	Percentage
Male	11	36.7
Female	19	63.3
Total	30	100

Table 1 63% of the respondents are female. The results regarding the age can be seen in Table 2.

Table 2: Data of Respondents Characteristics by age

Age (Depkes, 2009)	Total	Percentage
Early teenage (12-16 y.o.)	4	13.3
Late teenage (17-25 y.o.)	5	16.7
Early adulthood (26-35 y.o.)	6	20
Late adulthood (36-45 y.o.)	8	26.7
Early elderly (46-55 y.o.)	2	6.7
Late elderly (56-65 y.o.)	5	16.6
Total	30	100

Table 2 shows that most of the final adult respondents ranged from 36-45 years old (26.7%), The minority aged 46-55 years (6.7%). The results regarding the occupation can be seen in Table 3.

Table 3: Data of Respondents Characteristics by type of occupation

Occupations	Total	Percentage
Student	7	23.3
Housewife	8	26.7
Fisherman	9	30
Merchant	5	16.7
Civil servant	1	3.3
Total	30	100

Table 3 shows that most respondents work as fishermen (30%), but 3.3% works as civil servants. The results regarding the average calcium level of

groundwater and caries can be seen in Table 4.

Table 4: Mean Calcium Levels (mg/litre) and Incidence of Dental Caries

Variables	N	Mean
Calcium	30	126.75
Caries	30	2.2

Table 4 shows the average groundwater calcium content of 126.75 mg/litre (high category) and the DMF-T index average of 2.2 (low category). The results regarding the relationship of the calcium content of groundwater with dental caries can be seen in Table 5.

Table 5: Spearman correlation test results with variable levels of calcium and dental caries

Variable	Sig	Note
Calcium-Caries	0.029	There is a correlation

Table 5 shows a correlation of groundwater calcium content with dental caries with a significance value of 0.029 ($p < 0.05$).

Discussion

The Majority of respondents are female on the age between 36-45 years, working as a fisherman. Based on the results of a study that had been done, the average calcium water level was 126.75 mg/litre in which was categorised as high. There are four category regarding the calcium content (concentration) in water, ie low (< 60 mg/liter), medium (60 mg/liter-119 mg/liter), high (120 mg/liter-179 mg/liter), and very high (> 180 mg/liter) [13]. Several studies have shown a distinctive relationship of calcium, sodium, and fluoride content in a water source — the higher the fluorine level, the lower the calcium level. This might be caused by the exchange of Na with Ca in the underground water circulation [14]. Seawater is naturally water with a salt content of about 3.5%. Seawater contains salts, such as calcium` magnesium elements, belonging to elements of Group II A (alkaline earth), dissolved gases, organic materials and unsolved particles. The presence of salts affects the physical properties of groundwater in coastal areas [15]. Well water in the coastal area, based on geographical studies, undergoes an intrusion process from seawater so that the original freshwater soil turns into hard water. The intractable hard water of these seawater contains high calcium [16]. According to WHO standards, the average calcium content in drinking water is 75 mg/L, and the maximum allowed limit is 200 mg/L. Water with calcium levels the maximum limit can cause digestive problems, kidney problems, bladder stones, and urinary tract obstruction in humans [17]. Human activities can also affect water quality in coastal areas, especially in tourist-dense areas that still use groundwater as the main source of water [18]. Watu Ulo is also located between the sea and rice fields that utilise groundwater for irrigation purpose so that it can affect the hardness of groundwater in the region.

The average DMF-T score of 2.2 indicates the low category.According to the DMF-T index there are five categories of, very low (0.0-1.0), low (1.2-2.6), moderate (2.7-4.4), high (4.5-6.5), and very high (> 6.6) [19], [20]. Caries occurs because of four interplaying factors: diet, time, microorganism, and host. Caries occurs when all four factors contribute to the demineralisation process [21]. Teeth demineralisation may occur if saliva has an acidic pH level where enamel dissolves by acid resulting in partial loss of ions in the enamel by the following reaction [22], [23].

$$Ca_{10}(PO4)_60H_2+8H^+ \rightarrow 10Ca^{2+}+6HPO_4^{2-}+2H_2O$$

The DMF-T index of coastal communities in Watu Ulo, Sumberejo Village, Ambulu Sub-district, Jember Regency is low possibly because of the community behaviour in maintaining good oral hygiene as they consume a non-cariogenic nutritious diet.

Also, the average community in Watu Ulo, Sumberejo Village consumed groundwater with high calcium. Calcium plays a role in preventing the demineralisation process to decrease caries.

Spearman correlation results showed that there was a relationship of the calcium content of groundwater with dental caries. This occurs because calcium levels in groundwater are known to decrease dental caries which can be measured using the DMF-T index. Dental caries is inversely related to the concentration of calcium and fluoride contained in drinking water. This is supported by a study of Arvin et al., in 2018, stating that decreasing the calcium concentration from 120 mg/l to 33 mg/l can increase caries by 46% [5].

Another effect of groundwater calcium on caries suggests a positive relationship between calcium ions and fluoride levels in plaque. Calcium diffuses into the plaque and provides a bond for floride. The free ionic calcium ion in drinking water and fluoride causes the process of precipitation and dissolution of the fluoride ion into the biofilm fluid on the tooth surface to prevent the caries process [5]. Fluoride acts by inhibiting plaque bacterial metabolism that can ferment carbohydrates through alteration of apatite hydroxy in enamel to apatite fluorine by chemical reactions:

$$Ca_{10}(PO4)_60H_2 + F^- \rightarrow Ca_{10}(PO4)_6(OHF)$$

Apatite fluorine may cause the enamel to be more acid-resistant, thus inhibiting demineralisation and discontinuation of carious lesions [22]. WHO recommends that the ideal drinking water should contain at least 0.5-1.0 mg/L of fluoride [26].The

lowest fluoride concentrations in drinking water of 0.5 to 1 mg/litre effectively reducing the prevalence of caries and the concentration of 100 mg/litre of calcium has the same protective effect as 0.64 mg/litre fluoride [5]. Fluoride level of < 0.5 mg/L resulted in dental caries, 0.5 to 1 mg/L was the most acceptable body-safe level, while 1-3 mg/L resulting in dental fluorosis, 3-4 mg/L causing fragile bone, and levels > 4 mg/L cause knee deformities and even paralysis [26]. The optimum F concentration for dental health is generally between 0.5 and 1.0 mg/L [27].

Saliva is a major source of minerals that rebuilds demineralised enamel. The hydroxyapatite (HA) crystals in tooth enamel, consisting of Ca, Mg, and Pi (Phosphate) are more susceptible to dissolution by acids. If the pH is more than 4.5, the lost HA is immediately replaced by fluorapatite by the F ions available in oral biofilms which is more resistant to acid dissolution. This process leads to a decrease in demineralisation and is not considered as remineralisation since it is replaced by different minerals. In addition to the reduction of enamel demineralisation, F also increases remineralisation when pH rises. Ca assist this process by providing F with a place to bind with. Minerals in drinking water are the main source of Ca and Mg which are absorbed by the body. These elements play an important role in the body's physiological functions. Fluor has anticariogenic effects. The optimum Ca and Mg level in drinking water that is beneficial for health ranges from 40-80 mg/L for Ca and 20-30 mg/L for Mg. Ca concentration in saliva to helps repair early carious lesions ideally should not exceed 90 mg/L. Ca and F together caused 45% decrease in DMF-S index, supporting the role of Ca in teeth remineralisation [27].

The average calcium content in the sample was 126.75 mg/litre equivalent to 0.81 mg/litre fluoride, which showed an effective level to decrease the prevalence of caries. Calcium obtained from the diet will be absorbed by the body and affects serum calcium levels and also in saliva secreted by the salivary gland [28]. The total concentration of calcium in drinking water is 2 mmol/litre at 25°C, whereas, the whole human saliva contains about 1 mmol/litre of calcium. Human saliva also contains high concentrations of bicarbonate as protein and phosphorus, both of which will bind to calcium saliva. This causes free calcium ions in the saliva to be reduced to half of the total saliva calcium concentration so that the process of remineralisation is less than optimal. Free calcium ions will be more effective in enhancing the remineralisation process when binding to fluoride ions to form calcium fluoride (CaF_2). From both of these things, a higher intake of free calcium is required than the calcium in saliva [4], [5]. During the demineralization process, calcium is released from the enamel, dentine, and cementum before phosphate release. Therefore the use of calcium to inhibit the demineralisation process is considered more effective than the use of phosphate [29].

However, it is important to remember that individuals with high levels of calcium and phosphate in saliva are known to have higher resistance to caries, but are more susceptible to periodontal disease due to faster calculus buildup [30]. Therefore it is recommended to perform effective dental cleansing to prevent rapid formation of calculus as a result of high calcium intake.

Based on the research results, it can be concluded that there was a relationship of calcium levels in groundwater with dental caries. The higher Ca levels in groundwater the lower dental caries.

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ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2019 Jan 15; 7(1):139-142.
https://doi.org/10.3889/oamjms.2019.033
eISSN: 1857-9655
Dental Science

Knowledge and Interest in Treating Gingival Recession among Dental Practitioners in Saudi Arabia

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Citation: Bhat M, AlQahtani N, Khader M, Javali M, AlQahtani A. Knowledge and Interest in Treating Gingival Recession among Dental Practitioners in Saudi Arabia. Open Access Maced J Med Sci. 2019 Jan 15; 7(1):139-142. https://doi.org/10.3889/oamjms.2019.033

Keywords: Gingival recession; Dental practitioners; Periodontics; Knowledge; Interest; Miller's classification

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Received: 04-Nov-2018; **Revised:** 26-Nov-2018; **Accepted:** 30-Nov-2018; **Online first:** 14-Jan-2019

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

Abstract

BACKGROUND: Gingival recession is an enigma among clinicians due to multiple etiological factors and various treatment modalities.

AIM: Objective of this study was to evaluate the knowledge and interest among dental practitioners regarding the treatment of gingival recession.

MATERIAL AND METHODS: A survey was conducted to assess knowledge of gingival recession and interest and satisfaction of dental practitioners in periodontics. This survey was circulated among 250 dental practitioners throughout four months. The structured questionnaire consisted of 9 questions assessing the knowledge and interest of dental practitioners in periodontics; gingival recession per se.

RESULTS: Majority of the participants were general dentists. Among them, 46.23% had a habit of reading dental journals. Most of the participants had an opinion that improper tooth brushing (42.71%) is an important cause of the gingival recession. Only 34.17% had information about Miller's classification of gingival recession. Regarding general indication of root coverage procedures, 28.64% answered aesthetics was the most common indication. 39.7% mentioned that traumatic occlusion was a risk factor for gingival recession. A group of 29.65% mentioned that accidental toothbrush trauma leads to gingival recession.

CONCLUSION: The knowledge of gingival recession among the study participants was adequate. More specifically, the interest of participants in periodontics was 5.39 and satisfaction in treating periodontal cases was 5.47.

Introduction

The periodontal practice is swiftly shifting, as new evidence regarding cosmetic dental treatment is constantly increasing and extending further than replacement of teeth to include the soft tissue component of dentition and increase the lifespan of dentition using disease prevention or thorough treatment [1]. Also, the periodontal speciality is growing in diverse aspects ranging from newer advancements in diagnosis, treatment interventions, the use of regenerative techniques, and growth factors in various periodontal diseases [2].

One of the most common cosmetic concerns related to periodontium is a gingival recession [3]. It is a condition that affects the community and an enigma for dental practitioners because of numerous etiological elements and surplus treatment options present for its management. Local or generalized exposure of the root surface of teeth by the "displacement of marginal tissue apical to the cemento-enamel junction (CEJ)" is referred as gingival recession [4] and is often associated with problems such as cervical root abrasions, esthetic concerns [5], root caries [6] and root surface hypersensitivity [7] that make it a concern for patients.

As perio plastic surgeries are considered as sensitive technique procedures, the dentist should have thorough knowledge regarding gingival recession. The early diagnosis and treatment of gingival recession are very crucial for successful root coverage as the delay can worsen the expected outcome and in turn, may compromise the aesthetics. It is also essential to learn about possible gaps between scientific evidence and dental practice to adopt continuing education and to ensure that researchers include questions that are relevant to practising dentists.

A very limited scientific data is available regarding knowledge and interest in treating gingival recession among practising dentists in Saudi Arabia as general dental practitioners treat the major part of society, so their knowledge, attitude, and perception about the periodontal diseases and its management are of utmost importance.

The purpose of this study was therefore to evaluate the knowledge and interest among dental practitioners regarding treating gingival recession.

Material and Methods

A cross-sectional study was done among 250 dental practitioners in Saudi Arabia, throughout four months using the questionnaire by Grover V et al., [8]which was originally taken from the questionnaire used in the study conducted by Zaher et al., [9]. A panel of specialists evaluated the face validity of the questionnaire. The minor changes were made to make it more clear and understandable. The questionnaire was anonymous, and participation was voluntary. The approval for this study was taken from the Institutional Review Board.

The questionnaire consisted of 9 questions; most of them giving the possibility of multiple choices of answers. Initial information addressed the profile of the dentist. More precisely, about the dentist's age, years of practising after graduation, practising speciality, and preferred professional subjects. Furthermore, we recorded the habit of reading dental journals. The questions from 1 to 6 assessed the knowledge in the classification and aetiology of gingival recession as well as about the general indication of procedures for root coverage. Question 7 asked the dental practitioners about their habit of reading the dental journals (yes or no), question 8 estimated the interest in periodontics on a numerical scale from 1 (no interest) to 10 (high interest), and question 9 assessed the satisfaction in practising periodontics from 1 (no satisfaction) to 10 (high satisfaction).

The criteria for assessing knowledge (adequate or inadequate) are based on the 2nd quartile value (50th percentile) as cut off the score. The analyses were made with the SPSS (version 16.0) software. The results were expressed as percentages of the total. Level of significance was set at 5%.

Results

A total of 199 (79.6%) out of 250 dentists responded to the questionnaire. All the responses received and then evaluated. The data associated with the general characteristics of the participants are presented in Table 1. Mean age of the participants was 34.59 ± 8.62 years, and the mean professional experience was 7.97 ± 6.30 years.

Table 1: General characteristics of the participants

General characteristics		Number	%
Number of responders		199/250	79.6
Practising sector	Government	138	69.35
	Private	61	30.65
Years since graduation	1-5	90	45.23
	6 to 10	48	24.12
	11 to 15	30	15.08
	15 to 20	24	12.06
	> 20	7	3.52

The majority of the participants were general dentists (57.79%) while others were periodontists (10.05%), oral surgeons (7.54%), prosthodontists (7.54%), endodontists (5.03%), pedodontists (10.02%) or belonging to another specialty (2.04%). About 46.23% of the participants had a habit of reading dental journals, and the majority of them were general practitioners. Majority of the participants who were reading dental journals were above 32 years of age and with 6 to 10 years of experience ($P < 0.01$).

All the questions had one correct answer among multiple choices of answers. The responses of participants are presented in Table 2.

Table 2: Frequency of correct responses

No	Questions	Frequency	Percentage
1	The most common cause for Gingival Recession?	85	42.71
2	Do you know Miller's classification of gingival recession?	68	34.17
3	Indication for Root Coverage procedure?	57	28.64
4	Risk Factor for Gingival Recession?	79	39.7
5	The consequence of lip, oral and tongue piercing?	156	78.34
6	Tooth Brush Trauma and Gingival Recession?	111	55.8

According to study results, majority of the participants had an opinion that improper tooth brushing (42.71%) is the most common cause of gingival recession while others responded periodontal disease (28.14%), abnormal tooth position (16.58%) and high frenal attachment (5%) as the most common cause of gingival recession.

Results of the study show 34.17% of the

participants had information about Miller's classification of gingival recession, 25.50% did not know the classification, and 27.26% did not remember the classification and 13.07% of the participants knew another classification for recession.

Regarding general indication of root coverage procedures, the participants answered aesthetics was the most common indication (28.64%), whereas others answered dental hypersensitivity (28.14%), prevention of further progression of recession (23.12%) and preservation of vitality of the tooth (10.55%) and occlusal stability (8.54%) as the most common indication.

Amongst the total participants, 39.7% mentioned traumatic occlusion as a risk factor for gingival recession while the others mentioned tooth position (24.62%), tooth vitality (18.59%) and enamel hypoplasia (17.09%). Of those who mentioned traumatic occlusion as a risk factor for gingival recession, the majority were with 6 to 10 years of experience. When asked about the consequence of lip, oral and tongue piercing, the majority of the participants (78.34%) answered gingival recession.

Among the participants, 29.65% mentioned accidental toothbrush trauma resulting in gingival recession while other considered it as physiological (20.105), factitious (20.105), iatrogenic (29.15%).

Out of total participants, 55.8% had adequate knowledge on gingival recession while 44.2% were with inadequate knowledge (Figure 1).

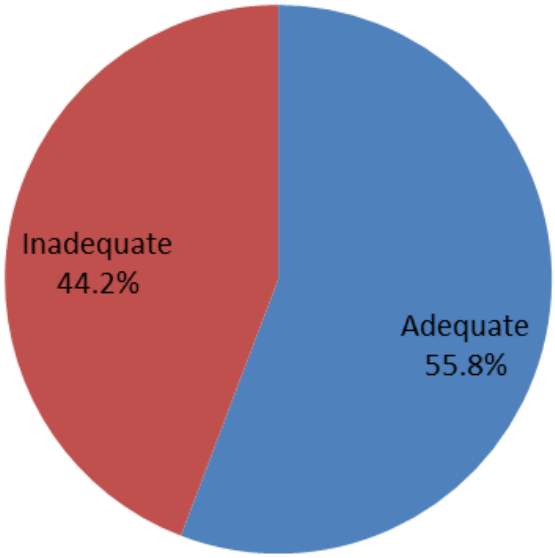

Figure 1: Knowledge regarding Gingival Recession among the subjects

The mean interest of participants in periodontics was 5.39 and satisfaction in treating periodontal cases was 5.47. The participants with 11 to 15 years of experience had more interest in periodontics and satisfaction in treating periodontal cases than the other groups, but the difference between the groups was not significant. A statistically significant relationship between the habit of reading dental journals and the interest in periodontics was also noted (Table 3).

Table 3: Relationship between the habit of reading dental journals and the interest in periodontics

		The habit of reading dental journals	Interest in periodontics
Spearman's rho	Correlation Coefficient	1.000	.661**
	Sig.	.	.000
	N	199	199

**. Correlation is significant at the 0.01 level.

Discussion

Gingival recession as a complex phenomenon makes the patients anxious and perplex for the practitioner who treats this situation. In day to day clinical practice, treatment of gingival recession depends on the practitioner's knowledge regarding classification, aetiology and treatment options. This survey evaluated the practitioners' knowledge of gingival recession and interest and satisfaction in treating periodontal cases.

The rate of response for this study was 79.6 % which was similar to previous studies [10], [11], [12], [13].In an attempt to encourage a high response rate and make the survey easy, the questionnaire was confined to 9 questions; most of them giving the possibility of multiple choices of answers.

According to the study results, the majority of the participants had an opinion that improper tooth brushing [11] (42.71%) is the most common cause of the gingival recession. This result was in consistent with an earlier study done by Zaher et al., [9]. 28.14% of the participants considered periodontal disease and 16.58% of participants considered abnormal tooth position as a major cause of the gingival recession. Stoner and Mazdyasna [12] have reported an association between high frenal attachment and gingival recession, while Powell and McEniery [13] found no correlation. In our study, 5% of the participant answered high frenal attachment as a major cause of the gingival recession.

Consistent with study results, 34.17% of the participants had information about Miller's classification of gingival recession, and 13.07% of the participants had information about a different classification for recession. However, there are various classification systems for gingival recession available in the literature [9], [14], [16]. Miller's classification [16] which is based on a prognostic evaluation of complete root coverage is still considered as the gold standard when deciding whether to attempt the root coverage for a certain

clinical case or not. Thus, it is important for practitioners to know this classification so that they can deliver proper treatment or refer gingival recession patients accordingly.

Amongst the total participants, 39.7% mentioned traumatic occlusion as a risk factor for gingival recession while the others mentioned tooth position (24.62%), tooth vitality (18.59%) and enamel hypoplasia (17.09%). Of those who mentioned traumatic occlusion as a risk factor for gingival recession, the majority were with 6 to 10 years of experience. Few studies [17], [18], [19] have mentioned possible risk factors of gingival recession. These studies have shown associations and possible factors, but they have not recognised the aetiological factors. Further studies are still required to explain the exact aetiology of gingival recession, to apply adequate preventive measures.

When asked about the consequence of lip, oral and tongue piercing, the majority of the participants (78.34%) answered gingival recession. Oral piercing is a cultural-causative factor of gingival recession [17], [19]. The differences that have observed among the various studies concerning the association between gingival recession and the examined variables could be attributed to numerous factors such as the heterogeneous population samples, the different study designs, etc.

The results revealed that almost 44.2% of the participants' knowledge on gingival recession was inadequate. The interest of participants in periodontics and satisfaction in treating periodontal cases was 5.47. So, there is an increased need for enhancing awareness among dental practitioners about the possible scope of periodontics so that timely specialist intervention is provided. Dental practitioner should pursue continued education through speciality teaching or certificate programs to stay informed about the newest research conclusions and novel treatment modalities to provide optimized care to the patient.

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ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2019 Jan 15; 7(1):143-147.
https://doi.org/10.3889/oamjms.2019.016
eISSN: 1857-9655
Public Health

Study On Social Support for Exercise And Its Impact on the Level of Physical Activity of Patients with Type 2 Diabetes

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Citation: Morowatisharifabad MA, Abdolkarimi M, Asadpour M, Fathollahi MS, Balaee P. Study On Social Support for Exercise And Its Impact on the Level of Physical Activity of Patients with Type 2 Diabetes. Open Access Maced J Med Sci. 2019 Jan 15; 7(1):143-147. https://doi.org/10.3889/oamjms.2019.016

Keywords: Diabetes; Social Support; Exercise; Health

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Received: 11-Oct-2018; **Revised:** 21-Nov-2018; **Accepted:** 22-Nov-2018; **Online first:** 04-Jan-2019

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

Abstract

BACKGROUND: Physical activity is one of the most important self-care approaches to controlling complications of type 2 diabetes. According to Bandura's social theory, factors such as social support are effective factors in the incidence of the behaviour.

AIM: This study aims to determine the level of physical activity, social support and their determinants.

METHODS: This descriptive study was performed on 250 patients with type 2 diabetes by Cluster-Random Sampling method in Rafsanjan City. Data were collected using the International Physical Activity Questionnaire and Social Support Questionnaire for Sport, that their validity and reliability were confirmed. The results were analysed by t-test, ANOVA and logistic regression.

RESULTS: This study showed that 46.8% of the patients were in the inactive group. Social support score for exercise was low in this group. The results indicated that social support and gender are predictors of physical activity, and with an increase in the social support score, the odds of having minimal physical activity increased 1.17 fold (OR = 1.167) and men were 4.18 times more likely to have minimal physical activity (OR = 4.183).

CONCLUSION: Considering the low level of physical activity and social support in diabetic patients, and the effect of social support on the prediction of physical activity, interventions are recommended to increase social support in this group.

Introduction

According to the World Health Organization, type 2 diabetes is the fourth leading cause of death in the world and the greatest challenge to today's modern life [1]. In the last decade, the fastest growth rate of diabetes was reported in the Middle East countries. And this increase includes individuals aged 45-64 years who are still socially and economically productive [2]. Diabetes is also prevalent in Iran, 7.7% of the adult population of Iran aged 25-64 years have diabetes, while half of the cases have not been diagnosed yet [3]. Evidence-based on the prevention of diabetes complications suggests that behavioural measures such as cessation of a smoking, healthy diet and physical activity are also necessary to prevent and reduce its complications, reduce blood sugar, lipids and blood pressure [4]. Although, diabetic patients are encouraged to carry out sufficient physical activity. Usually, they do not find much success in this task. Some of them cannot maintain their motivation to continue the physical activity, and there are numerous personal and environmental barriers that cause instability in physical activity [5]. According to Bandura's social cognitive theory, environmental factors are one of the important factors in the formation of behaviour, and Social support is considered as one of the influencing factors for the performance of physical activity [6]. Social support is considered as perceived support by others such as family and friends and is one of the factors associated with physical activity [7]. Duncan, according to the conclusions of some theories about social support, defined social support as any behaviour that helps an individual to reach his/her goals and consequences

[8]. These behaviours are categorised into four: emotional, tangible or practical, informative and supportive [9]. Social-emotional support involves expressing feelings, values and attitudes. Tangible support includes the provision of the necessary facilities for performing a behaviour, and information support include providing information, recommendations and guidelines for solving behaviour-related problems, and ultimately accompanying social support, including social belonging sense and presence of a companion to engage in activity [10].

Considering the chronic nature of diabetes, it is essential for these patients to change their behaviour and lifestyle. Social support is considered as an effective factor in self-care and acceptance of therapeutic procedures and lifestyle changes [11]. However, patients with type 2 diabetes have the least social support of physical activity among recommended behaviours [12].

Although there is no consensus, several studies indicate a positive impact of social support on the status of diabetic patients [13], and it is considered as one of the effective factors in initiating recommended behaviour in these patients [14]. Although the study of Morowati [15] showed that diabetic patients did not have favourable social support for self-care behaviours. Also, social support and family support had a low rate; they were able to predict self-care behaviours in diabetic patients, in these studies, self-care behaviours and social support were mentioned generally [16]. Until the present study, a study that specifically evaluates the social support for exercise and its dimensions, as well as its impact on physical activity behaviour in diabetic patients in the Iranian population, was not found.

This study aimed to evaluate the social support for exercise and the predictive power of social support in promoting the level of physical activity in diabetic patients. More interventional studies are recommended to assess the effects of social support for exercise in improving the level of physical activity in patients with diabetes.

Methods

This descriptive-analytical study was performed on patients with type 2 diabetes in health centres of Rafsanjan City, based on a similar study [15]. A group of 250 people entered the study. The random-cluster sampling method was used in this study, and four clusters were randomly selected from the eight health centres.

People with a previous diagnosis of type 2 diabetes were recruited. Patients were excluded from the study if they had a history of any psychiatric disorder, as well as the inability to carry out physical activity. The International Physical Activity Questionnaire was used to determine the level of physical activity that measures all physical activities in the environment including sports, working and daily activities. This standard questionnaire has been approved by the World Health Organization, and its reliability and validity have been approved in different countries [17]. In Iran, the Persian version of this questionnaire has been used in several cases, and its validity has been approved [18]. This self-report questionnaire examines three types of physical activity including walking, activity with moderate intensity and activity with high intensity.

The total score was calculated by the sum of duration and the number of days of the week spent on moderate and high-intensity activities and walking then turning them into metabolic equivalent per minute (METs), This questionnaire divides the individuals into three levels of activity: low, moderate and severe. The social support questionnaire of sport which was developed by Sallis et al., with 20 questions were used to determine the amount of social support of Exercise [19]. These questions were examined in the form of a five-point Likert scale on social support in two dimensions: social support of friends and support of family members. This questionnaire was translated by Noroozi et al. in Iran, and its reliability and validity were reviewed and confirmed by using exploratory and confirmation analysis procedures [20]. Data were analyzed by using SPSS18. The Kolmogorov–Smirnov test was used to examine the data normalisation. T-student, ANOVA, Chi-square and logistic regression tests were used for data analysis

Results

A group of 190 females (76%) and 60 males (24%) participated in the study. The guidelines recommended by (Sallies) were used to determine the level of social support. The results of social support study showed that the average score of social support was 29.42 ± 10.17. The study on social support dimensions showed that the maximum achievable score of social support of the family, as well as the score of verbal social support, was higher in this group (Table 1).

Table 1: Social Support Scores and Its Dimensions in Patients with type 2 Diabetes

Variable	Mean ± SD	Possible Range
Family social support	23.22 ± 7.34	0-60
Friends Social support	6.19 ± 3.86	0-20
Verbal support	6.84 ± 3	0-16
Practical support	4.22 ± 2.75	0-16
Emotional support	4.93 ± 3.14	0-20
Total social support	29.42 ± 10.17	0-80

T-test and variance analysis were used to investigate the effect of demographic factors on the rate of social support. The results showed that total social support score among individuals with different levels of education and income was significantly different (Table 2).

Table 2: Frequency distribution of social support regarding demographic characteristics

Variable		Mean ± SD Family social support	Mean ± SD Friends social Support	Mean ± SD Total social Support	P value
Gender	Male	24.45 ± 7.11	6.1 ± 3.95	30.55 ± 9.87	0.325
	Female	22.83 ± 7.39	6.22 ± 3.85	29.06 ± 10.26	
Age	25-39	20.93 ± 6.38	5.93 ± 3.3	26.86 ± 8.59	0.290
	40-49	24.23 ± 6.62	6.50 ± 4.02	30.73 ± 9.50	
	50-59	23.30 ± 7.38	6.23 ± 3.88	29.54 ± 10.23	
	60-65	21.60 ± 9.02	5.35 ± 3.70	26.96 ± 11.77	
Education	Elementary	20.43 ± 7.80	4.90 ± 4.17	25.36 ± 11.09	< 0.001
	Mid school	23.63 ± 6.40	6.56 ± 3.65	30.16 ± 8.86	
	Diploma	27.02 ± 7.02	7.04 ± 3	34.06 ± 9.54	
	Post graduate	23.33 ± 6.2	11.33 ± 6.80	34.66 ± 12.50	
Income	Weak	19.44 ± 6.50	4.23 ± 3.05	23.60 ± 9.19	< 0.001
	Moderate	24.30 ± 7.12	6.82 ± 3.74	31.15 ± 9.59	
	Good	24.30 ± 7.12	9.50 ± 2.81	41.62 ± 4.17	

The use of Post Hoc test showed that individuals with elementary education had lower social support scores than those with higher grades regarding educational level. Also, with an increase in income, the score of social support was significantly increased. The results of physical activity evaluation showed that 117 patients (46.8%) were in the inactive group based on the questionnaire scores and 133 patients (53.2%) were in the group with the minimal activity, and none of the patients was in the high-activity group. Probable factors affecting the level of physical activity in diabetic patients were analysed using the Analysis of Variance (ANOVA) and Chi-square test. The results showed that factors such as gender, education and income level had a remarkable effect on the rate of physical activity in diabetic patients. However, the age factor did not show any significant difference (Table 3).

Table 3: The relationship of demographic characteristics with physical activity in patients with type 2 diabetes

Variables		Inactive N(%)	Minimally active N(%)	P value
Gender	Male	16 (26.7)	44 (73.3)	< 0.001*
	Female	101 (53.2)	89 (46.8)	
Age	25-39	6 (40.0)	9 (60.0)	0.327
	40-49	27 (39.1)	42 (60.9)	
	50-59	68 (49.3)	70 (60.9)	
	60-65	16 (57.1)	12 (42.9)	
Education	Elementary	56 (71.8)	22 (28.2)	< 0.001*
	Middle school	50 (40.0)	75 (60.0)	
	Diploma and Above	11 (23.4)	36 (76.6)	
Income	Weak	52 (75.4)	17 (24.6)	< 0.001*
	Moderate	63 (36.4)	11 (63.9)	
	Good	6 (75)	2 (25)	

Logistic regression analysis was used to consider the predictive power of the factors affecting the physical activity behaviour in patients with diabetes. Using the Hosmer-Lemeshow test showed that the final model was well-fitted (p = 0.62). The results showed that the male gender (OR = 4.183) and high social support for exercise (OR = 1.167) were the predictors for promoting the level of physical activity in patients with diabetes from the level of inactive to minimal active. The income level and level of education were not confirmed as predictors of physical activity changes in patients with type 2 diabetes (Table 4).

Table 4: Regression analysis of variables as predictors of physical activity in Patients with type 2 Diabetes

Variable	B	OR	Wald	P value	95% C.I.for EXP (B) Lower Upper
Social Support	0.155	1.167	47.961	< 0.001 *	1.117 1.220
Gender	1.431	4.183	10.921	0.001 *	1.790 9.775
Education	0.709	2.033	2.547	0.176	0.851 4.858
Income	0.849	2.338	3.349	0.067	0.941 5.804

Discussion

The results of this study showed that the level of social support for exercise was not appropriate in type 2 diabetic patients. So that, the average score of social support in these patients was less than the half score of the questionnaire. However, the low level of social support is also mentioned in other studies. Studies conducted by Heidari and Morowati [15], [21] in Iran showed that the level of social support in diabetic patients is not appropriate, and in comparison with other recommended behaviours for the diabetic patient such as exercise and medical treatment, less amount of social support was reported for physical activity [22].

Low level of social support for physical activity can be due to different factors such as reduced awareness of individuals and families about the impact of physical activity on the improvement of the status of patients, as well as cultural perspectives and concerns about physical activity in patients with diabetes, especially elderly patients and women. With regards to the definition of social support in communicating with others, feeling of frustration and depression has also been mentioned as the reason for the reduction in this communication in patients. As indicated in several studies, one of the reasons for low social support is the existence of some level of depression [23]. On the other hand, some studies have shown that about half of patients with type 2 diabetes have some level of depression.

The mean score of practical social support was low in comparison with other areas [24]. These results are consistent with the results of other studies which showed that the highest social support was the verbal type and include a discussion on the benefits of sport and verbal encouragement. Although, according to the theory of social support, verbal induction has a positive effect on solving obstacles of the performance of behaviour and success in its implementation [25]. Due to the chronic and debilitating nature of diabetes as well as the complex nature of the onset of regular and continuous physical activity behaviour, providing practical support by family, friends, and essential

health systems are important. Assessing the influence of demographic factors on social support showed that, although social support score was higher in men, this difference was not statistically significant, which is consistent with the results of Daniel's study [26]. Of course, there are different views on the impact of gender on social support; in some studies, the level of social support of women is reported to be lower than that of men [27]. Some studies show that women are more dependent on social support in solving their problems [28]. It seems that friendship groups for exercise are increasing in Iranian women. The results of this study showed that with an increase in the level of education and income, the level of social support also increased. Access to financial resources, social value, as well as gaining more social support and widespread social networks are the most important effects of high educational level on health consequences [29]. In this study, Physical Activity Questionnaire was used to assess the level of physical activity. In this tool, the participants are divided into three levels: inactive, minimally active and highly active. The study showed that most people were at a minimal activity level. Studies also indicate that about 60-80% of people with type 2 diabetes do not meet the recommended levels of physical activity [30].

The results of this study indicate a lower level of physical activity in women with diabetes. Studies show that inactivity is common especially in elderly Iranian women [31]. This low mobility can be due to many social and individual factors. Among the social factors, the cultural limitations of physical activity in Iranian women, especially in an outdoor environment such as parks and green places, have been mentioned [32]. Other reasons include the involvement of multiple roles, home works and taking care of children and the family, which despite taking a large amount of women's time, do not lead to effective physical activity.

The present study has shown that the level of physical activity had a significant relationship with education and income, while age was not effective. This is consistent with Costanzo's study [33]. The effect of education on physical activity can be explained by the effect of awareness on self-care behaviours [34]. Although awareness does not necessarily lead to the adoption of behaviour, a higher level of education can be effective in physical activity by increasing the ability of understanding and accessing information necessary for carrying out physical activity. With regards to age, some studies have shown that lifestyle behaviours such as physical activity have been shaped since childhood and do not change with age [35].

On the other hand, there was no limit to the recommended physical activity for diabetic patients until 65 years for those who participated in this study. The use of a regression model showed that after adjusting for the confounding factors, two factors: gender and perceived social support, could predict the improvement of physical activity behaviour in patients with type 2 diabetes. The chance of having minimal physical activity in men is 4.18 times more than that of women. Also, those who had higher social support score, have 1.17 time's higher chance or 17% more likely chance to have minimal activity. Although, there is no specific mechanism for the impact of social support on self-care behaviors, some direct and indirect effects are mentioned. Social support can be directly influenced by the behavior of individuals, or it is indirectly effective by increasing self-efficacy and self-confidence, or by reducing individual stress and anxiety in self-care behaviors [36].

Given the complex nature of exercise in diabetes, social support provision from different sources including family, friends and health care providers would be helpful in this regard.

This study had some limitations including the descriptive nature of the study, which is necessary for interventional studies and to design and implement a more detailed examination of the impact of promoting social support in different dimensions on the amount of physical activity. On the other hand, in this study, the level of physical activity was assessed through self-reporting, which can be assessed by using the physiological dimensions of physical activity, such as cardiac fitness more precisely.

In conclusion, this study presents a low level of social support and physical activity in diabetic patients. On the other hand, social support was suggested as a predictor of physical activity behaviour, family and community interventions are recommended to improve the level of social support for exercise, to increase the level of physical activity in patients with type 2 diabetes.

Acknowledgements

The authors thank the Rafsanjan University of Medical Science.

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ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2019 Jan 15; 7(1):148-151.
https://doi.org/10.3889/oamjms.2019.037
eISSN: 1857-9655
Public Health

The Relationship between the Duration of Playing Gadget and Mental Emotional State of Elementary School Students

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Citation: Wahyuni AS, Siahaan FB, Arfa M, Alona I, Nerdy N. The Relationship between the Duration of Playing Gadget and Mental Emotional State of Elementary School Students. Open Access Maced J Med Sci. 2019 Jan 15; 7(1):148-151. https://doi.org/10.3889/oamjms.2019.037

Keywords: Mental, Emotional; Strength and Difficulties Questionnaire (SDQ); Gadgets; Elementary School

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Received: 21-Oct-2018; **Revised:** 21-Dec-2018; **Accepted:** 22-Dec-2018; **Online first:** 13-Jan-2019

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

Abstract

BACKGROUND: Technology has provided a type of facility in the world that currently makes the world like a virtual globe. One of the technologies currently used by humans is gadgets. Generally, children and adolescents nowadays often use gadget excessively. They use it excessively which may affect their social and emotional functions.

AIM: This study aims to determine the relationship between the duration of playing gadget and mental-emotional state of elementary school students.

METHODS: This study uses Strength and Difficulties Questionnaire (SDQ) which contains 25 questions that can be used for children aged 8 to 16 years. This research was conducted in August 2018 with fourth-grade students of Public Elementary School and Private Elementary School Medan as respondents. This study uses a cross-sectional study method with the chi-square test as a statistical hypothesis test. The number of respondents in this study was 103 students consist of 73 students of Public Elementary School and 30 students of Private Elementary School Medan.

RESULTS: The results of this study found a relationship between the duration of playing gadgets on mental-emotional at elementary school students with a probability value of 0.0001 ($p < 0.05$) and the relationship between the frequency of using gadgets and mental-emotional with a probability value of 0.001 ($p < 0.05$).

CONCLUSIONS: There is a significant relationship between mental emotional and the duration of playing gadgets and the frequency of using gadgets elementary school students.

Introduction

Information and communication technology systems have provided a kind of facility in the world that currently makes the world like a small globe globally [1]. One of the current technologies used by humans is gadgets. A gadget is an object or item created specifically in this advanced era with the aim of helping everything become easier and more practical compared to previous technologies [2]. Generally, children and adolescents nowadays often use intense gadgets. They use it excessively which can affect their social and emotional function. The majority of children and adolescents have smartphones, laptops, game consoles, tablets, iPod [3]. The use of electronic gadgets has greatly increased in the contemporary world, particularly among children, as a result of addiction [4].

Very early recognition gadget for children can have positive and negative impacts. These are influenced by several factors such as the frequency, duration, and supervision of parents. Using gadgets as a basic material for learning in children will have positive impacts such as increasing children's creativity and thinking. It can appear with parents and children, as well firm in providing time limits for children in playing gadget. Likewise the opposite, if the supervision of parents is lacking and there is no firm effort in providing time limits of playing gadget in children, can cause negative effects. The children

may develop to negative characters such as shy, lacking confident, lonely and stubborn [2].

The frequency or intensity of the children in using gadgets will affects their mental and emotional development. From a study conducted by the University of Western Australia, through a survey of 2,600 school students about the length of looking at the gadget screen, it was found that 45% of 8-year-old children and 80% of 16-year-old students spent more than two hours of playing gadget in a day [5].

There is no data about the correlation between mental and emotional condition with the duration and frequency of using gadgets among elementary school students in Medan, Indonesia which has been published yet. Thus, this study is seeking information whether mental and emotional condition is affected by the duration and frequency of using gadgets among elementary school students

Methods

This study is an analytical study using a cross-sectional approach. This study uses a questionnaire Strength and Difficulties Questionnaire (SDQ) which contains questions that can be used for children aged 8-16 years. SDQ has 25 items on psychological attributes, divided into 5 scales: emotional symptoms, behavioural problems, hyperactivity/inattention, peer relations problems, and prosocial behaviours that are answered based on 3 choices: never, rarely, and often [6].

The study was conducted in August 2018 with students of Public Elementary Schools and Private Elementary School Medan grade 4 as respondents. The population that the researchers set in this study were fourth-grade elementary students.

We carried out a simple random sampling technique by using computer draw the samples. Our respondents were elementary school students from Public Elementary Schools and Private Elementary School in Medan who were included in the criteria. The collected data were analysed using computer statistical software by using the chi-square test (χ^2). The statistical level was determined as significant if the p-value is < 0.05.

Results

In our study, we included 103 respondents consisting of 73 students (70.9%) from Public Elementary Schools and 30 students (29.1%) from Private Elementary Schools in Medan. Among the respondents 49.5% were boys, and 50.5 % were girls and the age distribution was majorly at the age of 8-9 years (52%). Based on the most used gadget type by the respondents are smartphones (52.4%) and followed by tablets (33%) and laptops (9.7%) The frequency of using gadgets was mostly 1-3 days (51.5%), 6-7 days (30.1%), and 4-5 days (18.4%), respectively. Based on the duration of gadget usage in a week, 43% of respondents used gadgets < 5 hours a week, 34% of respondents 6-10 hours a week, and 21% of respondents > 10 hours a week. Based on mental-emotional categories, we obtained 35.9% was normal and borderline categories and 28.2% were abnormal.

Table 1: Characteristics distribution of respondents, Characteristic Gadget, and mental-emotional category

Characteristics of Respondents		n	%
Gender	Male	51	49.5
	Female	52	50.5
Age	8-9 years old	54	52
	10-11 years old	49	48
School	Public Elementary Schools	73	70.9
	Private Elementary Schools	30	29.1
Gadget's type	*Smartphone*	54	52.4
	Tablet	34	33.0
	Laptop	10	9.7
	Etc	5	4.9
Number of Gadget Usage days in a week	1-3 day	53	51.5
	4-5 day	19	18.4
	6-7 day	31	30.1
Play Gadget Duration in a week	< 5 hour	45	43
	6-10 hour	36	34
	> 10 hour	22	21
Mental Emotional	Normal	37	35.9
	Borderline	37	35.9
	Abnormal	29	28.2

Based on SDQ criteria*.

Based on the research, it was found that the emotional mental results of the normal category based on female sex were 59.5% and men 40.5%. Borderline categories based on female sex were 43.2%, and men were 56.8%. The abnormal category based on female sex is 51.7%, and males are 48.3%. The normal emotional and mental category was 48.6% and 51.3% at the age of 8-9 years and 10-11 years, respectively. The borderline category among children ages of 8-9 year was56.7% and 10-11 years was 43.2%. The abnormal category in 8-9 years old was 51.7% and 10-11 years old was 48.2%.

Table 2: Mental Emotional Distribution of Respondents

Respondents based on Gender	Mental Emotional		
	Normal n (%)	Bordeline n (%)	Abnormal n (%)
Male	15 (40.5%)	21 (56.8%)	15 (51.7%)
Female	22 (59.5%)	16 (43.2%)	14 (48.3%)
Respondents based on Age	Normal n (%)	Borderline n (%)	Abnormal n (%)
8-9 years old	18 (48.6%)	21 (56.7%)	15 (51.7%)
10-11 years old	19 (51.3%)	16 (43.2%)	14 (48.2%)
Respondents based on School	Normal n (%)	Borderline n (%)	Abnormal n (%)
Public Elementary Schools	29 (78,4%)	22 (59.5%)	22 (75.9%)
Private Elementary Schools	8 (21.6%)	15 (40.5%)	7 (24.1%)

Based on elementary school type, it was found that most mental and emotional category of the public elementary school students was at the normal category (78.4%). Whereas in the private elementary school students, the mental and emotional category was mostly in borderline range (40.5%)

Table 3: Mental Emotional Relationships with the Frequency of Using Gadgets and Number of Hours in a Week

Number of Usage Gadgets days (/ week)	Mental Emotional		
	Normal n (%)	Borderline n (%)	Abnormal n (%)
1-3 days	27 (60)	6 (31.6)	4 (13)
4-5 days	18 (34)	8 (42.1)	11 (35.5)
6-7 days	8 (16)	5 (26.3)	16 (51.5)
Chi-Square			P = 0.001
Number of Gadget Usage Hours (/ week)	Normal n (%)	Borderline n (%)	Abormal n (%)
< 5 hours	26 (70.3)	15 (40.5)	4 (13.8)
6-10 hours	8 (21.6)	19 (51.4)	9 (31)
> 10 hours	3 (8.1)	3 (8.1)	16 (55.2)
Chi-Square			P = 0.0001

Based on the research, the results of the normal mental, emotional category based on the frequency of gadget usage in the week were 1-3 days a week at 60%. Mental-emotional with borderline categories based on the frequency of gadget usage in the most weeks is 4-5 days a week as much as 42.1%. The emotional mentality is not normal category based on the frequency of use of gadgets in a week which is the most is 6-7 days as much as 51.5% with P value = 0.001, indicating that there is a significant relationship between mental, emotional and frequency of playing gadget. Emotional mentality in the normal category based on the number of hours of gadget usage in the most week is < 5 hours a week at 70.3%, the most borderline category is 6-10 hours a week 51.4%, the abnormal category based on the most hours of gadget usage in the week is > 10 hours a week of 55.2% (p-value = 0.0001).

SDQ has 25 items on psychological attributes, divided into 5 scales: emotional symptoms, behavioural problems, hyperactivity/inattention, peer relations problems, and prosocial behaviours that are answered based on 3 choices: never, rarely, and often [6].

Table 4: Number of frequent answers by respondents

SDQ Scale	Number of frequent answers by respondents
Emotional symptoms	140
Behavioural Problems	89
Hyperactivity/lack of attention	160
Peer relationship problems	185
Prosocial behaviour	291

From the results of the study it was found that the most "frequent" answers on the prosocial behaviour scale were 291 answers from which of the 5 questions on the prosocial behaviour scale, problem number 1 had the most "frequent" answers.

Discussion

Based on the results of the study, the majority of the respondents were children of 8-9 years old. As many as 51.7% of respondents aged 8-9 years are considered an abnormal mental, emotional category. According to Mildayani (2017), children aged 6-17 years are playing gadgets in the specified time, when the time is up they will ask for extra time. This is one sign of the effects of addiction. Children with their strength will hit, become aggressive, or cry [7]. Research conducted by Wiguna et al., in 161 children and adolescents showed that 54.81% had problems with relationships with peers and 42.2% had emotional problems [8].

The study showed that most of the respondents were female. However, as many as 51.7% of male respondents were categorised as abnormal. According to Jarot (2015), the influence of the gadget on the level of violence is mostly males, because it is more aggressive and the level of emotion has not been controlled. So that fellow men often quarrel [9].

Based on the research obtained based on the type of gadget that has the most is a smartphone. According to research by Okky Rachman (2015), elementary school children who have the most types of gadgets are mobile phones, the second most choice is laptops because they are larger so they are more likely to be satisfied to play games [10].

Based on the results of the study, the highest distribution of respondents for the number of days of use of gadgets in a week is 1-3 days a week. However, in this study mental, emotional abnormalities obtained the highest frequency is 6-7 days of use in a week, whereas, the most duration of gadget usage is < 5 hours a week. However, in this study, the abnormally emotional category obtained the highest number in duration more than 10 hours.

According to Sari and Mitsalia (2016), the use of gadgets is categorised as high intensity when using a gadget with a duration of more than 120 minutes/day and in one use is > 75 minutes. Also, a day can be many times (more than 3 times usage) the use of gadgets with a duration of 30-75 minutes will cause addiction in the use of gadgets. Furthermore, the use of medium intensity gadgets if using a gadget with a duration of more than 40-60 minutes/day and intensity of use in one use 2-3 times/day for each use [11].

Based on the research, it was found that the emotional mentality of respondents with an abnormal category was 28.2%. According to Sundus M (2015) about the effect of using gadgets on children that using too many gadgets causes depression in children at certain ages. This also causes mental health problems in children in childhood and adolescence. They may act depressed, or we can see the worst of these symptoms of depression within a few days [12].

Other studies conducted by Mingli et al., (2015) say that children who use gadgets for more than 2 hours per day have an increased risk of depression, and that risk increases with increasing

screen time [13]. Ferguson (2017) found a small positive relationship but significant use of the duration of playing gadget with feelings of depression and delinquency in children when playing gadgets for more than 6 hours per day [14].

Another major concern regarding excessive use of gadgets in the age of children is a negative action. A study in Malaysia conducted in 2011 found that children became very dependent on gadgets, they would have negative sentiments if they lost their gadgets, the majority will say that they will be angry, sad, and insecure [4]. The use of gadgets for too long duration can affect aggressive levels in children. Also, the child becomes insensitive to the environment around him. Children who are too cool with their gadgets result in forgetting to interact or communicate with people around and their families, and that will have a negative impact on children's social development [2]. Many studies have explored the relationship between gadget use and mental emotionality in children, and the results are clear, when using gadgets increase, so too the risk of mental-mental problems including depression, anxiety, ADHD, mood disorders, and suicide [15].

In conclusion, based on the results of the study, the conclusions obtained are there is a significant relationship between the duration of playing gadgets on mental-emotional elementary school students. Mental-emotional elementary school students also have a significant relationship to the frequency of gadget usage in a week. There is a significant relationship between the respondent's mental emotionality on the number of hours of gadget usage in a week.

The advice given to parents and teachers is to limit the use of gadgets to children where the duration of playing gadgets should be less than 40 minutes/day and frequency < 3 times/day and 1-3 days/week. For health workers and schools can make counselling efforts on excessive use of gadgets and conduct early detection using the SDQ questionnaire.

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ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2019 Jan 15; 7(1):152-156.
https://doi.org/10.3889/oamjms.2019.028
eISSN: 1857-9655
Public Health

Socio-Economic Status Inequity in Self Rated Health in Patients with Breast Cancer

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Citation: Taheri M, Tavakol M, Akbari ME, Mohammadbeigi A, Abbasi M. Socio-Economic Status Inequity in Self Rated Health in Patients with Breast Cancer. Open Access Maced J Med Sci. 2019 Jan 15; 7(1):152-156. https://doi.org/10.3889/oamjms.2019.028

Keywords: Self-Related Health; Socio-Economic Status; Inequality; Concentration Index; Iran

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Received: 05-Nov-2018; **Revised:** 01-Dec-2018; **Accepted:** 15-Dec-2018; **Online first:** 14-Jan-2019

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

Abstract

AIM: We investigate the evaluation of socio-economic status (SES) inequality on self-rated health (SRH) at women with breast cancer.

STUDY DESIGN: Cross-sectional study

METHODS: The current study conducted on all 270 breast cancer patients that were admitted to one of the hospitals of Arak University Medical Sciences (Arak, Iran from April to July 2018) by census (using non-random sampling (accessible sampling). SES was calculated by asset-based questionnaire and Principle Component Analysis (PCA) was performed to estimate the families' SES. Concentration Index (C) and Curve (CC) was used to measure SES inequality in SRH. The data were analysed with Stata software.

RESULTS: The number of persons with good SRH by the level of SES was 165 (61.1%) and with poor SRH was 105 (38.9%). The number of persons with good SRH in comparison to same-aged people by level of SES was 135 (50%) and with poor SRH was 135 (50%). Concentration index of SRH in all level of SES was 0.061 (SE = 0.03). Also, Concentration index for SRH in comparison to same-aged people at different levels of SES was -0.044 (SE = 0.03).

CONCLUSION: The results of this study showed that there is inequality in SRH in a patient with breast cancer of the richest level of SES.

Introduction

Health is certainly a basic need in all human societies [1]. Health equity is a well-accepted ethical and human rights principle; that all humans have a high level of health [2]. So, measuring health inequality is the main part of assessing the performance of a health system. Despite the significant improvements in many health indices in different countries during the past decades, health inequality has not only remained but also increased in some of them [3], [4]. Measuring levels of health and its distribution is necessary for understanding the importance of the problem, evaluating the effect of interventions and monitoring progress [5].

In this study, we used SRH as an index to measure health [6] refers to a single item health measure that asks individuals to rate their health as excellent, good, moderate or poor. SRH is generally considered to be a valuable source of data on subjective health status and is popular due to its simplicity to collect [1], [2]. Compared to more detailed questionnaires as well as to clinical findings, SRH has been shown to have an approved validity and reliability in many studies [7]. Furthermore, its predictive value for mortality and morbidity in populations has been shown in some studies [8].

About 70% of deaths due to cancer occur in countries with a lower SES [9]. Over the past few decades, there has been a rapid growth in Asia's economic situation that has led to an increase in life expectancy and a reduction in mortality due to infectious diseases. In recent decades, the incidence of breast cancer has doubled or tripled in Japan, Korea and Singapore and has increased by more than 30% in China and India over the past few years [10], [11]. The SES and the level of education lead to a difference in the stage of breast cancer and subsequently its survival [12]. The SES refers to socio-economic factors such as education, income, and occupation, which can affect a person's or group's position in the community [13]. The relationship between inequality and health is one of the issues that is considered by many researchers. Inequality is an issue at the social level and imposes many costs on society [14].

Today, concentration index (CI) has the widest use in measuring inequity in health. This index expresses the magnitude of inequality in health or the use of health services in a single number that higher values represent higher levels of inequality [15].

The results of the studies indicate that the SRH, especially periodically, has a strong relationship with assessments of well-being, health outcomes and death [16]. So SRH has an important role in health improvement. To date, no study has been performed to assess the socioeconomic inequality in SRH in breast cancer with concentration index and decomposition method.

This study was conducted to evaluate socioeconomic inequality in SRH in women with breast cancer.

Material and Methods

In this cross-sectional study (April to July 2018) 270 breast cancer patients that were admitted to one of the hospitals of Arak University Medical Sciences (Arak, Iran) were entered to the study using census. The inclusion criteria included patients who had the ability of communication and passed at least 1 month from the diagnosis date. Not completing the questionnaire and suffering from severe psychological illnesses that can impair the patients' cooperation were considered exclusion criteria.

We conducted a pilot study on 20 samples, and the sample size was calculated as 250 patients with indexes of $\alpha = 0.05$, $d = 1.5$, $SD = 0.21$. To counteract the possibility of sample loss during the study, 270 patients were requested to participate in the study.

After explaining the purpose of the study and the way of completing the questionnaire, the informed consent form was signed by qualified patients. Then, the necessary explanation, regarding the objectives of the study, was given to patients and the questionnaires were distributed among them.

The protocol of the study was approved by the ethics committee of the Shahid Beheshti University of Medical Sciences grant number IR.SBMU.RETECH.REC.1396.839.

Data collection was done by three questionnaires. At first, demographic and individual information of people including age, education, place of residence, etc. To examine the household social status an asset-based questionnaire used. This questionnaire including 10 questions: the level of woman education, the education of the spouse, the area of the infrastructure by households, the price per square meter of residential land, facilities and amenities (the personal car and computer) and the household income. The correlation of these factors with a total score was obtained 0.87 and the reliability was 0.88 [17].

The SRH was examined by two questions: 1) In general, what would you say your health is? It was measured with a Likert's type 5-point scale ranging from 'excellent' (score 1) to 'poor' (score 5). 2) How would you assess your general health status in comparison with your own age? Which included these responses: much worse, worse, slightly worse, not better, not worse, a little better, better and much better. Reliability and validity of this questionnaire have been assessed in other studies [18], [19], [20].

The Principle Components Analysis (PCA) was used to measure the SES. PCA is a multivariate statistical technique for reducing a set of consistent variables to a small number of non-consistent variables. The first component of the analysis of the most variance is explained among the variables and thus it is considered as an index of the SES of each individual (household). This component provides a score for each household, which reflects the SES of that household and can be used in analyzes [21], [22].

The inequity in the different levels of Socio-Economic Status (SES) in the studied patients affected to cancer was assessed by the Concentration (C) Index. C is constructed based on Concentration Curve (CC). The CC represents the SRH versus the concentration percentage of the y axis that are organized according to the SES of the poorest to the richest (axis x). CC will be a 45-degree line, which will be called the "equality line". If the SRH has more accumulation among the poor, the CC will be placed above the equality line, indicating the existence of inequality. According to the definition C is the area under the CC multiply by 2. Therefore, if the equity line and CC fit together, the C will be zero. When the CC is above the equity line, C has a negative sign and if it is bottom the equity line has a positive sign. The C

changes between the two -1 and +1 ranges [23], [24]. The C index is a common inequity measure in health outcomes and has been used continually in recent studies [23], [25], [26], [27], [28]. The C was calculated by the Kakwani *et al*., formula [23]. [Formula 1].

$$C = \frac{2}{\mu}\sum_{t=1}^{T} f_t \mu_t R_t - 1,$$

In this formula, μ is the mean of the SRH in studied patients with cancer and μ_t is that for the t[he] group. Also, f_t is the group share of patients. Also, R_t is the relative rank of the t[th] educational level of the participating patients, which was obtained through formula 2:

$$R_t = \sum_{t=1}^{T} f_r - \frac{1}{2} f_t,$$

Therefore, R_t indicates the cumulative proportion up to the midpoint of each SES group interval. The correspondence confidence interval for C is calculated based on Wagstaff and Van Doorslaer method [23], [29], [30]. This method has been used in other studies [30], [31], [32], [33] and is as given below.

$$Var(C) = \frac{1}{n}\left[\sum_{t=1}^{T} f_t\, a_t^2 - (1+C)\right] + \frac{1}{n\mu^2}\sum_{t=1}^{T} f_t\, \sigma_t^2 (2R_t - 1 - C)^2$$

In this formula σ_t^2 is the variance of μ_t,

$$a_t = \frac{\mu_t}{\mu}(2R_t - 1 - C) + 2\text{-}q_{r-1} - q_t \quad ,$$

and $q_t = \frac{1}{\mu}\sum_r^t \mu_r f_r$, which is the ordinate of L (P), q_0 = 0 and $p_t = \sum_{r=1}^{t} f_r R_r$

Results

Distribution of SRH by the level of SES has shown in Table 1. According to these results, 165 (61.1%) of women were with good SRH, and 105 (38.9%) persons were with poor SRH. 70% of persons with good SRH was in the richest level of SES. Between SES and SRH was a statistically significant relationship (p-value < 0.05).

Table 1: Distribution of Self rated health by level of SES

Education Level	Good SRH	Poor SRH	*p*-value
Poorest	48 (53.3)	42 (46.7)	
Middle	54 (60)	36 (40)	0.046
Richest	63 (70)	27 (30)	
Total/average	165 (61.1)	105 (38.9)	

Distribution of Self rated health in comparison to same-aged people by level of SES shown in table 2. According to these results, 135 (50%) of women were with good SRH, and 135 (50%) persons were with poor SRH. Also, distribution of persons in levels of SES was almost the same. Between SES and Self rated health in comparison to same-aged was not a statistical significant relationship (p-value > 0.05).

Table 2: Distribution of Self rated health in comparison to same-aged people by level of SES

Education Level	Good SRH	Poor SRH	*p*-value
Poorest	51 (56.7)	39 (43.3)	
Middle	42 (46.7)	48 (53.3)	0.301
Richest	42 (46.7)	48 (53.3)	
Total / average	135 (50)	135 (50)	

Concentration index, Standard error of C, and confidence interval of C, for SRH in different levels of SES shown in table 3. Concentration index of SRH in all level of SES was 0.061 (SE = 0.03). Also, this index for the poorest level of SES was 0.012 (SE = 0.053), for middle level of SES was 0.048 (SE = 0.052) and for the richest level of SES was 0 (0.048). The concentration index and 95% confidence interval for SRH was 0.061(-0.055 to 0.176) (Table 3), while table 4 showed that the C index for SRH in comparison to same-aged people was estimated as -0.044 (-0.124 to 0.036).

Table 3: Calculation of Concentration index, Standard error of C, and confidence interval of C, for SRH in different levels of SES

Group	f%	SE P	Quintile %	CUM-Quin	f-mu	Cum-f-mu	C Index
Poorest	0.533	0.053	0.333	0.333	0.178	0.178	0.012
Middle	0.6	0.052	0.333	0.667	0.2	0.378	0.048
Richest	0.7	0.048	0.333	1	0.233	0.611	0
Total/average	0.611	0.03	1		0.611		0.061

Concentration index, Standard error of C, and confidence interval of C, for SRH in comparison to same-aged people at different levels of SES shown in table 4. Concentration index for SRH in comparison to same-aged people at different levels of SES was -0.044 (SE = 0.03). Also, this index for the poorest level of SES was -0.022 (SE = 0.052), for middle level of SES, was -0.022 (SE = 0.053) and for the richest level of SES was 0 (0.053).

Table 4: Calculation of Concentration index, Standard error of C, and confidence interval of C, for SRH in comparison to same-aged people at different levels of SES

Group	f%	SE P	Quintile%	CUM-Quin	f-mu	Cum-f-mu	C Index
Poorest	0.567	0.052	0.333	0.333	0.189	0.189	-0.022
Middle	0.467	0.053	0.333	0.667	0.156	0.344	-0.022
Richest	0.467	0.053	0.333	1	0.156	0.5	0
Total/average	0.5	0.03	1		0.5		-0.044

Discussion

Few studies have evaluated the socio-economic inequality in SRH. Our study is the first study that evaluates socioeconomic inequality in SRH

by the concentration index and decomposition methods. The results of our study showed a direct correlation between SRH inequalities with different levels of SES. In this study, two questions were asked for SRH. Also, the concentration index was evaluated at all three levels of SES. In the general SRH question, the number of 0.012 indicates an inequality in it. The positive sign shows that SRH is higher in people with higher SES. In the middle level of SES also was 0.048 that shown individuals with a higher SES have more SRH. In the question of health evaluation than same age C index was -0.022 both for weak and moderate SES which indicates the inequality at the SES levels in this question.

Similar to our results were reported in other studies. Cabieses et al. showed a significant concentration of above average SRHS favouring richer people in Chile in both years, which was less pronounced in 2013 than 2000. (Erreygers corrected CI 0.165 [Standard Error, SE 0.007] in 2000 and 0.047 [SE 0.008] in 2013). To help interpret the magnitude of this decline, adults in the richest fifth of households were 33% more likely than those in the poorest fifth to report above-average health in 2000, falling to 11% in 2013 [34]. Income is closely and strongly associated with health [35]. Previous research highlights the multidimensional effects of poor income in healthy population [36]. Absolute poverty directly affects health, including self-reported health [37]. Jung also showed how socio-demographic, socioeconomic, cancer related, and health information factors are associated with SRH by health information seeking/avoiding behaviour in a survey of 502 post-treatment cancer patients. Information avoiding behaviour, however, does not exhibit a negative contribution toward the relationship between SRH and SES [38]. McFadden et al. also showed the prevalence of poor or moderate (lower) self-rated health increased with increasing age in both men and women. There was a strong social class gradient: in manual classes, men and women under 50 years of age had a prevalence of lower self-rated health similar to that seen in men and women in non-manual social classes over 70 years old. Even after adjustment for age, educational status, and lifestyle factors (body mass index (BMI), smoking, physical activity and alcohol consumption), there was still strong evidence of a social gradient in self-rated health. There was a strong gradient of decreased SRH with age in both men and women [39].

SRH is generally considered to be a valuable source of data on health status, popular due to its simplicity to collect and its strong association with future mortality [40]. The social class gradient for chronic diseases such as cancer disease is well recognised [41].

Some qualitative studies have evaluated the processes through which individuals evaluate their health status [42], [43]. It appears that there may be important differences in people's perception of health between socioeconomic groups. Men and women from higher social groups appeared to use a larger number of factors when assessing their health, including aspects such as being fit and active and the absence of illness, as well as aspects of well-being such as happiness and feeling in control [43].

This study has some limitations. The cross-sectional design limits conclusions on causality. Also individuals with major medical conditions that could potentially have confounded the relationship between SRH and SES. Similar to many inequality studies we use the measurement of the current status for assessment of SES (44), although the most emphasis is on the measurement of life-course SES [45]. Despite this limitation, this study provided good evaluate of SES inequality in SRH.

In conclusion, the inequality of SES affects self-rated health. High level of SES has more SRH. Also the level of SRH related to the level of SES. Regarding the importance of self-rated health in the process of improving the health of breast cancer patients, and based on the findings of this study, the impact of socio-economic inequalities on self-rated health is needed to make fundamental decisions and changes in health policy and to improve socio-economic status and to eliminate inequalities in the health field.

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ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2019 Jan 15; 7(1):157-161.
https://doi.org/10.3889/oamjms.2019.039
eISSN: 1857-9655
Public Health

Characteristics and Factors Associated with Medical Waste Management Behaviour in Private Dental Health Services in Pekanbaru City, Indonesia

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Citation: Dewi O, Sukendi S, Ikhwan YS, Nazrianti E. Characteristics and Factors Associated with Medical Waste Management Behaviour in Private Dental Health Services in Pekanbaru City, Indonesia. Open Access Maced J Med Sci. 2019 Jan 15; 7(1):157-161. https://doi.org/10.3889/oamjms.2019.039

Keywords: Medical waste; Private dental healthcare; Waste management behaviour; Training; Facilities; Personal protective equipment

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Received: 22-Oct-2018; **Revised:** 15-Dec-2018; **Accepted:** 23-Dec-2018; **Online first:** 14-Jan-2019

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

Abstract

BACKGROUND: Medical waste is a problem when its amount is accumulated as well as the way the private dental healthcare still manages improperly.

AIM: This study aims to define types and the number of medical wastes, also to analyse behaviour toward waste management and its associated factors.

MATERIAL AND METHODS: The research used a quantitative analytic approach and cross-sectional design with 149 private dental practice populations in total. There were 60 dentists obtained using systematic random sampling in Pekanbaru. Data processed by conducting summation medical waste and counting the percentage of behaviour's variables. Data collected within 20 days were processed with dental waste laboratory tests and chi-square analysis.

RESULTS: The result showed that dental, medical wastes average was 0.3 ± 0.07 kg/day which is 69% infectious, 27% toxic, and 4% radioactive. Overall results showed associated factors related to waste management behaviour were knowledge, training attainment, availability of facilities, and the use of personal protective equipment.

CONCLUSION: The numbers of medical waste from dental health services in Pekanbaru were still low. More than half the Dentist had poor behaviour in dental, medical waste management. It is recommended to the dental profession organisation to cooperate with City Health Office to hold management training on medical waste in dental health care to educate and raise dentists' awareness to be able to manage the waste of dental health services properly and by the regulations.

Introduction

According to Environmental Protection Agency, medical waste is all of the residual material generated by health care facilities, such as hospitals, clinics, blood banks, dental practices, veterinary clinics, and medical and laboratory research facilities. Dental practice was one of the health-care facilities that generating as medical waste. Every year, the number of dentists were increased, based on data from the Indonesian Medical Council, the total of dentists in 2018 are 31,630 dentists and 3,786 dentist specialist [1].

Study of 80's Individual Dental Health Care Services in Bandung showed that the numbers of medical waste were reached 142.77 gram per dental practices per day. These wastes consist of 80.45% infectious and potentially infectious waste, 14.25% non-infectious waste, and 5.3% other residual waste. The average waste density of private dental health services is 83.076 kg/m [2].

The waste problem becomes complicated due to health practitioners waste management commitment has not been well established and the

existence of public misperception about medical waste. Health practitioners are still not conducting medical waste management efforts according to the regulations. As stated by Maironah, the reaction of health workers in handling medical waste at the Ulin Banjarmasin Regional General Hospital is still below the standard (61.58% knowledge, 67.31% attitude and 67.30% action) [3]. In Indonesia, the rules applied based on the regulation of The Minister of Health No.1204/2004 and The Minister of Environmental regulation No.56/2017 about The System of Medical Waste Management in Health Services [4].

The result of Khanderwal's study in India, there were 80% dentists who agreed with waste management, while the rest was no response, the ones sorting waste before disposal were only 34% while directly disposing to general waste container were 42% [5]. As many as 68% are unwilling to implement waste management properly due to financial reasons. Nearly 14% of dentists were not aware of various waste categories, and 12% were unaware of the colour coding used to sort out the waste. About 26% of dentists made the mistake of removing sharp objects and dental tissue, and 32% did not dispose of expired drugs properly [6]. This may be caused by several factors that influence the behaviour of waste management on dentists and the other dental officers. Medical waste management on private dental healthcare consists of sorting medical waste based on the label of the type of waste (infectious, toxic and hazardous materials), a collection of storage, transportation, management, and disposal of medical waste [7]. According to Lawrence Green's Theory, behaviour influenced by a variety of factors such predisposing factors (knowledge, attitude), enabling factors (funds, waste collected instrument, personal protective equipment) and reinforcing factor (waste management training) [8].

According to data from Indonesian Dentist Organization (PDGI), the total of dentists in Pekanbaru City is 427 and 306 of it have their dental healthcare practices which are 149 of it are running their private practice [9]. In the early stage, the researcher conducted a survey for 15 dentists in Pekanbaru. It's showed that 11 dentists were managed the wastes without sorting between medical and domestic wastes. And there's only one dentist who delivers infectious wastes to the hospital to be destroyed by the incinerator. Based on this survey, this is then done by this study.

This study aims to define types and the numbers of medical wastes and analyse factors associated with the behaviour of medical waste management in personal dental health services in Pekanbaru.

Methods

This study was a quantitative analytic study with a cross-sectional design. All dentists (149 dentists) with personal practices were selected as populations in this research. Based on a survey in 15's dental practices, it's showed the numbers and types of wastes for 20 days. Data analysis have calculated the average of medical wastes and the percentage of medical wastes based on types such infectious wastes, toxic wastes, and radioactive wastes.

The factors and behaviour of waste management data were conducted with interviewed and observations in selected 60's dentists by systematic random sampling. The questionnaire was delivered to respondents consists of 15 items for knowledge, 9 items for attitude, and 4 items for practices. Every questionnaire was verified by validity and reliability tests. There's also questions related funds, facilities availability, training and personal protective equipment. All of the data were coded and analysed. The results were calculated based on sums and percentages of variables with SPSS vers.21 software and chi-square analysis with the level significance of p-value less than 0.05

Results

There were 60 respondents, consists of 16.7% male (n = 10) and most of them were woman 83.3% (n = 50). Around 76.7% (n = 46%) already had dental practices later than 5 years with the average ages 41-45 years old with 28.3%. The details showed in Table 1.

Table 1: Characteristic's Respondent

No	Characteristic's Respondent	Total (n = 60)	Percentage	95% CI	
				Min	Max
1	Age (years)				
	25 – 30	4	6.7	0.054	0.079
	31 – 35	10	16.7	0.155	0.178
	36 – 40	13	21.6	0.204	0.328
	41 – 45	17	28.3	0.270	
	46 – 50	11	18.3	0.172	0.194
	51 – 55	3	5	0.043	0.057
	56 – 60	2	3.4	0.028	0.040
2	Sex				
	Male	10	16.7	0.155	0.178
	Female	50	83.3		
3	Medical Wastes Management Training	52	86.7	0.863	0.871
	Never	8	13.3	0.010	0.0163
	Ever				
4	Personal Protective Equipment				
	No	10	16.7	0.155	0.178
	Yes	50	83.3	0.828	0.838
5	Funds Availability				
	No	50	83.3	0.828	0.838
	Yes	10	16.7	0.155	0.178
6	Availability of Waste Management Facilities	34	56.7	0.557	0.577
	No or Inadequate	26	43.3	0.427	0.439
	Yes and Adequate				

The total of medical wastes obtained was 4.62 kg/day with the average of medical wastes generated of each dentist was 0.3 kg ± 0.07 kilogram per day. Based on types of dental, medical wastes were such infectious waste 69%, toxic waste 27%, and radioactive waste 4%. The comparison of medical waste based on its percentages is explained in Figure 1 below.

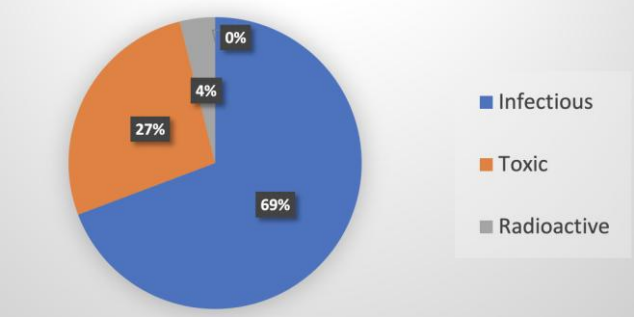

Figure 1: Percentage of Personal Dental Medical Waste Types on Health Services in Pekanbaru

Respondent's knowledge of wastes management was calculated based on the correct scores of 15 items question if the answer more than 75% were corrected then concluded in the high category. On the other hand, if the answer less than 75% categorised low. The results showed that the percentage of high wastes management's knowledge equal with the low knowledge at 50%

Respondent's attitude of waste management was calculated with the correct scores of 9 items question if the answer more than 75% were corrected then concluded in agree category. On the other hand, if the answer less than 75% categorised disagree. The results showed that the percentage of the respondent's attitude was agreed about wastes management were 98% (n = 59). In the other 1.7% (n = 1) respondents were disagree.

Table 2: The percentage of knowledge, attitudes and the act of waste management medical in Pekanbaru

No	Variables	Frequency (n – 60)	Percentage
	Knowledge		
1	less	30	50
	good	30	50
	Attitude		
2	disagree with medical wastes management	1	1,7
	agree with medical wastes management	59	98,3
	agree with the medical waste management		
	The practice of Wastes Management		
	a. Shorting		
	Yes	32	53,3
	No	28	46,7
	b. Storing		
	< 24 jam	36	60
3	≥ 24 jam	14	40
	c. Transporting/Disposal		
	Deliver to incinerator	19	31,7
	Disposal to final storage	36	60
	Buried	3	5
	Burning	2	3,3

Factors related to Medical Wastes Management's Behavior.

Respondents practice of wastes management consists of sorting, store, and transport/destroyed. The categorical for practice divided into correct and incorrect practice based on wastes management regulations of Ministry of Health and Environmental. The appropriate action includes sorting infectious wastes, toxic and radioactive materials, waste storage less than 24 hours to hospital or centre of health services for incinerated the wastes to destroyed. The results showed that there were 31.7% (n = 19) were appropriate and the others 68.3% (n = 41) were inappropriate with the medical wastes regulation. The percentage of knowledge, attitude, and practice are explained in Table 2.

Table 3 showed that variables which strongly influence the behaviour of medical wastes management. Behaviours are influenced by 3 factors such as predisposing factors (knowledge, attitude), enabling factors (funds, wastes collected equipment, personal protective equipment), and reinforcing factors (wastes management training). The results showed that factors which significantly related were knowledge, training, and personal protective equipment, and the availability of wastes management facilities.

Table 3: Factors that influence the behaviour of personal dental, medical wastes management in Pekanbaru

Factors	Wastes Management Behavior				
	Inappropriate	Appropriate	Total	P value	POR (95% CI)
Knowledge					
Less	26 (86.7%)	4 (13.1%)	30 (100%)	0.006	6.5 (1.8-23.2)
Good	15 (50 %)	15 (50%)	30 (100%)		
Attitude					
Disagree	1 (100%)	0	1 (100%)	1.00	-
Agree	40 (67.6%)	19 (31.7%)	59 (100%)		
Participate in Training					
Never	40 (76.9%)	12 (23.1%)	52 (100%)	0.001	23.3 (2.6-208.9)
Ever	1 (12.5%)	7 (87.5%)	8 (100%)		
Personal Protective Equipment					
No or Inadequate	10 (100%)	0	10 (100%)	0.023	1.61 (1.29-2.004)
Yes and Adequate	31 (62%)	19 (38%)	50 (100%)		
Funds Availability					
Not available	39 (78%)	11 (22%)	50 (100%)	1.001	14.18 (2.83-76.6)
Available	2 (20%)	8 (80%)	10 (100%)		
Facilities availability					
Not available	34 (100%)	0	34 (100%)	< 0.001	3.71 (1.97-6.99)
Available	7 (26.9%)	19 (73.1%)	26 (100%)		

Discussion

Most personal dental health services in Pekanbaru were operated by women who have a length of employment over five years. The numbers of women dentists were four times higher because of many people interested in studying in the faculty of dentistry. Women dominate it because the dentist is a feminism occupation. There have been increased numbers of women dentist in Brazil and many countries around the world [10]. The mechanism of behaviour to chooses work related different between men and women, and a tendency to take care of others is partaking of women than in men. Men tend to select dentistry as a possibility of good business, while women get it to base their decisions on the

relations of with others and be emphatic and more able to communicate with a patient. They appear to be less in a hurry and are willing to discuss disease and worry their patients with that which is care and human than a dentist man.

Medical waste produced by dentists in Pekanbaru is still under the average of produced waste in Mumbai India is about 0,5-1,0 pounds per day [11]. This number is still higher than Wulandari's study with the average of licensed personal dental healthcare services in Bandung was about 0.14 pounds each practice dentist every day [2]. This means a personal dentist still produce medical waste in a considerable amount.

Based on its types, personal dental health services generated wool or cotton and infectious gauze contaminated with blood and saliva, toxic wastes as dental acrylic gypsum and radioactive material such amalgam as teeth fillings and unplugged amalgam. Based on Kooviland's study, the most potential infectious waste products are paper towels (gauze) contaminated with blood (3.88 kg), toxic film waste of x-ray blast (2.75 kg) and the most chemical waste is dental printing materials (3.99 kg) [12].

Disposal of infectious medical wastes if other domestic mixed with rubbish have caused them to be contaminated so that there is a selection process the transmission of disease, the material also can cause pollution on the ground. This means that dental, medical waste health services containing the germs of a disease, a toxin and hazardous materials (mercury) can insult the environmental, health, and social economic if they are not being managed correctly.

The results of the descriptive analysis study show that the knowledge of a dentist to all information waste management is equal. The results are higher than Machandra's study that only 42.5% a dentist who has of good knowledge about waste management [13]. Dentist's attitude who agreed to personal dentist medical in health services is very dominant compared with a dentist who disagrees to do waste management. This is indicated from Sanjev's study that 90% dentist agrees with the encoding colour on the receptacle collection waste [14]. The act of dentists to manage are experiencing high levels that have not been by the regulations that had determined by the government in Indonesia. Sorting waste based on its kind still has not been carried out. Waste storage is still done more than 24 hours. The extermination of waste is experiencing high levels of in a conventional manner that is thrown on the trash domestic, bury and burn the waste.

The Lawrence Green's theory, behaviour influenced by 3 factors namely predisposing (knowledge, attitude), enabling (funds, a waste collector, personal protective equipment) and reinforcing factor (training waste management). Knowledge has a signature to manage the action; this is possible because for knowledge is the basis of a person to make decisions and take action right or wrong. And so attitude, attitude is an unobserved directly that uncertain realised be an action. To realise the attitude to be the kind of the act of they are also required to some factors enabling as well as reinforcing factors [8]. In this study, there was not an effect of attitude toward waste management behaviour, although there is some approval without the availability of fund and facilities, then an appropriate behaviour of waste management will not be possible.

In this study, enabling factors like the associated waste management and personal protective equipment significantly affect behaviour manage the appropriate regulations. While enabling factor in the form of funds are not affect behaviour significantly. Supplementary equipment to waste management commonly used by dentists in Pekanbaru including the trash domestic are black, a box for storage waste sharp as needles, scalpel and sewing needles. Personal protective equipment commonly used by dentist and the dental hygienist is gloves, goggles and a mask.

Reinforcing factors such as training management waste ever followed by dentists are still few (13.3) per cent with another 86.7 per cent dentist in Pekanbaru gone after said there is no waste management training. According to Notoatmodjo, there are factors related to increasing training employees that have capability or skill occupying a job or a particular task. Training in general stress to psychomotor ability, although based on knowledge and attitudes. The purpose of training medical management waste is that health workers have sufficient knowledge and skills in the management of waste, and possible environmental health care facilities, safety and security for the officers and the community. Based on the data, that a dentist who had followed the training is a dentist who works at the Centre of Health Services and Hospitals as civil servants. They can attend the program for Training programs done by the City Health Department and Provinces.

Availability of facilities such as a labelled rubbish container, covered and secured trash cans made of tough and easy to clean material, safety boxes to stored sharp, hazardous, and toxic materials were all significantly associated (OR = 3.2; p-value = 0.0; CI 95% {1.97 – 6.99}). This means the respondents who did not have facilities that meet the requirements were 3.7 times more likely not manage the wastes properly compared to the ones who have the facilities. This is in line with Rahno's study that the availability of waste management facilities was associated with its management behavior, therefore, maintaining environmental health would not a priority to develop better healthcare if it's facilities were unavailable and not adequate [15].

In conclusion, the numbers of medical waste from dental health services in Pekanbaru were still low. In the future, if dental, medical wastes are not well managed, it will be a potential risk for raising health problems and environmental health risk. Especially, in health, most of the medical wastes are potentially infectious, toxic, and radioactive. More than half the Dentist had poor behaviour in dental, medical waste management because most of them didn't follow the Indonesian regulation of waste management well. This behaviour was affected by knowledge, training, facilities availability, medical wastes management materials, and personal protective equipment.

It is recommended to all dentists specifically on the private dental health services of Pekanbaru to reduce their medical wastes and to use safe and environmental-friendly materials and tools for dental and oral care. It is also suggested to not only sorting out between medical and non-medical wastes and storing waste on a suitable container but also disposing of it according to applicable provisions and professional organisations. The latter must be in collaboration with Pekanbaru City Health Office to conduct training on medical waste management especially for dentists in private dental health care services. Those are all important efforts to improve the behaviour of better waste management on dental practices.

Acknowledgement

We are grateful to all dentists in Pekanbaru who voluntarily helped this research. This study was successful received ethical clearance from Ethical Committee-Universitas Riau No. 106/UN.19.5.1.1.8/UEPKK/2018, to protect human rights and welfare subject of medicine and health study.

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ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2019 Jan 15; 7(1):162-173.
https://doi.org/10.3889/oamjms.2019.010
eISSN: 1857-9655
Review Article

The Diagnostic and Clinical Approach to Pediatric Myocarditis: A Review of the Current Literature

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Citation: Bejiqi R, Retkoceri R, Maloku A, Mustafa A, Bejiqi H, Bejiqi R. The Diagnostic and Clinical Approach to Pediatric Myocarditis: A Review of the Current Literature. Open Access Maced J Med Sci. 2019 Jan 15; 7(1):162-173. https://doi.org/10.3889/oamjms.2019.010

Keywords: Myocarditis; Cardiomyopathy; Myocardium; Ventricular dysfunction; Cardiac magnetic resonance; Endomyocardial biopsy; Immunomodulation; Immunosuppression

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Received: 01-Oct-2018; **Revised:** 01-Dec-2018; **Accepted:** 02-Dec-2018; **Online first:** 04-Jan-2019

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

Abstract

Myocarditis is an inflammatory disease of the myocardium with a broad spectrum of clinical presentations, ranging from mild symptoms to severe heart failure. The course of patients with myocarditis is heterogeneous, varying from partial or full clinical recovery in a few days to advanced low cardiac output syndrome requiring mechanical circulatory support or heart transplantation. Myocarditis is a very heterogeneous disease, especially in the pediatric age group as worldwide disease myocarditis has been defined by the World Health Organization/International Society and Federation of Cardiology as an inflammatory disease of the heart muscle diagnosed by established histological, immunologic, and immunohistological criteria. Pediatric myocarditis remains challenging from the perspectives of diagnosis and management. Multiple etiologies exist, and the majority of cases appear to be related to viral illnesses. Enteroviruses are believed to be the most common cause, although cases related to adenovirus may be more frequent than suspected. The clinical presentation is extremely varied, ranging from asymptomatic to sudden unexpected death. A high index of suspicion is crucial. There is emerging evidence to support investigations such as serum N-terminal B-type natriuretic peptide levels, as well as cardiac magnetic resonance imaging as adjuncts to the clinical diagnosis. In the future, these may reduce the necessity for invasive methods, such as endomyocardial biopsy, which remain the gold standard. Management generally includes supportive care, consisting of cardiac failure medical management, with the potential for mechanical support and cardiac transplantation. Treatments aimed at immunosuppression remain controversial. The paediatrics literature is extremely limited with no conclusive evidence to support or refute these strategies. All these summarised in this article and the listed current literature showed that there is no consensus regarding aetiology, clinical presentation, diagnosis, and management of myocarditis in pediatric patients.

Introduction

Myocarditis, the inflammation of the muscular walls of the heart, remains a diagnostic challenge in the clinical setting because of the variability of presentations in the pediatric population. It has the potential for significant morbidity including diminished cardiac function and cardiac failure, occasionally necessitating aggressive circulatory support [1]. Dilated cardiomyopathy is a significant sequela of myocarditis and is a common indication for cardiac transplantation. Myocarditis is also identified as the cause of sudden unexpected death in young patients. Nevertheless, this condition remains somewhat enigmatic from the perspective of diagnosis, and there exists considerable variation in management practices. This article aims to summarise the current research and emphasise pertinent aspects of myocarditis for the general paediatrician [2].

Definition

In 1995, the World Health Organization created the following definition: ‘‘Inflammatory cardiomyopathy is defined by myocarditis in association with cardiac dysfunction. Myocarditis is an

inflammatory disease of the myocardium and is diagnosed by established histological/immunological, and immunohistological criteria. Idiopathic, autoimmune, and infectious forms of inflammatory cardiomyopathy are recognised'' [3]. Notably, this inflammation occurs in the absence of ischemia.

Epidemiology

The true incidence of myocarditis in the pediatric population is difficult to measure due to the wide variety of presentations and the lack of diagnostic protocols [4]. Over half of all cases of myocarditis occur in patients below the age of 40 years. A post-mortem analysis showed a prevalence of 3.5-5% [5], but the rate of clinically significant cases more closely approximates 0.1-0.6% [6]. Patients of all ages may be affected, but the majority of cases occur in infants and teenagers; more than one-half of all cases are seen in the first year of life [7]. The reason for this unusual bimodal age distribution is currently unclear. By screening electrocardiograms in Japan, the approximate incidence of myocarditis is estimated to be about 7 per 60,000 (0.012%) asymptomatic children [8], [9]. A recent retrospective review reported the prevalence of myocarditis to be 0.5 cases per 10,000 emergency department visits [10]. Children in this study were diagnosed using history, clinical exam, and various supportive investigations. An approximate incidence may lie between 0.15% and 0.6% in the overall population based on postmortem histology [11]. As myocarditis is also associated with sudden cardiac death in young patients, this may further confound estimates of incidence and prevalence [12], [13].

Aetiology

The multiple etiologies of myocarditis are summarised in Table 1 [14]. It is caused primarily by numerous infectious agents, but it may also accompany autoimmune disease, hypersensitivity reactions, and toxins [15]. Infectious exposures, especially viruses, account for the vast majority of pediatric myocarditis; in Canada and the United States, whereas globally the most common cause is Trypanosoma cruzi (Chagas disease) [16]. The majority of paediatric cases are believed to be due to adenoviruses and enteroviruses, such as coxsackieviruses A and B, parvovirus, echovirus, and poliovirus [17], [18]. The relative frequency of cases related to these viruses is variable, and there is much discussion as to which is more common. In the past, enteroviruses, especially the coxsackieviruses, were considered the most common aetiology. A recent study suggested that adenovirus may be a more significant pathogen than previously recognised [19]. Among patients given a clinical diagnosis of myocarditis, those with positive adenovirus polymerase chain reaction were less likely to have a corresponding histological diagnosis of acute myocarditis compared with the enterovirus group (40% versus 79%) [20]. This suggests that adenovirus is associated with a lesser degree of inflammation in the myocardium. This may suggest a higher prevalence of adenovirus than suspected [21].

A subsequent study reviewed etiologies for biopsy-confirmed myocardial inflammation and found parvovirus B 19 to be the most common, followed by enterovirus, human herpesvirus 6, and then adenovirus [21], [22]. Following the H1N1 Influenza A pandemic, there were case reports of associated myocarditis. Of the 80 confirmed viral infections reported by one group, there were four cases of associated myocarditis, three of which presented with fulminant myocarditis: one patient had a fatal outcome due to acute atrioventricular block, and two required extracorporeal membrane oxygenation support due to severely reduced left ventricular systolic function.[23,24] Extracorporeal membrane oxygenation is implemented in many paediatrics tertiary centres for either respiratory failure or cardiac compromise in cases of H1N1 infection. Further population data are required to clarify the clinical course of H1N1-associated myocarditis [25].

Table 1: Various causes of myocarditis

Aetiology	Examples
Infectious	*Viral*: adenoviruses, echoviruses, enteroviruses (eg, coxsackieviruses), herpesviruses (human cytomegalovirus, Epstein-Barr virus, human herpesvirus 6), hepatitis C virus, human immunodeficiency virus, influenza A virus, parvovirus B19 *Bacterial:* chlamydia, Corynebacterium diphtheria, Klebsiella, Salmonella, Legionella, Mycobacterium tuberculosis, mycoplasma, staphylococcus, streptococcus A, Streptococcus pneumonia, Tryponema Pallidum, Haemophilus influenzae *Fungal:* actinomyces, aspergillus, candida, cryptococcus *Helminthic*: Echinococcus granulosus, Trichinella spiralis *Protozoal:* Toxoplasma gondii, Trypanosoma cruzi *Rickettsial:* Coxiella burnetti, Rickettsia typhi *Spirochetal:* Borrelia burgdorferi, leptospira, Treponema pallidum
Autoimmune diseases	Celiac disease, Churg-Strauss syndrome, Crohn disease, dermatomyositis, giant cell myocarditis, hypereosinophilic syndrome, Kawasaki disease, lupus erythematodes, lymphofollicular myocarditis, rheumatoid arthritis, sarcoidosis, scleroderma, ulcerative colitis
Hypersensitivity reactions	Penicillin, ampicillin, cephalosporins, tetracyclines, sulfonamides, antiphlogistics, benzodiazepines, clozapine, loop and thiazide diuretics, methyldopa, smallpox vaccine, tetanus toxoid, tricyclic antidepressants
Toxic reactions to drugs	Amphetamines, anthracyclines, catecholamines, cocaine, cyclophosphamide, 5-fluorouracil, phenytoin, trastuzumab
Toxic	Amitriptyline, Amphotericin B, Cannabis, Carbon monoxide, Cyclophosphamide, Electric shock, Ethanol, Hymenoptera, Isoniazid, Lead, Lidocaine, Methyldopa, Nonsteroidal anti-inflammation, Phenytoin, Snake or scorpion venom,
Others	Arsenic, copper, iron, radiotherapy, thyrotoxicosis

Pathology and pathophysiology

There are several proposed mechanisms of myocardial injury in viral myocarditis [4], [5], [21]. The

inflammatory process begins when the body's immune cells (the cells that fight infection) penetrate the heart tissue. These immune cells become activated and produce chemicals that can cause damage to the heart muscle cells. There are thickening and swelling of the heart muscle. All four chambers of the heart may be affected and become enlarged [24], [25]. These include the excessive immune-mediated destruction of the myocardium by infiltrating immune cells targeting the infected cardiomyocytes; autoimmune-mediated destruction of cardiac cells by circulating autoantibodies; and direct virus-induced cardiac myocyte injury. It is proposed that three phases of viral myocarditis exist, beginning with a period of viral replication, followed by acute injury to the myocytes, and subsequent evolution of dilated cardiomyopathy, associated with profound alteration in the extracellular matrix of heart muscle [4], [8], [9], [22].

Mechanism of developing the myocarditis after infection of cardiac endothelial cells or cardiac myocytes by virus leads to direct cellular damage. A subsequent innate and adaptive immune response develops that can evolve into resolution and healing or dilated cardiomyopathy resulting from severe initial injury, persistent inflammation, or persistent viral infection. Adapted from Schultheiss et al., [5] with permission of the publisher.

Damaged muscle cells may heal over time, or there may be cell death followed by scar formation. If this process is extensive and a large portion of the heart is involved, the heart's ability to pump blood is impaired. As a result, the key organs and tissues in the body do not get enough oxygen and nutrients and cannot get rid of waste products. This is often referred to as congestive heart failure. Up to 27% of dilated cardiomyopathy cases in children may be attributable to viral myocarditis [23], [24].

Clinical presentation

Cardiovascular syndromes observed with pediatric myocarditis are sudden death, arrhythmias, chest pain, myocardial infarction, acute heart failure with a dilated cardiomyopathy phenotype.

Sudden death

Sudden death in the pediatric population is commonly associated with myocarditis, and it may be the presenting feature of myocarditis, and in these cases, the diagnosis is often made histologically. It is suggested that some cases may be the result of inflammatory infiltrates that act as arrhythmogenic foci, leading to fatal arrhythmia. Studies of sudden infant death syndrome have linked infection with viruses such as enterovirus, adenovirus, parvovirus B19, and Epstein-Barr virus and myocarditis to sudden infant death syndrome victims [25], [26]. A broad clinic-pathological classification system can be used in which there are four categories of myocarditis in the setting of a viral illness. Myocarditis accounted for ≈ 9% of sudden deaths in young athletes in the United States in whom a confirmed cardiovascular event was documented. Despite the existence of echocardiographic and endomyocardial biopsy criteria for myocarditis and resulting cardiomyopathy, expert opinion currently suggests that the diagnosis of ‘‘probable’’ myocarditis is based on clinical judgement [27].

Arrhythmias

Symptoms such as palpitations and syncope occur in pediatric myocarditis patients even in the absence of heart failure or demonstrable reduction of left ventricular function. Pediatric ventricular arrhythmias in structurally normal hearts and ventricular tachyarrhythmias in athletes have been associated with myocarditis [28], [29]. Myocarditis should always be considered in a child with acquired complete heart block. Lyme carditis and Chagas disease have been associated with complete heart block. Although the majority of children may recover atrioventricular conduction, most patients need implantation of a permanent pacemaker [30], [31], [32].

Chest Pain/Myocardial Infarction

More than 20 years ago, it was recognised in adults and children that myocarditis might mimic myocardial infarction with severe symptomatic chest pain, characteristic ECG findings, and elevation of serum creatinine kinase, in the presence of normal coronary angiograms. Coronary spasm has been observed with this presentation in adults [33], [34]. Parvovirus B19 has been found in the myocardium of such patients, as well as adenovirus and Epstein-Barr virus [35]. In a study of 4436 patients presenting to a pediatric emergency department with chest pain, 24 had a confirmed cardiac origin, of whom four were diagnosed with myocarditis [36]. A recent study of pediatric patients, presenting with myocarditis and a chest pain/myocardial infarction pattern, found that all had elevations of cardiac troponin I (peak range, 6.54–64.59 ng/mL), in the presence of normal values of erythrocyte sedimentation rate and C-reactive protein. Echocardiograms demonstrated a mild reduction in left ventricular function in 57% of the patients, and 5 of 6 patients demonstrated cMRI findings consistent with myocarditis. The prognosis was good with a resolution of cardiac abnormalities within a few weeks, similar to the adult experience [37], [38].

Acute heart failure with dilated cardiomyopathy phenotype

The classic presentation of myocarditis is the development of symptoms of heart failure with a dilated cardiomyopathy phenotype a few weeks after a history compatible with a viral illness, including fever, myalgias, and respiratory or gastrointestinal symptoms. Myocarditis accounts for 30% to 35% of children with dilated cardiomyopathy phenotypes in the Australian and North American pediatric cardiomyopathy registries and for 22% of new-onset left ventricular dysfunction in the United Kingdom [38, [39], [40].

Fulminant myocarditis is a distinct subset of acute myocarditis characterized by heart failure with severe hemodynamic compromise requiring inotropic or mechanical circulatory support and at least 2 of the following criteria: fever, distinct onset of heart failure symptoms within a 1- to 2-day period, and a history consistent with viral illness within the 2 weeks before hospitalization. Fulminant myocarditis is associated with symptoms of significant left ventricular dysfunction and unexpected cardiac failure within 2–3 weeks of the onset of viral infection [40], [41]. Fulminant myocarditis has been described in children with mortalities varying from 48.4% in Japan to 9% in France. Despite the severe presentation, outcomes are substantially better than in adults with acute myocarditis. Acute myocarditis presenting with severe heart failure, arrhythmias, and lack of responsiveness to supportive care after 1 to 2 weeks leads to concern for giant cell myocarditis, which can be diagnosed by biopsy and has a grim prognosis, although is responsive to immunosuppression [42].

Acute myocarditis in children has been associated with a good prognosis with a good chance for ultimate recovery of left ventricular dysfunction [16], [17]. Within the North American Pediatric Cardiomyopathy Registry (PCMR), 372 myocarditis patients diagnosed by biopsy (n = 119) or clinical criteria (n = 253) were compared with 1123 patients diagnosed with idiopathic dilated cardiomyopathy. Outcomes were similar in the biopsy and clinically diagnosed myocarditis patients and substantially better than in children diagnosed with idiopathic dilated cardiomyopathy. These results are similar to an estimated 58% spontaneous recovery in acute adult myocarditis gleaned from an adult meta-analysis [43].

‘‘Subacute myocarditis’’ can be described as moderate left ventricular dysfunction and has less distinct clinical features of the disease.

‘‘Chronic active myocarditis’’ is associated with moderate left ventricular dysfunction, indistinct symptoms, and endomyocardial biopsy reveals ongoing inflammation, myocardial damage, and active scar tissue.

‘‘Chronic persistent myocarditis’’ is described in the setting of normal left ventricular function and palpitations or atypical chest pain, with persistent inflammation on biopsy [44].

Myocarditis in children is associated with a high rate of congestive heart failure, hospitalisation, intensive care unit stay, and use of inotropic support at the time of diagnosis compared with children with idiopathic dilated cardiomyopathy. A recent study of hospitalised patients in the United States found that nearly half of the patients required inotropic support, 37.5% required mechanical ventilation, and 7.4% required extracorporeal membrane oxygenator (ECMO) support [23], [45].

Diagnosis

History and physical examination

The diagnosis of pediatric myocarditis is especially challenging because of the variable clinical presentation, ranging from asymptomatic patients with only subtle findings on an electrocardiogram to fulminant cardiac failure and sudden death. Diagnosis remains largely based on clinical judgement, supported by ancillary tests; therefore, a high index of suspicion is necessary. Clinical features are often those of congestive cardiac failure and myocarditis is identified as the most common cause of new-onset cardiac failure in previously well children [22], [46]. Even patients with mild symptoms are at risk of deterioration, and therefore early diagnosis is important in establishing appropriate monitoring and supportive care.

The majority of patients present with a resting tachycardia, but other cardiac-specific signs such as pallor, hypotension, oedema, and hepatomegaly occur in only a minority of cases. Chest pain, syncope, and palpitations may also be presenting complaints. Fever may or may not be present. There are several paediatric studies that have examined the most common signs and symptoms of patients with myocarditis. Shortness of breath, respiratory distress, or an abnormal respiratory exam were commonly observed [22], [47]. In two studies, 84% of patients required more than one visit to a physician within 14 days before the diagnosis of myocarditis or dilated cardiomyopathy was made [23].

Children were often given an initial diagnosis of asthma or pneumonia [5]. Resting tachycardia is also an important subtle feature, and although this is not always a consistent finding, it may be the only finding of mild disease [5], [48]. Isolated vomiting is observed, and this may be due to gut ischaemia secondary-to-low cardiac output. Isolated gastrointestinal symptoms of anorexia, abdominal pain, and vomiting may also occur. Hepatomegaly is a

common feature on physical exam and may present as non-specific abdominal pain [5], [23]. In contrast to adults with myocarditis, chest pain is seldom reported in younger children though it may be a more common feature in adolescents. A case series of several teenage patients diagnosed with myocarditis described symptoms of severe chest pain, in combination with elevated cardiac troponin-I, ST-segment, and T-wave changes [24], [48].

Investigations

Despite many diagnostic modalities, suspicion of myocarditis is mainly based on clinical criteria. Diagnostic workup should include baseline investigations listed in Table 3. Abnormal troponin levels may support a diagnosis of myocarditis, but there are limited data in the current pediatric literature [29], [49]. An elevated creatine kinase level may also be observed in myocarditis, although it is a non-specific marker.

It has been suggested that, in comparison with creatine kinase, cardiac troponin-T may provide better sensitivity for detecting micronecrosis in myocarditis because of a proportionally higher and longer-lasting elevation of serum levels [30], [50]. In the Myocarditis Treatment Trial, elevations of cardiac troponin I occurred more frequently than did elevations of creatine kinase muscle and brain subunits in patients with biopsy-proven myocarditis [31], [51]. There was one pediatric study that illustrated a cut-off point for a cardiac troponin T level of 0.052 nanograms per millilitre for diagnosing acute myocarditis with a sensitivity of 71% and specificity of 86%, although there was no correlation between cardiac troponin T level and ejection fraction [32], [52].

Use of the N-terminal segment of the B-type natriuretic peptide prohormone levels may be helpful in the setting of myocarditis, especially concerning recovery. In one study, elevated N-terminal B-type natriuretic peptide levels correlated well with clinical status and echocardiographic findings in patients with dilated cardiomyopathy or myocarditis [33], [53], [54]. N-terminal B-type natriuretic peptide levels had 78% sensitivity and 100% specificity for the diagnosis of persistent left ventricular dysfunction. Patients who recovered had N-terminal B-type natriuretic peptide levels that were within normal limits [55].

Chest X-ray findings often include cardiomegaly and may demonstrate pulmonary oedema, infiltrates, or pleural effusions [6], [34].

Electrocardiogram ECGs are virtually always abnormal in children with myocarditis, found in over 93% of patients but a normal ECG does not rule out the possibility of the disease [23], [56]. ECG abnormalities, however, are widely variable, and there is not one specific abnormality that occurs with enough frequency to be a specific marker. The most common abnormalities include sinus tachycardia, criteria for ventricular hypertrophy, ST segment, and T-wave abnormalities. Premature contractions and a wide variety of tachyarrhythmias and bradyarrhythmias occur in myocarditis, including complete atrioventricular block, atrial and ventricular delays and prolongation of QT intervals may also occur. The combination of an electrocardiogram with chest X-ray in the setting of possible myocarditis has very good sensitivity for the diagnosis [4], [13], [57].

Echocardiographic findings may include increased ventricular end-systolic or diastolic dimensions, reduced shortening or ejection fractions, atrioventricular valve regurgitation, and regional wall motion abnormalities [36]. Echocardiography is also important to rule out coronary abnormalities as a cause of depressed myocardial function. Abnormal right ventricular function on echo may be associated with an increased likelihood of adverse outcomes including cardiac transplantation or death [37]. Other methods of non-invasive imaging are being investigated in the setting of myocarditis.

Nuclear medicine techniques have not been used widely in paediatrics, in part due to concerns of excessive radiation exposure for children [38].

Cardiovascular magnetic resonance imaging is a widely accepted tool to assess myocarditis. This technique provides a detailed not only functional and morphological assessment of the heart, but also reliable visualisation of tissue markers of myocarditis including oedema, inflammation, and fibrosis [41], [43]. Previously, oedema could not be well imaged; however, cardiac magnetic resonance is now able to visualise myocardial oedema without using radiation or contrast agents. Currently, cardiac magnetic resonance may be considered the single best non-invasive imaging study for this indication, and standardised protocols for cardiac magnetic resonance imaging of myocarditis are available [42]. In acute myocarditis, cardiac magnetic resonance assessment of myocardial oedema is performed using T2-weighted short TI inversion recovery imaging in which regions of myocardial oedema appear bright, whereas fat and blood signals are suppressed.

The sensitivity and specificity of T1-weighted images in patients with suspected chronic myocarditis were 62% and 86%, respectively, using immunohistological methods as the gold standard [39]. Similar to myocardial oedema, early enhancement normalises approximately 4 weeks after the clinical presentation and therefore may be used as a marker of the acuity of myocardial injury [23]. In a small proportion of patients, the early enhancement may persist and is correlated to reduced cardiac function. It is not currently known whether persistently elevated early enhancement truly reflects an ongoing

viral infection. The cardiac magnetic resonance technique of gadolinium late enhancement allows visualisation of myocardial necrosis and fibrosis associated with myocarditis.[58] Gadolinium accumulates in regions of fibrosis, leading to increased signal intensity in late enhancement images. In the setting of myocarditis, the pattern of myocardial fibrosis varies considerably but typically involves patchy focal fibrosis of the sub-epicardium or mid-myocardial wall, rarely extending to the sub-endocardium [41], [43]. The presence of myocardial fibrosis detected by cardiac magnetic resonance late enhancement is more common in males and younger patients, and the pattern of myocardial injury sustained in young patients tends to be more regional and more severe, with a higher incidence of irreversible myocardial scarring [28]. This pattern of myocardial injury may explain why younger patients are at risk of adverse cardiac outcomes — the presence of gadolinium late enhancement 4 weeks after the acute presentation is also correlated with reduced left ventricular ejection fraction and clinical symptomatology at 30-month follow-up [58].

To standardise cardiac magnetic resonance imaging to diagnose myocarditis, the International Consensus Group on Cardiovascular Magnetic Resonance created new criteria, known as the ‘‘Lake Louise Criteria’’. These recommendations describe indications for cardiac magnetic resonance in patients with suspected myocarditis, cardiac magnetic resonance protocol standards, terminology for reporting cardiac magnetic resonance findings, and diagnostic cardiac magnetic resonance criteria for myocarditis. These guidelines report that the greatest balance of diagnostic sensitivity and specificity for acute myocarditis is achieved with the presence of any two or more of the following three criteria: myocardial oedema (T2 short TI inversion recovery), hyperaemia/capillary leak (early enhancement), or myocardial fibrosis (late gadolinium enhancement) [59], [60].

The gold standard for the diagnosis of myocarditis remains endomyocardial biopsy; however, this approach has significant limitations [26]. According to the Dallas Criteria, endomyocardial specimens are considered diagnostic of active myocarditis, if routine light microscopy reveals infiltrating lymphocytes and myocytolysis [26]. Borderline or ongoing myocarditis is defined as lymphocytic infiltration in the absence of myocytolysis. A biopsy is considered negative, if both lymphocytic infiltration and myocytolysis are absent [27], [61].

A potential reason to proceed with biopsy is that the presence of immune complexes and complement deposits supports an immune-mediated process; therefore, the management may differ from that of the case of viral infection. For example, immune suppression is not indicated in cardiomyopathy but may be worthwhile in viral myocarditis. A biopsy may be useful in the setting of eosinophilic infiltrates, which could suggest a hypersensitivity response to an exogenous agent. In this case, once the histology is confirmed, the offending agent can be removed, and the clinical picture may improve. A significant limitation of cardiac biopsy is that myocarditis affects the myocardium in a patchy fashion, and therefore negative biopsy may not exclude disease [62], [63].

There remains controversy as to whether or not to proceed with biopsy, especially since biopsy can be associated with arrhythmia and perforation of cardiac tissue. Pediatric literature also suggests that the highest rate of complication occurs in patients being evaluated for myocarditis, as well as those requiring inotropic support [49], [63]. Despite the overall complication rate being relatively low (1-6%), with mortality between 0.0% and 0.4%, risk-benefit analysis occurs on a case-by-case basis [49], [64].

Table 2: Diagnostic classification for patients with myocarditis

Criteria	Pathological Confirmation	ECG or Imaging
Possible subclinical acute myocarditis In the clinical context of possible myocardial injury without cardiovascular symptoms but with at least 1 of the following Biomarkers of cardiac injury raised ECG findings suggestive of cardiac injury Abnormal cardiac function on echocardiogram or cardiac MRI	Absent	Needed
Probable acute myocarditis In the clinical context of possible myocardial injury with cardiovascular symptoms and at least 1 of the following: Biomarkers of cardiac injury raised ECG findings suggestive of cardiac injury Abnormal cardiac function on echocardiogram or cardiac MRI	Absent	Needed
Definite myocarditis Histological or immunohistological	Needed	Not needed

Treatment

Despite improved understanding of the pathogenesis of myocarditis, treatment strategies remain controversial, and the general approach is supportive. The stabilisation of children who present with hemodynamic compromise and early involvement of pediatric cardiology specialists are crucial. Treatment of congestive cardiac failure may include diuretics, afterload reduction, inotropic support, anticoagulation, arrhythmia management, and ventilatory support [64], [65].

Aggressive administration of intravenous fluids initially may exacerbate cardiac failure. Transfusions of packed red blood cells may be required to optimise oxygen carrying capacity.

The use of inotropes such as dopamine, dobutamine, and milrinone may be necessary. Afterload reduction, if it can be tolerated, with

phosphodiesterase inhibitors such as milrinone can be extremely useful. It is also suggested that the early introduction of angiotensin-converting enzyme inhibitors, as well as beta-blockers, once the acute decompensatory phase has begun to resolve, may be beneficial. These medications may help prevent the remodelling that evolves to dilated cardiomyopathy as well as relieving vasospasm to some degree [17], [18]. In pediatric patients, carvedilol was suggested in this regard but has not been studied extensively [67]. Anticoagulation may be recommended if the ejection fraction is severely decreased or in the setting of atrial arrhythmias [55]. Some patients may require ventilatory support as a means of reducing cardiac demand. Ventilation and oxygenation may be best managed with continuous positive airway pressure or other non-invasive methods, for several reasons. Increased intrathoracic pressure via nasal or mask continuous positive airway pressure may help to reduce left ventricular afterload by reducing transmural myocardial wall tension, without causing the hypotension associated with pharmacologic afterload reduction. Intubation medications can cause hypotension and acute cardiovascular collapse; therefore, continuous positive airway pressure may also circumvent this and is an excellent adjunctive therapy for decompensated cardiac failure [43], [55]. Those with more severe cases of myocarditis may require circulatory support in the form of extracorporeal membrane oxygenation or ventricular assist devices.

Immunomodulation, immunosuppression and antiviral therapy

Animal studies have suggested that myocarditis has a 3-phased course [31]. Phase 1 involves initial direct myocardial injury from the actively replicating virus or the innate immunological response from infection of cardiac myocytes, fibroblast, or endothelial cells. Phase 2 is marked by activation of antigen-specific immunity involving T cells, B cells, and antibody production. Various chemokines are present that may contain the inflammatory response but extend tissue injury. Development of autoantibodies and persistent T-cell activation can be induced by antigens intrinsic to the myocardium that cross-react with viral peptides (molecular mimicry). Ultimate outcomes may vary. Negative immune modulation may occur rapidly after the elimination of the infectious pathogens, leading to a cessation of the inflammatory response with a complete recovery or little long-term myocardial damage. However, phase 3 may occur in which acute myocarditis leads to a chronic dilated cardiomyopathy. This may result from severe myocardial injury caused by the acute event; an ongoing inflammatory, autoimmune process that may occur without the persistent presence of virus in the myocardium (inflammatory dilated cardiomyopathy); or ongoing direct injury from virus with or without a persistent myocardial inflammatory response (viral heart disease) [7], [8], [66], [67].

An area of significant controversy involves the use of immune suppression and immune modulators in the context of myocarditis. There are large-scale adult studies concerning immune suppression, and overall the data have shown some limited, short-term response to these therapies. In two large adult prospective studies, immunosuppression including prednisone with or without azathioprine or cyclosporine was used [68], [69]. With prednisone alone, there was a modest improvement in left ventricular function in patients with evidence of inflammation on biopsy; however, this improvement was not sustained. The Myocarditis Treatment Trial found no significant improvement in function and no difference in survival when compared with controls [2], [70].

Despite the existence also of a handful of uncontrolled studies that showed benefit with various immunosuppressive regimes, some meta-analyses of adult studies did not show a significant beneficial effect of immunosuppression [6], [71]. It is always difficult to extrapolate the findings of adult studies to the pediatric population. Pediatric studies are limited, and there are no randomised controlled trials. Many used prednisolone and did find some benefit, though not always statistically significant, by adding a second agent. In one trial, patients were stratified into either standard treatment, or given one of three immunosuppressive therapies, prednisolone, prednisolone and azathioprine, or prednisolone and cyclosporin A [70]. The groups receiving immunosuppressive treatment with a second agent, in addition to the prednisolone, showed improved hemodynamic parameters, as well as histological improvement in inflammation [72]. Some small case series suggested some clinical benefit to immunosuppressive treatment, again using corticosteroids with an additional agent; however, most were small, uncontrolled studies [22] A meta-analysis of pediatric studies between 1984 and 2003 systematically reviewed the impact of immunosuppressive therapy in children with myocarditis and overall found no consistent benefit [73. This included nine pediatric studies, all of which were small and very heterogeneous in design. Many of the studies were retrospective and uncontrolled, introducing significant bias.

Intravenous immunoglobulin was also examined as a potential immunomodulating therapy to decrease inflammation potentially. Despite some studies observing a trend towards improved cardiac status following treatment, there are no randomised controlled trials using intravenous immune globulin in children with myocarditis. A few authors conducted a

comprehensive multi-database search including patients of all ages with myocarditis treated with intravenous immune globulin. They concluded that if there is evidence that ongoing, active infection may be causing persistent cardiac dysfunction, intravenous immune globulin may be helpful [74]. Even if post infectious inflammation is the most likely aetiology, intravenous immune globulin may still be beneficial by preventing the formation of cytokines [16], [17]. There is one multi-centre adult randomised controlled trial using intravenous immune globulin [75]. They found no significant difference in rates of survival, cardiac transplantation, use of ventricular assist devices, improvement in ventricular function, or functional capacity at 1 year of follow-up. A Cochrane review of intravenous immune globulin in dilated cardiomyopathy or myocarditis showed no benefit in adults; however, some case reports and case series suggested that adults treated with intravenous immune globulin did exhibit improved cardiac function [15], [16], [28], [76]. Some case reports and series in children have shown improved ventricular function and clinical improvement after high-dose intravenous immune globulin, however with small numbers.[77] Overall, it was felt that the validity of many of these studies was compromised due to the lack of control groups. There was one case report of coxsackievirus A19 myocarditis in which intravenous immune globulin was not effective, and another case series found no additional benefit by adding intravenous immune globulin to steroid treatment [78].

Studies using immunosuppressive or immunomodulating treatments, therefore, were difficult to translate into an effective, routine therapy for children or adults with myocarditis. Many patients with myocarditis have spontaneous improvement. It is difficult to know if the observed improvement after treatment with immunosuppression or intravenous immune globulin is attributable to treatment versus the natural course of the disease. Potentially, some aetiologic types of myocarditis respond differently, and certain histologic characteristics may make specific patients better responders than others [9], [11], [79]. Further studies are needed to determine the possible utility of immunosuppressive or immunomodulating therapy.

Therapy for advanced heart failure/cardiogenic shock in pediatric myocarditis

Acute pediatric myocarditis is commonly associated with severe, progressive heart failure. The majority of patients receives care in an intensive care unit at presentation and is treated with intravenous inotropes [80]. Mechanical circulatory support is frequently required when pharmacological therapy is ineffective, as reflected in evidence of elevated blood lactate levels and evidence of end-organ dysfunction [32]. Most commonly, ECMO support is used. ECMO is currently used in ≈ 20% of American children hospitalised with myocarditis [81]. Some single-centre studies have reported hospital discharge rates of ≈ 80% in pediatric myocarditis requiring ECMO support, with ≈ 60% of the patients experiencing myocardial recovery. Multicenter data from the Extracorporeal Life Support Organization (ELSO) registry demonstrated a lower hospital discharge rate of 61% in 10 years from 1995-2006 [51], [82].

ECMO provides biventricular circulatory support but does not decompress the left ventricle. Patients placed on ECMO will initially demonstrate a stunned, left ventricle with no effective ejection, which can lead to a need for decompression of the left ventricle via a left-sided vent or atrial septostomy in as many as 30% of cases–to avoid pulmonary venous hypertension and pulmonary haemorrhage [83]. Evidence of improved left ventricular ejection usually appears less than a week after the initiation of ECMO. Although ECMO can provide effective short-term (< 2 weeks) support, survival was < 50% in myocarditis patients requiring > 2 weeks of support in the ELSO registry. Factors associated with death on ECMO have included the presence of arrhythmia on support, the need for dialysis, and higher stages of end-organ hypoperfusion, as reflected in serum lactate, creatinine, and aspartate aminotransferase levels [84], [85]. In 1 centre's experience, the absence of the virus in the myocardium or evidence of myocardial inflammation was associated with a greater chance for recovery [86].

Ventricular assist devices (VADs) have revolutionised the care of adults with advanced heart failure. VAD support, usually in the form of left ventricular assist devices as opposed to biventricular assist device support, is being increasingly–used in pediatric myocarditis [78], [87]. Continuous-flow VADs, used in adults, are limited to use in older children and adolescents, although the Heartware device has been used in children as small as 0.8 m^2. Initial experience with the use of these devices in the pediatric population is favourable, with low mortality and morbidity rates, similar to the adult experience [88], [89].

The primary use of pediatric VADs is as a bridge to heart transplantation. In the Berlin EXCOR trial, the mortality in patients on the device was 8%, and 87.5% of patients placed on the device received transplantations. This experience is similar to the recent experience in pediatric patients using adult continuous-flow VADs [90]. The overall mortality rate in patients on the device in the United States during the period of the trial was 26% with a transplantation rate of 67%, reflecting the ability of centres to use the Berlin EXCOR on a compassionate-use basis during the conduct of the trial [91], [92]. Lower patient weight (especially < 5 kg), elevated serum bilirubin, lower

estimated glomerular filtration rate, and use of biventricular assist device support was associated with mortality with the device. These encouraging outcome results were tempered by high rates of neurological adverse events (29%, primarily thromboembolic stroke), major bleeding (44%), and major infection (44%) [93].

Outcomes and prognosis

As described, it is difficult to estimate the true incidence and prevalence of myocarditis because of the broad spectrum of symptoms. It is said that the prognosis for children with viral myocarditis tends to be more positive than the outcomes with dilated cardiomyopathy [2], [88]. Survival rates for paediatric patients with myocarditis can be as high as 93% [81]. However, a large, multi-centre study including all age groups showed that there was significant mortality in neonates and infants (33-45% survival, 23-32% improvement) with better outcomes in children between 1 and 18 years of age (78-80% survival, 46-67% improvement) [18].

A study of 28 children with a diagnosis of myocarditis also observed that only 17 survived to hospital discharge with variable degrees of improved heart function, whereas the remaining 11 patients developed intractable cardiac failure leading to cardiac transplant listing (seven cases) or death (four cases) [45]. Predictors of a poor outcome were found to be ejection fraction less than 30%, shortening fraction less than 15%, left ventricular dilatation, and moderate-to-severe mitral valve regurgitation.98 The authors of several case series involving children requiring mechanical support for myocarditis have reported survival rates of 67-83% [67], [78], [79]. An updated review of the extracorporeal membrane oxygenation registry revealed a survival rate of 61% for children supported through acute myocarditis [91]. This is comparable to, and perhaps better than, survival rates of children supported with extracorporeal membrane oxygenation after repair or palliation of congenital cardiac disease, in which survival rates range between 37% and 42% [86]. Hetzer reported that of 21 children supported with the Berlin Heart Excor device for myocarditis or cardiomyopathy, 90% survived to hospital discharge [87]. Collective experience with ventricular assist devices in this setting, however, is quite limited. The prognosis in biopsy-proven myocarditis may depend on the severity of symptoms, histologic classification, and biomarkers. Acute fulminant myocarditis is associated with better overall survival [56], [93]. Giant cell myocarditis, although rare, is associated with especially poor outcomes and a reported median duration of survival, if untreated, of 5.5 months [81]. It also carries an 89% rate of death or transplant. Myocarditis may account for nearly half of all children with dilated cardiomyopathy [84], [91]. The outcomes for patients with acute viral myocarditis are much better than cases with established dilated cardiomyopathy [92]. For this reason, a high index of suspicion for the disease, and early effective supportive care are crucial. Myocarditis remains a relatively common indication for transplant listing.

Overall, 1-8% of patients with acute myocarditis eventually go on to transplant [25]. Owing to the potential for recovery, even with severe disease at presentation, patients are not typically listed for cardiac transplant unless a recovery is considered extremely unlikely despite reasonable management and a period of observation.

Conclusions

Acute myocarditis comprises a wide clinical spectrum including the asymptomatic patient with subclinical myocardial dysfunction to severe cardiac failure or sudden cardiac death. In paediatrics, the symptoms and signs can mimic many other common diseases and a high index of clinical suspicion is imperative. Treatment of myocarditis remains supportive and should be initiated even before solidifying the diagnosis. Biopsy remains the gold standard for diagnosis; however, it has significant limitations. Cardiac magnetic resonance imaging has become a widely accepted non-invasive imaging modality for myocarditis and standardized protocols are now available for myocarditis. There exist controversial data and no consensus with respect to immunosuppressive or immunomodulating therapy for myocarditis. Similarly, there is no foundation of evidence to indicate a routine treatment strategy other than standard cardiac failure management for these patients. Most patients with myocarditis have spontaneous improvement in their cardiac function and symptoms; however, there remains significant morbidity and mortality associated with this condition. Additional studies are a necessity to clarify the most appropriate management options in pediatrics to improve outcomes.

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ID Design Press, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2019 Jan 15; 7(1):174-178.
https://doi.org/10.3889/oamjms.2019.027
eISSN: 1857-9655
Review Article

Evaluation of Serum Interleukin-6 Levels in the Renal Transplant Recipients: A Systematic Review and Meta-Analysis of Case-Control Studies

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Citation: Omrani H, Jasemi SV, Sadeghi M, Golmohamadi S. Evaluation of Serum Interleukin-6 Levels in the Renal Transplant Recipients: A Systematic Review and Meta-Analysis of Case-Control Studies. Open Access Maced J Med Sci. 2019 Jan 15; 7(1):174-178. https://doi.org/10.3889/oamjms.2018.369

Keywords: Chronic kidney disease; Transplantation; Serum; Cytokine; Interleukin-6

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Received: 28-Aug-2018; **Revised:** 06-Nov-2018; **Accepted:** 07-Nov-2018; **Online first:** 14-Jan-2019

Funding: This research did not receive any financial support

Competing Interests: The authors have declared that no competing interests exist

Abstract

AIM: The purpose of this meta-analysis was the assessment of the serum IL-6 levels in the renal transplant recipients compared to the healthy controls.

MATERIAL AND METHODS: Four databases including PubMed, Web of Science, Scopus, and Cochrane Library were searched up to July 2018 without language restriction. The quality of studies was evaluated using the Newcastle-Ottawa scale (NOS). A continuous random-effects meta-analysis was used by RevMan 5.3 using the mean difference (MD) and 95% confidence intervals (CIs). Also, a regression model was done by Comprehensive Meta-Analysis version 2 (CMA v2).

RESULTS: Out of 615 studies identified in the databases, 15 studies included and analysed in the meta-analysis. The studies were reported from 1994 to 2018. The meta-analysis included 1035 renal transplant recipients and 682 healthy controls. The pooled MD of the serum IL-6 levels in the transplant recipients compared to the healthy controls was 3.25 pg/mL [95%CI: 2.17, 4.32; P < 0.00001; I2 = 98% (P < 0.00001)]. Meta-regression analysis showed that one of the reasons of heterogeneity is the year of publication (Correlation coefficient (r) = 0.208, p-value = 0.00002).

CONCLUSION: An elevated serum IL-6 level in the renal transplant recipients compared to the healthy controls showed that the serum level of this marker could be used for the evaluation of inflammation in ESRD patients undergoing renal transplantation.

Introduction

Chronic kidney disease (CKD) is the usual name for various disorders impacting renal structure and functions [1]. The incidence of cardiovascular disease in patients of end-stage renal disease (ESRD) is 10–20-fold that in the general population [2]. Renal transplantation (RT) is the treatment of choice for ESRD patients [3], [4]. Acute rejection is a common complication after RT and is associated with reduced graft survival [5]. Cardiovascular and cerebrovascular diseases are the leading causes of death following renal transplantation [6], [7] that the main underlying reason is inflammation [7]. Inflammation is generally controlled by following changes in concentrations of C-reactive protein (CRP), cytokines, and chemokines [8]. Interleukin-6 (IL-6) is reportedly responsible for acute complications in patients with ESRD such as fever, headache, and hypotension [9]. This cytokine is also a reliable marker of disease severity and inflammatory organ injury in rheumatoid arthritis [10] and oral lichen planus [11]. Therefore, it has been found to act as both a pro-inflammatory and anti-inflammatory cytokine [12]. Also, the serum IL-6 levels increase depending on the reduction of renal function

[13].

The meta-analysis aimed to assess the serum IL-6 levels in the renal transplant recipients compared to the healthy controls.

Material and Methods

The meta-analysis created according to a guideline for the preferred reporting items for systematic review and meta-analysis (PRISMA) literature search [14].

The research protocol was supported by the Ethics Committee of Kermanshah University of Medical Sciences, Kermanshah, Iran (Ethical code: IR.KUMS.REC.1396.597). A comprehensive search was used with the databases of PubMed, Web of Science, Scopus, and Cochrane Library up to July 2018. A combination of terms “kidney transplantation" or "renal transplantation" or "kidney transplant" or "renal transplant" and “serum” and "interleukin-6" or “IL-6” was used in the search without language restriction. The studies were selected for evaluation of the serum IL-6 levels in the renal transplant recipients compared with the healthy controls. The studies included in this meta-analysis had to: (I) use a case-control design; (II) report the serum IL-6 levels in the renal transplant recipients (III) report the controls without renal transplantation and any systematic disease (the healthy controls). Before transplantation, the more renal transplant recipients were on renal replacement therapy (pretransplant dialysis) and had immunosuppressive treatment.

One reviewer (M.S) searched the articles and then selected the relevant publications and other authors reviewed them independently. We applied a regular protocol and recording information for data extraction from each publication including the first author’s name, the year of publication, the country which the study was reported, number/the mean age/male (%) of the cases and healthy controls, immunosuppressive regimen, measured method of IL-6 level, and transplantation evolution.

The quality of studies was evaluated using the Newcastle-Ottawa scale (NOS) [15], in which the maximum total score is 9 for the case-control study. The quality evaluation was done by one author (M.S) for each study.

To compare serum IL-6 levels in the renal transplant recipients compared with the healthy controls, a continuous random-effects meta-analysis was done by Review Manager 5.3 (RevMan 5.3, The Cochrane Collaboration, Oxford, United Kingdom) using the mean difference (MD) and 95% confidence intervals (CIs). Heterogeneity among studies was evaluated with the Q, and the I^2 statistic and results were defined as heterogeneous for $P < 0.10$ or $I^2 > 50\%$ [16] and *P*-value (2-sided) < 0.05 was estimated statistically significant in the meta-analysis study. Also, the publication bias was estimated by funnel plot using Begg’s and Egger’s tests. The unit of measurement of IL-6 levels was pg/mL. The meta-regression analysis is a technique used to evaluate heterogeneity between the studies. This statistical approach determines whether there is a significant association between the study period and the pooled MD of the serum IL-6 levels. A regression model by Comprehensive Meta-Analysis version 2 (CMA v2) was done with the p-value and regression coefficient (r) to evaluate the strength of this association.

Results

Out of 615 studies identified in the databases, after excluding duplicate studies, 344 studies were screened that 312 studies were excluded. Out of 32 studies that their full-texts were assessed for eligibility, 17 studies were removed with the reasons (Figure 1). At last, 15 studies included and analysed in the meta-analysis.

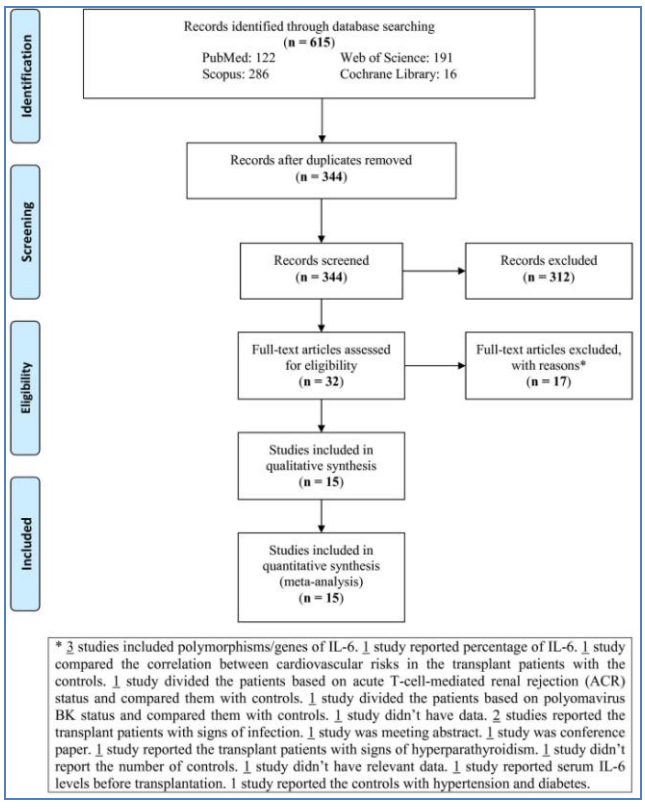

Figure 1: Flowchart of the study

The features of 17 studies that have been included in the meta-analysis are shown in Table 1. The studies were reported from 1994 to 2018. Three studies [17], [18], [19] were reported from Poland, two

[7], [20] from Turkey, two [15], [16] from Iran, and also Canada, Germany, the USA, Argentina, Spain, Connecticut, India, and China (one study each) [6], [8], [21], [22], [23], [24], [25], [26]. The meta-analysis included 1035 renal transplant recipients and 682 healthy controls. Six studies [3], [8], [20], [24], [25], [27] reported pretransplant dialysis in all or more recipients. Eight studies [6], [8], [17], [18], [19], [22], [25], [27] reported stable transplantation as inclusion criteria in the recipients and two other studies [23], [26] showed acute rejection and stable evolutions. The mean age, male percentage, immunosuppressive regimens, and measured method of IL-6 level are shown in Table 1.

Table 1: Characteristics of the studies included in the meta-analysis (n = 15)

	Country	No. of Transplant recipients/ mean age, year/No. of males	No. of healthy controls/ mean age, year/ No. of males	Pretransplant dialysis in all or more recipients	Immunosuppressive regimen	Measured Method of interleukin-6 level	Transplantation evolution
Nickerson, 1994 [21]	Canada	38/46.1/24	31/matched/matched	-	Azathioprine, Prednisone, and cyclosporine	ELISA kits (R & D systems, Quantikine human IL-6 immunoassay, Minneapolis, MN)	-
Waiser, 1997 [22]	Germany	145/-/-	20/-/-	-	Cyclosporine and methylprednisolone	ELISA kits (R&D Systems, Quantikine human IL-6 immunoassay)	Stable
Preston, 2002 [8]	USA	10/-/-	10/-/-	Yes	-	ELISA kits (MMP-2 and MMP-3, Amersham Life Science, Buckinghamshire, England; IL-6, Biosource International, Inc., Camarillo, Calif., USA)	Stable
Malan Borel, 2003 [23]	Argentina	13 rejection:11 stable/-/-	20/-/-	-	Prednisone, cyclosporine or FK506	ELISA kits (the Quantikine immunoassay (R & D Systems Inc., Minneapolis, USA)	Acute rejection vs. stable
Lauzurica, 2005 [24]	Spain	178/53/117	40/-/-	Yes	Corticosteroids or azathioprine and Cyclosporine or tacrolimus	An immunofluorimetric automatized method in Immulite-1 (DPC-Dipesa)	-
Malyszko, 2006 [17]	Poland	96/52.7/48	33/50.6/-	-	Cyclosporine, prednisone, and azathioprine	(high-sensitivity assays) from Bender MedSystem (Vienna, Austria)	Stable
Malyszko, 2008 [18]	Poland	90/46.2/43	30/44.8/14	-	Cyclosporine/tacrolimus, azathioprine/mycophenolate mofetil plus prednisone.	kits from Bender MedSystems (Vienna, Austria)	Stable
Shaqman, 2010 [25]	Connecticut	90/53/48	72/51/12	Yes	Prednisone, and cyclosporine	ELISA kits (Diagnostic Products, Los Angeles, CA)	Stable
Malyszko, 2013 [19]	Poland	62/44.3/35	24/51.6/-	-	Calcineurin inhibitor in combination with mycophenolate mofetil and prednisone.	Enzyme immunoassay (EIA) using a commercially available kits from Bachem (St. Helens, UK).	Stable
Sonkar, 2013 [26]	India	40 rejection:50 stable/32.3/-	90/30.4/-	-	Cyclosporine, azathioprine, and prednisone.	ELISA kits (Beckman Coulter Inc, Marseille, France)	Acute rejection vs. stable
Xue, 2014 [6]	China	25/44.3/12	25/45.9/15	-	Cyclosporine, mycophenolate mofetil, and prednisone.	ELISA kits (Boster Biological Engineering (Huhan, China).	Stable
Colak, 2015 [20]	Turkey	45/40.7/24	36/42.1/19	Yes	Calcineurin inhibitors, steroids and mycophenolate mofetil	ELISA kits (Camarillo, CA, USA)	-
Avci Çiçek, 2016 [7]	Turkey	82/41.8/43	81/46.7/-	-	-	commercial kit (eBioscience, Austria)	-
Eskandari Naji, 2017 [3]	Iran	30/48.5/18	30/50.4/15	Yes	Cyclosporine, CellCept, and prednisolone	ELISA (DIA source, Belgium, catalog number: KAP1261)	-
Argani, 2018 [27]	Iran	30/49/18	30/50/15	Yes	Prednisolone, calcineurin inhibitor, and CellCept	ELISA kits (IBL International GmbH, Germany).	Stable

Figure 2 is shown the forest plot of random-effects analysis that the pooled MD of the serum IL-6 levels in the transplant recipients compared to the healthy controls was 3.25 pg/mL [95%CI: 2.17, 4.32; P < 0.00001; I^2 = 98% (P < 0.00001)]. Therefore, the serum IL-6 level in the transplant recipients was significantly higher than the healthy controls.

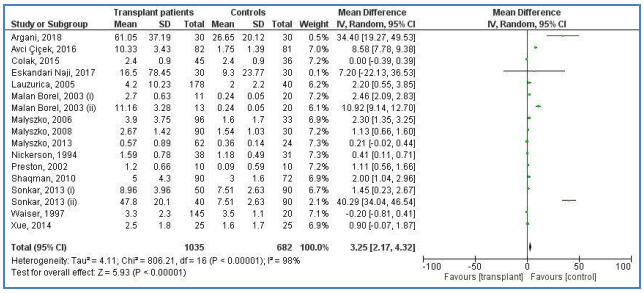

Figure 2: Forest plot of random-effects of serum interleukin-6 levels in the renal transplant recipients compared to the healthy controls. i: the renal transplant recipients with stable evolution, and ii: the renal transplant recipients with acute rejection evolution

Table 2 shows the quality score for each study included in the meta-analysis. The mean score was 5.8 for all case-control studies.

Table 2: Quality ratings for the studies included by Newcastle-Ottawa quality assessment scale (n = 15)

The first author, year	Selection	Comparability*	Outcome	Total score
Nickerson, 1994 [21]	2	0	2	4
Waiser, 1997 [22]	2	0	2	4
Preston, 2002 [8]	2	0	2	4
Malan Borel, 2003 [23]	4	1	2	7
Lauzurica, 2005 [24]	3	0	2	5
Malyszko, 2006 [17]	3	1	2	6
Malyszko, 2008 [18]	3	2	2	7
Shaqman, 2010 [25]	3	1	2	6
Malyszko, 2013 [19]	2	0	2	4
Sonkar, 2013 [26]	3	2	2	7
Xue, 2014 [6]	3	2	2	7
Colak, 2015 [20]	4	2	2	8
Avci Çiçek, 2016 [7]	3	0	2	5
Eskandari Naji, 2017 [3]	2	2	2	6
Argani, 2018 [27]	3	2	2	7

*One star if two groups matched via age and another star if two groups matched via sex or body mass index (BMI).

Figure 3 shows the funnel plot of studies entered to the analysis. Egger's test (p-value (2-tailed) = 0.042) reveal, but and Begg's test (p-value (2-tailed) = 0.067) didn't reveal a significant sign of publication bias between the involved studies.

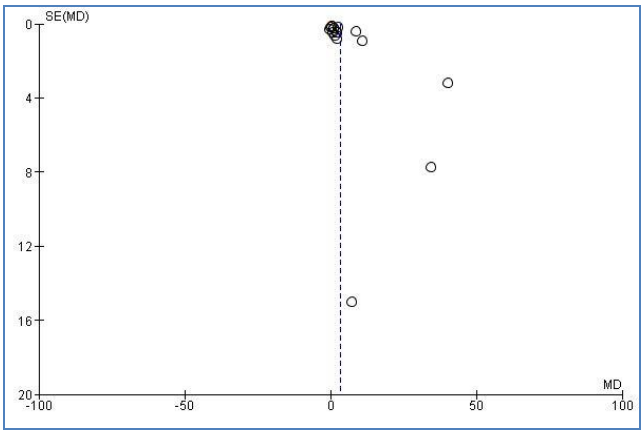

Figure 3: Funnel plot of random-effects of serum interleukin-6 levels in the transplant recipients compared to the healthy controls

Meta-regression analysis identified a significant statistical relationship between the pooled MD of serum IL-6 levels in the renal transplant recipients compared with the healthy controls and the year of publication. This means that one of the reasons for heterogeneity is the year of publication (Correlation coefficient (r) = 0.208, p-value = 0.00002). Significant positive correlation showed that there was an increase in the MD of the serum IL-6 with time (Figure 4).

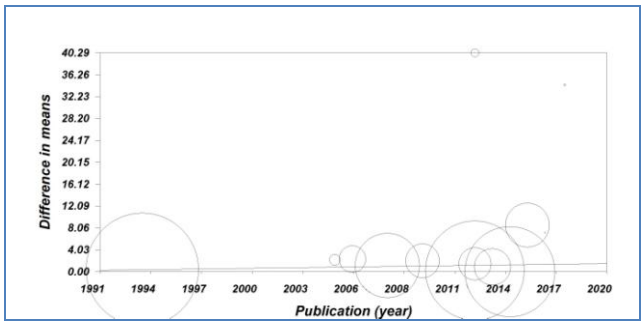

Figure 4: Meta-regression analysis the study period versus the mean difference of the serum IL-6 levels

Discussion

The elevated serum IL-6 levels in the renal transplant recipients compared to the healthy controls have been shown in this meta-analysis study. The Th1 cytokine pattern is generally related to the rejection of the transplantation; a Th2 cytokine pattern may lead to transplant tolerance and stable graft survival. [13]. Inflammation is the most important underlying mechanism in cardiovascular disease and other systemic diseases [1]. Increased IL-6 leads to chronic inflammatory conditions such as the synthesis of acute phase proteins like CRP, hypercoagulability and accelerated atherosclerosis. [7], [28], [29], [30], [31] that can cause to the loss of renal function [7]. Also, some mechanisms including volume overload and oxidative stress can cause the usual increase in inflammatory symptoms (including IL-6) [32].

Serum levels of IL-6 increased significantly after renal transplantation [33]. The results received show an obvious relationship within the serum levels of IL-6 and graft rejection [23]. Levels of IL-6 have been greatly reduced in desirable evolution and raised with increasing renal failure. Therefore, the level of IL-6 can be used as an indicator of graft evolution and as a prerequisite for proper evaluation of renal biopsy [23]. The changes in the serum levels of IL-6 in transplanted patients can lead to several factors that affect the production of cytokines. Also, IL-6 may act a function in the close correlation between the high incidence of inflammation, cardiovascular disease, and malnutrition in HD patients [34]. CRP elevation may only represent the end stage of substantial inflammation, and other inflammatory markers may be more sensitive indicators of early inflammation injury, almost in HD patients [8]. Therefore, levels of CRP can correlate with IL-6 levels [9]. Serum levels of IL-6 in HD patients are significantly higher than in renal transplant recipients [20], [35]. The injury to the endothelium might trigger the release of IL-6 in renal transplant recipients [26]. This increase in IL-6 could be the result of increased activation of nuclear factor-kappa B, which is an inducible transplantation factor necessary for the activation of multiple important inflammatory cytokine genes like to the IL-6 gene [36]. 1) Pretransplant dialysis in the recipients. 2) Different immunosuppressive treatment in the recipients. 3) Differences in the measured method of IL-6 levels. 4) The sample size was small in some studies. 5) There was high heterogeneity in the analysis. 6) Low quality of more studies. Therefore, these limitations cause a bias between the studies.

In conclusion, about the limitations, there was an elevated serum IL-6 level in the renal transplant recipients compared to the healthy controls. The results may indicate potential usefulness of the serum level of this marker as an indicator of immunologic risk and can be used for the evaluation of inflammation in ESRD patients undergoing renal transplantation. *HRO* and *SG* conceptualised this study, designed the methodology for data collection, collected and analysed the data, and wrote the manuscript. *SVJ* and *MS* extensively supported the development of the study concept, data analysis, and the writing, editing and finalising of the manuscript. *All authors* read and approved the final manuscript.

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