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Basic Science

TP53 Mutation in Correlation to Immunohistochemical Expression of P53 Protein in Patients with Hepatocellular Carcinoma

Dafina Nikolova[1], Viktorija Chalovska-Ivanova[1], Magdalena Genadieva-Dimitrova[1], Aleksandar Eftimov[2], Rubens Jovanovik[2], Vesna Janevska[2]

[1]University Clinic of Gastroenterohepatology, Faculty of Medicine, Ss Cyril and Methodius University of Skopje, Skopje, Republic of Macedonia; [2]Institute of Pathology, Faculty of Medicine, Ss Cyril and Methodius University of Skopje, Skopje, Republic of Macedonia

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***Correspondence:** Dafina Nikolova. University Clinic of Gastroenterohepatology, Faculty of Medicine, Ss Cyril and Methodius University of Skopje, Skopje, Republic of Macedonia. E-mail: dafina.janevska@gmail.com

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Abstract

BACKGROUND: Mutations causing p53 inactivation are among the most common genetic alterations in human malignant tumours including hepatocellular carcinoma. Detection of p53 gene mutations in patients with hepatocellular carcinoma (HCC) should provide relevant data for the patients from the Republic of Macedonia and should allow the survivals additional therapeutic option as is gene therapy.

AIM: We aimed to detect p53 gene mutations in HCC tissue, and to correlate them with the immunoexpression of p53 protein and multiple clinicopathologic characteristics of a tumour.

MATERIAL AND METHODS: We analysed thirty patients with HCC for multiple clinic-pathological characteristics. Tumour tissue samples were immunostained for p53 and detection of p53 gene mutations was performed by polymerase chain reaction followed by Sanger sequencing.

RESULTS: Changes in p53 gene sequence were detected in four patients (13.33%), one of them a polymorphism and the other three were missense point mutations with p53 immunoexpression of 50%, 0%, 0% and 90%, respectively. All patients with p53 mutations had cirrhosis. Two of them had Hepatitis B infection, moderately differentiated tumour and T2 status. There was one case with a well-differentiated tumour and one with T4 status. All of them were with vascular invasion. The size of the tumours was in the range of 2.5 cm to 16 cm. All 3 mutations were located in exon 7.

CONCLUSION: Mutations in p53 gene are not always associated with obviously altered immunoexpression of p53 protein. Detection of p53 gene mutations is necessary in each case because the new therapeutic modalities offer to apply gene therapy.

Introduction

Tumour suppressor gene p53 plays a key role in cell cycle regulation, cell proliferation and apoptosis following DNA damage. p53 is responsible for maintenance of genomic integrity [1] [2]. Inactivation mutation of p53 is among the most common genetic alterations in human cancers, including hepatocellular carcinoma (HCC). The mutational spectrum of p53 gene differs in HCC from different geographic regions. This difference comes due to differences in population exposition to Aflatoxin B1 and Hepatitis B infection (HBV). Transversion of guanine to thymine at the third position of codon 249 is a common finding in patients with HCC from southern Africa and southern China [2].

In contrast, in regions where exposure to Aflatoxin B1 is low, there are almost no mutations in codon 249, or they occur less significantly [2] [3] [4] [5] [6] [7]. Population in the Republic of Macedonia is exposed to Hepatitis B infection with the incidence of 7.8 in the 2013 year [8], and the same year Aflatoxin M1and B1 contamination is found in raw milk and feed [9]. In the neighbouring countries, the contamination of raw milk with Aflatoxin Ma and B1 was detected

earlier [10]. According to EUROCAN the incidence rate of liver cancer and intrahepatic bile duct cancer in the Republic of Macedonia is 8.4, and the mortality rate is 11.1 [11].

Mutations of p53 gene or positive immunostaining for mutated p53 protein can be used as a significant indicator of poor prognosis in patients with HCC [12] [13]. Modern therapeutic possibilities such as surgical resection, liver transplantation, percutaneous ablation and transcatheter chemoembolization provide a better prognosis for patients, but the overall survival rate in patients with HCC remains low [11]. Hence, great importance for patients with HCC is the development of multidisciplinary interventions and new therapeutic approaches, including biotherapy. The efficacy of chemo and radiotherapy may be elevated by an endogenous or exogenous normal type of p53. There are currently several in vitro and in vivo studies with products for modulating the p53 status [14].

Advexin (for adenoviral p53), ONYX-015, CNHK200, SCH58500 are products in clinical development, while Gendicine-a gene therapy product mainly built from normal p53 and modified adenovirus is already approved for commercial use by the Chinese State Food and Drugs Administration [15] [16] [17] [18] [19]. Due to the new therapeutic possibilities, and because the presence of the mutant p53 gene in HCC is an indicator of a poor prognosis, the detection of the mutant gene and the presence of its protein are extremely important for patients with HCC.

This study aims to determine the mutations of the p53 gene and correlate them with the immunohistochemical expression of its product, as well as to correlate them with the clinical and pathological characteristics of a tumour in 30 cases of HCC in patients from the Republic of Macedonia.

Material and Methods

We analysed 30 patients with histologically proven HCC, diagnosed and treated at the University Clinic of Gastroenterology and Hepatology and the University Clinic of Abdominal Surgery in Skopje

Following parameters were determined by echosonography and computed tomography images: the dimension of tumour node, the multiplicity of the tumour nodes, the presence of tumour emboli in the large vessels and the presence of cirrhosis in the surrounding liver tissue. Serological tests for hepatitis B and C infection were performed in all patients. All patients were followed up until the death.

During the diagnostic procedure of HCC at the Institute of Pathology in Skopje, the grade of differentiation, vascular invasion, T category of pTNM classification (AJCC 2017) and the presence of cirrhosis in the surrounding, peritumoral liver tissue was determined. Additionally, immunohistochemical staining for p53 and PCR for the detection of p53 gene mutations were performed.

Immunohistochemical stainings with an antibody against p53 (Monoclonal Mouse, Anti-Human, Clone DO-7, DACO, dilution 1:50) using Avidin-Biotin immunoperoxidase technique were made. For the visualisation of the antigen-antibody reaction, LSAB and En-Vision kit from DAKO was used. The results of immunostaining were determined as a percentage of positive nuclear signals for p53.

For the determination of p53 gene mutation polymerase chain reaction (PCR) with subsequent Sanger sequencing of exons 2-11 of the p53 gene were performed. The primers for each exon are shown in Table 1.

Table 1: Nucleotide sequences of primers used for amplification (p53 priming sequences)

Oligonucleotide	Oligo sequence (5' to 3')	Product size
p53Ex2-3F	tctcatgctggatccccact	365
P53Ex2-3R	agtcagaggaccaggtcctc	
p53Ex4F	tgaggacctggtcctctgac	413
p53Ex4R	agaggaatcccaaagttcca	
p53Ex5-6F	tgttcacttgtgccctgact	467
P53Ex5-6R	ataacccctcctcccagaga	
p53Ex7F	cttgccacaggtctccccaa	263
P53Ex7R	aggggtcagaggcaagcaga	
p53Ex8-9F	ttgggagtagatggagcct	445
P53Ex8-9R	agtgttagactggaaacttt	
p53Ex10F	caattgtaacttgaaccatc	260
P53Ex10R	ggatgagaatggaatcctat	
p53Ex11F	agaccctctcactcatgtga	245
P53Ex11R	tgacgcacacctattgcaag	

DNA was extracted from deparaffinized tissue sections in polypropylene tubes using Dynabeads DNA kit (Invitrogen, ThermoFisher Scientific).

Evaluation of the quality and quantity of the isolated material was made using horizontal agarose gel electrophoresis and spectrophotometer (Thermo Scientific Evolution 260 Bio spectrophotometer).

Polymerase Chain Reaction (PCR)

For amplification of the DNA regions of interest, standard polymerase chain reaction was performed. The PCR reaction was performed on AutoQ server thermal cycler (Quanta Biotech).

Detection of TP53 gene mutations

Detection of TP53 gene mutations in the amplified exons was carried out with Sanger DNA sequencing on automated genetic analyser ABI 310 Genetic Analyzer (Applied Biosystems®).

Sequencing of the amplified DNA fragment

The technique of DNA sequencing was performed using the commercial Big Dye Terminator v.1.1 Cycle Sequencing Kit. Two µl of the PCR product were used to perform the sequencing reaction, 2 µl of 2.5 X Big Dye Terminator v.1.1 Cycle Sequencing, 1 µl 5 X Big Dye Sequencing Buffer and 1 µl of 10 pmol forward or reverse primer in a total volume of 10 µl.

After the sequencing reactions competed, capillary gel electrophoresis of the purified mixture was performed on automated genetic analyser ABI 310 under the conditions of the BDx_Standard_Seq_Assay_POP4 module. The electrophoresis products were subsequently analysed with the protocol KB_310_POP4_BDTv1.1 through the software Sequencing Analysis v5.4 and TP53 gene mutations were confirmed through the SeqScape v2.7 software.

Results

Ten patients out of 30 (33.33%) were female, and 20 (66.66%) were male, aged 38 to 76, with a mean age of 59.13 years. Twenty-three patients (76.66%) were serologically positive for hepatitis B, two patients (6.6%) were seropositive for hepatitis C, and 5 (16.66%) patients were seronegative to both B and C hepatitis. Liver cirrhosis was detected in 28 (93.33%) patients. In the most of the patients the local growth was determined as T2 - 13 cases (43.33%), in 5 (16.66%) patients the local growth was determined as T1, in 10 (33.33%) patients it was T3 and one patient (3.33%) had T4 status of the local growth. Vascular invasion was detected in 20 (66.66%) patients. Six patients (20%) had multiple HCC nodes in the liver, and the remainder 10 patients (33.33%) had solitary tumours. The smallest tumour node measured 3.5 cm and largest 16 cm. Clinicopathological characteristics of the analysed group of patients and immunoexpression of p53 in HCC tissue are shown in Table 2.

Immunohistochemical staining with the antibody against p53 showed positivity in the range from 0% to 90% in the cell nuclei (Figure1). Changes in p53 gene sequence were detected in four patients (13.33%), but one of them a polymorphism, and the other three were missense point mutations. So, p53 gene mutation was found in 3 (10%) out of 30 patients, highlighted in Table 2, under the numbers 10, 20, and 21.

All 3 mutations were heterozygous point mutations, in exon 7. No mutations were found in codon 249. The polymorphism in codon 247 (exon 7) was detected in patient 7. Missense point mutations in codon 260 (exon 7) was detected in patient 10, in codon 245 (exon 7) in patient 20, and in codon 242 (exon 7) in patient 21 (Figure 2).

Two patients with missense mutations were male, and one was female at the age of 38 to 75 years, mean 60.66 years. Two of the patients had hepatitis B infection, and one was negative. All patients had cirrhosis and solitary tumours ranging in size from 2.5 cm to 16 cm. All showed the presence of vascular invasion. Two of the tumours were moderately differentiated; one was well differentiated. Two of them showed a T2 tumour local growth, and one was T4 tumour status. The percentage of the p53 immunohistochemical expression ranged from 0 to 90 per cent.

Table 2: Clinicopathological characteristics of the patients and p53 immunoexpression

Patient	Gender	Age	Hepatitis B	Hepatitis C	Cirrhosis	Tumour node	Tumour size**** Cm	Vascular invasion	G	T	P53 %	Survival Months
1	f*	42	+	-	+	M***	12	-	2	3	5	8
2	m**	63	+	-	+	1	7	+	2	2	20	1
3	m	66	+	-	+	1	11	+	2	2	25	26
4	m	57	+	-	+	M	9	+	2	3	30	21
5	f	59	+	-	+	1	3.5	+	2	2	50	23
6	m	76	+	-	+	M	3.5	-	1	2	20	70
7	m	65	+	-	+	1	3.5	+	2	2	50	13
8	m	66	+	-	+	1	3.8	-	2	1	50	24
9	f	75	-	+	+	M	12	+	3	2	30	1
10	f	38	+	-	+	1	10	+	2	4	90	12
11	f	73	+	-	+	1	2.5	+	2	1	85	62
12	m	53	+	-	+	1	9	+	2	2	80	1
13	m	52	+	-	+	1	9	-	3	1	30	17
14	m	56	+	-	+	1	3	-	1	1	25	13
15	m	74	1	-	+	1	10	+	1	1	50	13
16	f	59	+	-	-	1	15	+	2	3	20	9
17	f	71	-	-	+	1	10	+	1	3	80	9
18	f	57	+	-	+	M	10	+	2	4	70	3
19	m	67	+	-	+	M	6	-	1	2	90	25
20	m	75	+	-	+	1	2.5	+	1	2	0	8
21	m	69	-	-	+	1	16	+	2	2	0	4.5
22	m	42	+	-	+	1	12	-	2	3	90	24
23	m	61	+	-	+	1	10	+	1	3	80	7
24	m	50	-	+	+	1	11	+	2	3	90	6
25	m	48	+	-	+	1	14	-	1	2	70	6
26	f	67	-	-	+	1	12	-	1	2	40	11
27	f	48	+	-	+	1	10	+	3	3	1	7
28	m	65	-	-	+	1	7	+	1	3	20	8
29	m	52	+	-	-	1	6.5	-	1	3	10	23
30	м	58	-	-	+	1	3	+	2	2	80	2

* Female, ** Male, ***Multiple tumour nodes in the liver. **** If multiple nodes present - the greatest dimension of the greatest node is shown in the table.

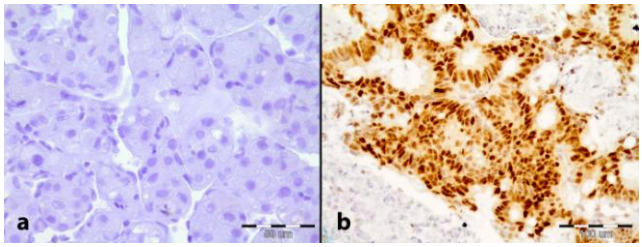

Figure 1: Micro-image of immunohistochemical staining for p53 in HCC tissue. a) Negative immunostaining for p53 (patient No. 20) (10 x 40) b). Variable intensity of immunostaining in about 90% of the tumour tissue in this microscopic field. The surrounding non-coloured tissue represents a cirrhotic tissue in which a tumour infiltrates (patient No. 10), (10 x 20)

In tumour samples where p53 missense mutations in codons 245 and 242 were detected, the immunoexpression of p53 was 0% (cases 20 and 21), and the tumour sample with a p53 missense mutation in codon 260 (case 10) showed an immunoexpression of p53 in approximately 90% of the tumour cells.

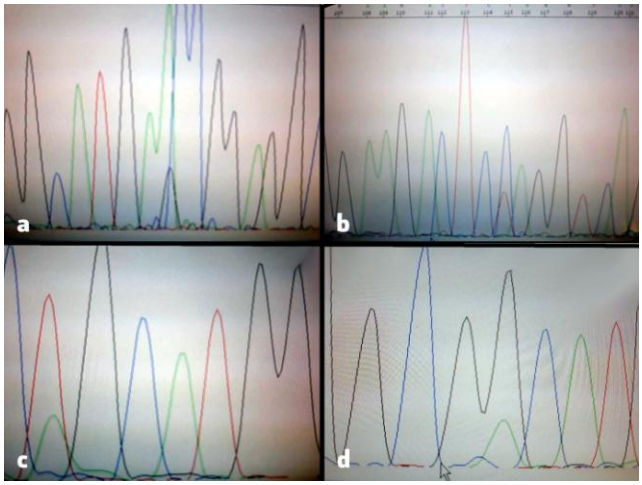

Figure 2: a) AAC> AGC LYS247SER (Patient 10) b) Polymorphism TCC> TCT SER260SER (patient7) c) GGC> GAC GLY245ASP (Patient 20) d) TGC> AGC CYS242SER (Patient 21)

The survival time of the entire group was 1 to 70 months, mean survival 15.25 months. Patients with missense mutations in p53 gene had a mean survival of 8.16 months (min. 4.5; max. 12), while those without p53 mutation had a mean survival time of 16 months (min. 1; max. 70; SD = 16.58). However, intergroup differences were insignificant ($P > 0.05$), probably due to the small number of cases in the group with mutated p53.

Discussion

The p53 gene is located on the 17th chromosome (17p13.1) and encodes phosphoprotein, formed by 393 amino acids. It consists of 4 units (domains): a) a domain that activates transcription factors, b) a domain that recognises specific DNA sequences, c) a domain responsible for tetramerisation of the protein, and d) a domain that recognises damaged DNA. The protein responds to different cellular stresses to regulate the expression of target genes, thereby induces cell cycle arrest, apoptosis, ageing, DNA repair, or changes in metabolism [20] [21].

The level of p53 protein in normal cells is low, and the wild type of p53 is a labile protein. Cellular stress can trigger a rise in the p53 protein to fulfil its function as a "genome keeper" [20] [21] [22].

P53 gene mutations are the most commonly reported somatic mutations in human neoplasms. Reports have been published in which mutations in the p53 gene, or positive immunostaining for p53, are associated with a higher grade of neoplasms and a more advanced stage in various types of malignant tumours. In many reports p53 mutations are considered a strong marker that predicts an increased risk of local relapse, failure of therapy and poor survival in many types of human neoplasms, such as breast cancer [24], colorectal cancer [25], cancer of esophagus [26], lung [27], ovarian cancer [28] and head and neck cancer [29].

Several studies suggest that p53 mutations are involved in determining the differentiation, proliferative activity, and progression of the HCC. p53 mutations are also associated with marked HCC invasiveness and may influence the postoperative course and occurrence of relapses [20] [21] [22] [23]. Additionally, p53 mutations or overexpression of the mutant p53 protein can be used as a significant indicator of a poor prognosis [30] [31] [32] [33] [34] [35] [36]. However, for the predictive evaluation of HCC, the p53 effect should be considered in correlation with other significant factors such as tumour size, Child-Pugh score, TNM stage and vascular invasion [14].

Positive immunoexpression of the mutant p53 protein is detected in 37% HCC, but the overexpression of the p53 protein does not always depend on the p53 mutation [37] [38] [39].

Hence, mutations in the p53 gene in patients with HCC, in each case are of particular importance, especially for the development of new modalities of therapy and the application of gene therapy. Today's therapy for patients with HCC is with limited efficacy. The development of gene therapy for HCC, such as the use of apoptotic genes, genes that code anti-angiogenic factors or immunomodulatory molecules, gives hope for a longer survival of patients with HCC [15] [16] [17] [18].

In this study, three mutations of the p53 gene were found in three different patients out of 30

analysed. In two of the tumour samples with a p53 missense mutation, we did not find any immunoexpression of the p53 protein, although the mutation was present. In the third case, the immunoexpression of the pathological p53 was 90% of cells.

The survival time of patients with confirmed mutations in this study was shorter than the survival time of the remaining patients, however statistically insignificant ($P > 0.05$). So, the detection of the mutant p53 gene is necessary for each patient with HCC, to build a strategy for application of appropriate therapy.

Detection of mutations in the p53 gene in survivors will also provide a gene therapy option with p53 products already in commercial use or in development [15] [16] [17] [18].

Compliance with Ethical Standards

All procedures performed in studies involving human participants were by the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

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Basic Science

HER2 Positive Gastric Carcinomas and Their Clinico-Pathological Characteristics

Ljubomir Ognjenovic[1*], Gjorgji Trajkovski[1], Stojan Gjoshev[1], Aleksandar Shumkovski[1], Darko Dzambaz[1], Dragan Hadzi-Manchev[1], Goce Volcevski[1], Igor Fildishevski[2], Dafina Nikolova[3], Gordana Petrushevska[4], Vesna Janevska[4], Vlado Janevski[1]

[1]University Clinic of Abdominal Surgery, Faculty of Medicine, Ss Cyril and Methodius University of Skopje, Skopje, Republic of Macedonia; [2]University Clinic for Surgery St. Naum Ohridski, Skopje, Republic of Macedonia; [3]University Clinic of Gastroenterohepatology, Faculty of Medicine, Ss Cyril and Methodius University of Skopje, Skopje, Republic of Macedonia; [4]Institute of Pathology, Faculty of Medicine, Ss Cyril and Methodius University of Skopje, Skopje, Republic of Macedonia

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***Correspondence:** Ljubomir Ognjenovic. University Clinic of Abdominal Surgery, Faculty of Medicine, Ss Cyril and Methodius University of Skopje, Skopje, Republic of Macedonia. E-mail: ljognjenovic@gmail.com

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Abstract

BACKGROUND: HER2 protein expression in gastric carcinoma, in correlation with existing, acknowledged prognostic factors which include the parameters that determine the TNM stage of the disease, could become the basis for ongoing research in the field of molecular targeted and personalised therapy.

AIM: To determine the expression of the HER2 protein in gastric carcinoma and to correlate the expression of a HER2 protein with clinicopathological characteristics of the disease.

MATERIAL AND METHODS: The data of HER2 protein expression and the parameters of the TNM classification were obtained from the histopathological reports of the Institute of Pathology in Skopje, and for the clinical stage we used patient's files from the University Clinic for Abdominal Surgery in Skopje.

RESULTS: The analysis of the correlation of HER2 protein expression and TNM classification parameters pointed out a significant correlation between HER2 protein expression and intragastric localisation of gastric carcinoma (P = 0.005), and the tumour grade of differentiation (P = 0.034). There was also a positive correlation between HER2 protein expression pattern and positive lymph nodes in patients with gastric carcinoma (P = 0.03). The expression pattern of HER2 +++ was significantly more common registered in patients with positive lymph nodes (P = 0.03)

CONCLUSION: The expression of HER2 protein could represent a biological marker with prognostic and predictive value in patients with gastric carcinoma. Considering the high mortality rate in patients with gastric carcinoma and lack of international standardised therapeutic approach, research of the role and significance of HER2 overexpression and Trastuzumab therapy may prove useful in the development of new therapeutic strategies.

Introduction

Gastric carcinoma is an aggressive disease that has a daunting impact on global health. Despite the decline in incidence and mortality rate in recent years, gastric carcinoma remains one of the leading causes of cancer-related deaths worldwide, especially in developing countries. According to the recent statistical database, gastric carcinoma, with 930,000 new diagnosed and 700,000 diseased per year, is included with 8% out of 10% cancer-related deaths per year among the world population [1] [2] [3].

Because the most patients present with advanced disease the survival rate in patients with gastric carcinoma remains low, besides the evolution of new and sophisticated surgical techniques and the development of supplementary preoperative, neoadjuvant and adjuvant chemotherapy protocols. Recognition of complete recovery after surgical treatment is present only in early stages of diagnosed gastric carcinoma. According to the fact that, even in developed countries, primary detection of gastric carcinoma is in the nonresectable stadium of the disease, the systemic therapy is the main option for treatment that will only prolong the duration of survival [4] [5] [6]. In spite of the surgical treatment and systemic/adjuvant chemotherapy, survival rate in

patients with advanced stage of gastric carcinoma remains low, as a consequence of which the medical treatment of patients in advanced stage of gastric carcinoma demands novel therapeutic possibilities.

Understanding the molecular basis of cancer will facilitate the development of novel molecular target therapies, which interfere with different signal cascades involved in cellular proliferation, differentiation and survival. For this purpose, new research reports for the influence of new biomarkers such as microRNA, microsatellite instability, different types of cytokines (IL1, IL6, IL10, IL11, TNF, X12), CyclinD, Bcl2, p53 and other, including the HER2 protein in carcinogenesis, and as e target molecules for new therapeutic modalities are more often published [7] [8] [9] [10] [11] [12] [13] [14] [15] [16] [17] [18].

The data about HER2 protein expression are dual, and the reported results in the literature are different, as a consequence of which the evaluation of HER2 protein expression values in gastric carcinoma in correlation with other clinicopathological prognostic factors is currently ongoing process [7] [15] [16] [17].

HER2 protein expression in gastric carcinoma, in correlation with existing acknowledged prognostic factors which include the parameters that determine the TNM stage of the disease, could become the basis for ongoing research in the field of molecular targeted and personalised therapy.

This research aims to determine the correlation between tissue expression of the HER2 protein and the stage of the disease, the parameters of TNM gastric carcinoma classification and the histologic grade of gastric cancer.

Material and Methods

One hundred and forty-nine patients with gastric carcinoma surgically treated at the University Clinic for abdominal surgery in Skopje were included in the study. The operative material was analysed at the Institute of Pathology, Medical Faculty in Skopje.

Before the surgical treatment at the University Clinic for Abdominal Surgery in Skopje, an imaging technique procedure, gastroscopy and preoperative evaluation and preparation were obtained. For every patient, a standard operative procedure, according to the tumour localisation with loco-regional and systemic lymphadenectomy was performed. Sixty-one patients underwent subtotal gastric resection with lymphadenectomy, and 88 patients underwent total gastrectomy with lymphadenectomy.

Following the surgical treatment, a substitution therapy in the post-operative period was applied, using different solutions. The substitution therapy includes correction of electrolyte disbalance with electrolyte solutions, correction of anaemia with transfusion, correction of hypoproteinemia with plasma and pure albumin solution, correction of coagulation factors deficiency with fresh frozen plasma and antibiotic therapy was performed if necessary. Every patient had a controlled postoperative dietary regime, antithrombotic prophylaxis and controlled analgesia for pain management.

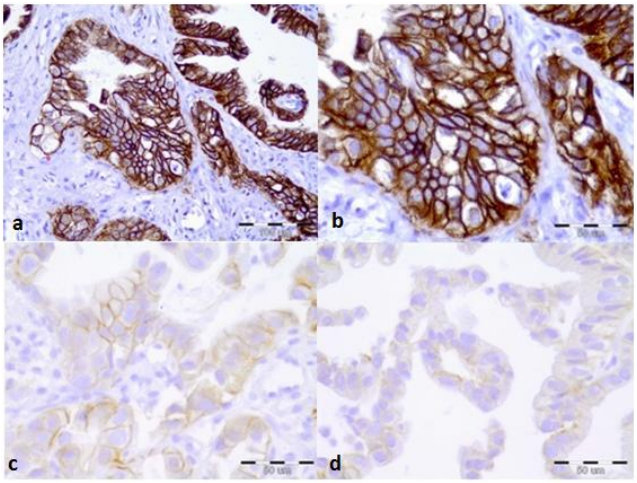

Figure 1: Immunohistochemical HER2 protein expression histological patterns. a) Her2+++ pattern of expression (x 200); b) same pattern of expression at higher magnification (x 400); c) Her2 ++ pattern of expression (x 400); d) Her2 + pattern of expression (x 400)

Data of HER2 protein expression and the parameters of the TNM classification (AJCC Cancer Staging 2017) were obtained from the archive histopathological reports of the Institute of Pathology in Skopje, and for the clinical stage, we used patient's files from the University Clinic for Abdominal Surgery in Skopje.

Immunohistochemical stainings were made with a standard procedure using Immunoperoxidase LSAB + system and specific primary monoclonal HER2-antibody (Ventana Medical Systems, Roche, and-HER2/neu Rabbit Monoclonal Primary Antibody Clone 4B5).

HER2 protein overexpression was defined in 4 histological patterns [18] (Figure 1):

0 No membranous staining or staining of < 10% of the tumour cells

+ Staining is weak or detected in only one part of the membrane in ≥ 10% of the cells

++ Moderate/weak complete or basolateral membranous staining in ≥ 10% of the cells

+++ Strong complete or basolateral membranous staining in ≥ 10% of the neoplastic cells

HER2 ++ and + expression are additionally

determined with Fluorescent in Situ Hybridization (FISH) method

To make a correlation between tissue HER2 protein expression and the disease stage, parameters of the TNM classification and histologic tumour grade HER2 expression was carried out in 2 steps. The first step, the patients were divided into two groups, according to the HER2 protein expression, as patients with positive HER2 expression and patients with negative HER2 immunostaining, excluding the importance of protein expression scheme. In the second step, the same analyses were repeated, except for the fact that HER2 positivity was analysed according to the scheme mentioned above (+, ++, +++).

Descriptive statistical methods were used for statistical analysis of the data. The rate of the interdependence of the analysed parameters was obtained with linear correlation. Statistical program SPSS for Windows 19.0 was used.

Results

The analysed group consisted of 149 patients, with the mean age of 65.19 ± 10.1, 108 (72.48%) of which were female and 41 (27.52%) male.

Clinical and histopathological characteristics of the patients and their cancers are shown in Table 1.

Table 1: Clinical and pathological characteristics of the analysed patients

Parameter	N (%)
Localization	
Cardia	61 (40.94)
Corpus	37 (24.83)
Antrum/Pylorus	51 (34.23)
T	
1	5 (3.6)
2	19 (12.75)
3	41 (27.52)
4	84 (56.38)
Nodal involvement	
Negative	36 (24.16)
Positive	113 (75.84)
Nodal status (TNM classification)	
0	36 (24.16)
1	27 (18.12)
2	30 (20.13)
3	56 (37.58)
Distant metastases	
No	136 (91.27)
Yes	13 (8.72)
Grade	
1	2.68)
2	64 (42.95)
3	81 (54.36)
Stage	
I	11 (7.38)
II	37 (24.83)
III	88 (59.06)
IV	13 (8.72)

The most common intragastric location of gastric carcinomas was cardia in 61 (40.94%) patients, followed by antral/pyloric carcinoma location in 51 (34.23%) patients and corpus location in 37 (24.83%) patients. According to the T state (local tumour growth), more than half of the examined patients 84 (56.38%) were in T4 state of the disease.

Presence of positive regional lymph nodes was detected in 113 (75.84) patients, and negative in 36 (24.16%) patients. The majority of the patients that comprised the analysed group had poor differentiated gastric carcinoma 81 (54.36%), and 88 (59.06%) were in Stage III of the disease.

Immunohistochemical staining with HER2 antibody showed HER2 protein expression in 44 (29.53%) carcinoma tissue, of which (6.71%) with HER2+, 7 (4.69%) with HER2++ and 27 (18.12%) with HER2+++ expression pattern.

Table 2: HER2 expression in analyzed patients

Variable	N (%)
Her2 expression (pattern)	
-	105 (70.47)
+	10 (6.71)
++	7 (4.69)
+++	27 (18.12)
Her2 positivity	
Negative cases	105 (70.47)
Positive cases	44 (29.53)

HER2 + expression pattern in gastric carcinoma was more frequent in female patients (27.59% vs 13.33%), while HER2+++ expression pattern was more frequently represented in male patients (73.33% vs 55.17%) in the group of 44 positive cases.

There was the insignificant difference in the distribution of HER2 protein expression according to the gender of the patients (P = 0.4).

Patients with positive HER2 protein expression in gastric carcinoma were with a median age of 65.5 ± 9.8 years, and patients with no HER2 protein expression were with a median age of 64.4 ± 10.7 years, without significant difference between the groups (P = 0.55).

Presence of HER2 protein was obtained in 16 (26.23%) carcinomas with a location in gastric cardia, 11 (29.735) in the corpus and 17 (33.33%) carcinomas with the antral/pyloric location, with no significant difference in HER2 protein expression, according to the location of gastric carcinoma (P = 0.71).

The analysis of different HER2 expression patterns showed:

HER2+ protein was more frequently present in gastric carcinomas with a location in the corpus (54.55%) about antrum/pylorus location (17.65%) and cardia (6.25%).

HER2++ expression was most frequently registered in carcinomas located in cardia (31.25%) and was absent in gastric corpus.

HER2+++ protein expression was more frequent in antral/pyloric location, in correlation with

cardia and corpus location (70.59%, 62.5%, 45.45% respectively).

Statistical analysis confirmed the presence of a significant difference in HER2 protein expression pattern according to the location of the gastric neoplasm (Table 3).

Table 3: HER2 protein expression in gastric carcinoma according to the location

Localization	n	Her2 expression pattern			P-value
		+	++	+++	
Cardia	61	1 (6.25)	5 (31.25)	10 (62.50)	0.005**
Corpus	37	6 (54.55)	0	5 (45.45)	
Antrum/Pylorus	51	3 (17.65)	2 (11.76)	12 (70.59)	

**P < 0.05; **P < 0.01.*

We did not find significant differentiation in HER2 expression according to the local growth (T status) neither when analysed only HER2 negative cases with HER2 positive, nor when we analysed HER2 negative carcinomas with different HER2 positive patterns (+,++,+++) (P = 0.54, P = 0.63 respectively.

HER2 protein expression was insignificantly different in patients with gastric carcinoma with positive and negative lymph nodes. Additional analysis of different HER2 expression pattern regarding positive and negative lymph nodes showed that HER2 +++ was significantly more frequent in patients with positive regional lymph nodes (P = 0.03). HER2 +++ pattern was found in 33.33% of patients with negative lymph nodes and 68.57% of patients with positive lymph nodes.

Statistically, a significant difference was found in the carcinomas with different grade (G) in patients with positive and negative HER2 expression. More frequent HER2 expression was detected in patients with moderately differentiated carcinomas in comparison to the patients with poorly differentiated carcinomas (40.63% vs 20.99%, P = 0.034), (Table 4).

Table 4: Distribution of HER and HER negative patients according to the grade of carcinoma differentiation

Grade	n	Her2 expression		p-value
		Negative	Positive	
G1	4	3 (75)	1 (25)	0.034
G2	64	38 (59.38)	26 (40.63)	
G3	81	64 (79.01)	17 (20.99)	

**P < 0.05; **P < 0.01.*

Additional analysis of HER2 expression patterns (+, ++, +++) confirmed non-significant difference in HER2 expression patterns according to the tumor differentiation (P = 0.28), but there was a statistically negative significant correlation between HER2 expression pattern and tumor differentiation (P = 0.01), meaning that HER2+++ pattern was significantly related to well-differentiated carcinomas (Figure 2).

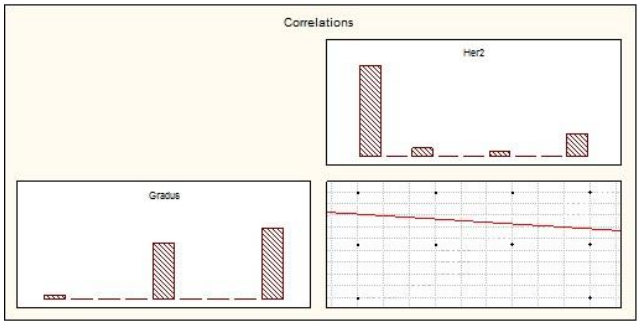

Figure 2: Correlation between the grade of differentiation and HER2 protein expression

HER2 protein expression was not significantly related to the Stage of the disease neither an analysis of positive and negative cases nor in HER2 expression patterns analysis (P = 0.56; P = 0.61, respectively).

Discussion

TNM classification of gastric carcinoma is the most valuable prognostic factor in patients with this disease. The classification contains the following elements: T – local tumour growth, N- lymph node involvement and M- the distant metastatic spread of the primary disease. The grade of differentiation of a tumour is also a part of the classification. However, there is a variable prognosis among the patients at the equal stage of the disease. Therefore, a request for revealing additional parameters for better identification of biological subgroups imposes itself. Biological predictive factors are obtained from genetic process, which is considered to be the key step in the development of gastric carcinoma (HER2, E-cadherin, EGFR, microsatellite instability, changes in few factor expression, including thymidylate synthase, beta-catenin, mucin-Ag, p53, COX-2, matrix metalloproteinases, and receptors for vascular endothelial factor) [7].

HER2 protein (HER2/neu, ErbB-2) is a 185-kDa transmembrane tyrosine kinase receptor, coded by HER2 proto-oncogene, located on the long arm of the 17-the chromosome. HER2 protein is a member of epidermal growth factor receptor family. This receptor family consists of 4 members: HER1 (also known as EGFR), HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4), which share the same molecular structure on their extracellular domain for ligand binding and intracellular part with tyrosine-kinase (TK) activity (with the exception of HER3). HER2 does not bind to anyone knew ligand and is considered to be ligand-free co-receptor for all other members of ErbB family. In fact, HER2 presents a peripheral partner for hetero-dimerisation of other members of HER family. Ligand binding to HER family cellular receptor induces homo-

dimerisation and hetero-dimerisation of EGFR to other types of HER proteins (HER2 is a part of the hetero-dimerisation process). Various ligand binding activity for extracellular domain initiates a signal transmitting (transducing) cascade, which may have an impact on the cell proliferation, apoptosis, cell adhesion, migration, angiogenesis and differentiation [7] [8] [9] [19] [20].

Many authors suggest an important HER2 role in various cancer types development. HER2 expression and/or amplification is recorded in invasive type of breast carcinoma [19], endocrine cancer [20] colon carcinoma [21], bladder carcinoma [22], ovarian carcinoma [23], endometrial [24] and cervical carcinoma [25], lung carcinoma [26], head and neck carcinoma [27], esophageal [28] and gastric carcinoma [29].

The reported data about the overexpression of HER2 in gastric carcinoma are diverse, depending on characteristics of the analysed groups and HER2 overexpression is reported in a range from 2% to 34%. The difference in expression is dependent on the localisation of a tumour in the stomach (gastroesophageal function or other localisation), the histological subtype (diffuse, intestinal, mixed, and unknown), and differentiation of a tumour.

Although according to some authors, there is no correlation between over-expression of HER2 and the disease prognosis, other authors found an association between HER2 overexpression and worse prognosis. It is considered that HER2 expression is in positive correlation with the tumor size, serous invasion and lymph node metastases, and also with poor prognosis of the patients for the 10-year survival period [7] [14] [29] [30] [31] [32] [33] [34] [35] [36).

The results from this study confirmed a significant correlation between tissue expression of HER2 and intragastric tumour location, lymph node metastasis and grade of tumour differentiation. The correlation between HER2 expression and the grade of differentiation is determined as negative, which means that HER2 expression is significantly associated with the more differentiated carcinomas, i.e. well-differentiated carcinomas.

Trastuzumab (Herceptin, Genentech, San Francisco, CA), is a monoclonal antibody that interferes with HER2 receptor (EGFR 2 receptor blocker), which in combination with chemotherapy protocols improves the outcome and survival in patients with different types of carcinomas, including the gastric carcinoma [19] [37] [38], but yet some patient may develop resistance to therapy with this antibody. For that reason, ongoing research on HER2 receptor are still required [38] The results from The International randomized study carried out in 2010, (Trastuzumab for gastric cancer treatment), revealed that Herceptin human monoclonal antibody, trastuzumab antibody anti-HER2 significantly prolong the overall survival, in comparison to chemotherapy protocol alone, in patients with HER2 overexpression [20] [37] [38] [39]. So, this research may be the basis for continuous research of the importance of HER2 expression in gastric carcinoma, with scientific and also medical call practice contribution.

In conclusion, this research determined significant difference in HER2 expression in gastric carcinoma with antro/pyloric localiztion, in comparison to carcinoma located on gastric cardia or corpus.

Statistically significant negative correlation of HER2 expression in gastric carcinoma and histologic grade of a tumour is also cinfirmed. According to these results, HER2 expression is significantly corelated to more aggressive disease and possibility of poor outcome.

We also found significant correlation between HER2 expression pattern HER2+++ and metastasis in regional lymph nodes.

The correlation of HER2 expression and above mentioned parameters highlights the possibility of HER2 expression being a valuable biologic marker with prognostic importance in gastric carcinoma patients. Considering the high mortality rate in patients with gastric carcinoma and lack of international standardized therapeutic approach, research of the role and significance of HER2 overexpression and Transtuzumab therapy may prove useful in development of new therapeutic strategies and treatment possibilities.

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Basic Science

Assessment of Laser Biostimulation in Induction of Ovulation

Mohamed A. El-Nourya[1], Mahmoud B. El-Begawyb[2], Mona E. Morsyc[3], Ehab M. Al Sherbineyd[4*]

[1]Department of Obstetrics & Gynecology, National Institute of Laser Enhanced Science (NILES), Cairo University, Cairo, Egypt; [2]Department of Pathology, Faculty of Veterinary Medicine, BeniSeuf University, Cairo, Egypt; [3]Department of Photobiology, National Institute of Laser Enhanced Science (NILES), Cairo University, Cairo, Egypt; [4]Department of Obstetrics & Gynecology, Nasr City Hospital for Surgery, Ministry of Health & Population, Cairo, Egypt

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***Correspondence:** Ehab M Al Sherbineyd. Department of Obstetrics & Gynecology, Nasr City Hospital for Surgery, Ministry of Health&Population, Cairo, Egypt. E-mail: elsherbiniehab@yahoo.com

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Abstract

AIM: This study aimed to evaluate a new modality of low power laser biostimulation in rat ovaries, in comparison with the conventional medical therapy by clomiphene citrate that depends on up-regulation of the hypothalamic-pituitary-ovarian axis to induce ovulation.

STUDY DESIGN: A Prospective experimental study carried out from January 2014 to February 2016.

SETTING: University-based photobiology laboratory.

MATERIALS AND METHODS: Seventy-two (72) Female-Wistar Albino rats were used in this study, divided into three groups: 17 rats used as a control group, 19 rats received clomiphene citrate (Clomid group), 36 rats exposed to diode laser 660 nm wavelength (laser group).

RESULTS: Biochemical assessment of serum Estradiol and serum Progesterone was done in the three study groups. Serum Estradiol & Progesterone levels were statistically significantly higher in clomiphene and laser treated groups than non-interventional controls, while no significant difference between clomiphene and laser groups as regard of both hormones.

CONCLUSION: This study shows that ovarian laser biostimulation is a new encouraging method for induction of ovulation, at least in animals. This had been proven biochemically by a significant increase in serum Estradiol and serum Progesterone.

Introduction

In humans as well as animals including rats, the reproductive function of the ovaries is controlled through complex feedback mechanisms, involving releasing factors from the hypothalamus and FSH and LH from pituitary gland. The resulting estrogen and progesterone produced by the ovarian follicles and corpora lutea, respectively, are the basis for the oestrous and menstrual cycles and also for normal reproductive function [1]. Many differences exist between menstrual and oestrous cycles, in humans, the reproductive cycle, called the menstrual cycle, last approximately 28 days, in rodents this cycle, called the oestrous cycle, lasts approximately 4-5 days. Rats display, most of the time, regular cycles; they are easy to manipulate, and the cycle is not disturbed easily even with the routine stress in the animal facility [2]. Another difference is that animals that have oestrous cycles reabsorb the endometrium if conception does not occur during that cycle. Animals that have menstrual cycles shed the endometrium through menstruation instead [3].

The estrus cycle is characterised as proestrus, estrus, metestrus (or diestrus I) and diestrus (or diestrus II) [4]. Vaginal smear cytology is used for the determination of the estrus cycle phases [5]. The characterisation of each phase is based on the proportion of three types of cells observed in the vaginal smear: epithelial cells, cornified cells and leukocytes [6].

Clomiphene citrate (CC) is a non-steroidal selective estrogen receptor modulator (SERM) of the triphenylethylene group that has become the most

widely prescribed drug for ovulation induction to reverse anovulation or oligo-ovulation [7]. It was described on the 19th WHO Model List of Essential Medicines [8], the most important medications needed in a basic health system.

Clomiphene binds to the E2 receptors in the hypothalamus (interfering with recycling of receptors) to create a state of hypo-estrogenicity, thereby causing an enhanced gonadotropin-releasing hormone (GnRH) release followed by an increased secretion of gonadotropins which induces ovulation [9].

Ovulation is known to occur in 70% of cases, while pregnancy occurs only in about 25-30% of the cases. The low pregnancy rate is due to the antiestrogenic effects of CC on the cervix, which would make it difficult to sperm penetration and on the endometrial growth which would, therefore, be unreceptive to the embryo [10].

Some studies have suggested that clomiphene citrate if used for more than a year, may increase the risk of ovarian cancer [11].

This, however, is disputed, and some feel there is no significant increase in risk [12]. It is not recommended by the manufacturer to use clomiphene for more than 6 cycles [13].

The clinical effect of light can be classified as direct and indirect, depending on whether the light causes an effect to occur within the irradiated tissue or whether a nervous or neuroendocrine signal is generated in the irradiated area and causes a systemic effect in another part of the body [14].

Low power lasers do not have a thermal effect on tissue. Photons may influence the proliferation of cells [15].

Low-level laser therapy (LLLT) is a form of laser medicine used in physical therapy and veterinary treatment that uses low-level lasers or light-emitting diodes to alter cellular function [16].

Photobiology works on the principle that, when the light hits certain molecules called chromophores, the photon energy causes electrons to be excited and jump from low-energy orbits to higher energy orbits. In nature, this stored energy can be used by the system to perform various cellular tasks, such as photosynthesis [17].

Cellular targets are mitochondria with the effect of increased adenosine triphosphate production, modulation of reactive oxygen species, and initiation of cellular signalling [18].

The final enzyme in the production of ATP by mitochondria, cytochrome-C-oxidase does appear to accept energy (photoacceptor) from laser-level lights, making it a possible candidate for mediating the properties of laser therapy [19].

The effects of LLLT appear to be limited to specified wavelengths of laser [20]. The typical wavelength is in the rage of 600-1000 nm (red to near infrared) [21].

Administering LLLT below the dose range does not appear to be effective [22]

The depth of penetration of laser light depends on the light's wavelength, mode of the laser, power density, technical design of the apparatus and the treatment technique used [23].

Material and Methods

All Institutional and National Guidelines for the care and use of experimental animals were followed.

Eighty-two adult female-Wistar Albino rats (Rattus norvergicus) were used in this study since they are the most commonly used experimental animals and easily available, selected at the average fertile period at 10-15 weeks old and 180-220 gm body weight.

The ovaries of two sacrificed female rats were subjected to diode laser 660 nm wavelength using an apparatus laser vex inc.-DPSSL II with contact control. The laser beam was delivered using a fibre-optic that was introduced directly through the vagina.

To estimate the accurate dose reaching the ovaries, a very sensitive power meter (Coherent-Laser Check = Model 1098293) was placed behind the ovaries to measure the amount of power delivered to the ovaries through the vaginal vault.

Many measurements were done until we obtained the recommended power, in which at a current of 210 m AMP, the power at the tip of the fibre-optic was 9.06 mW and those reaching the ovaries was 5.03 mW, and this was the targeted power [24] [25] (Figure 1).

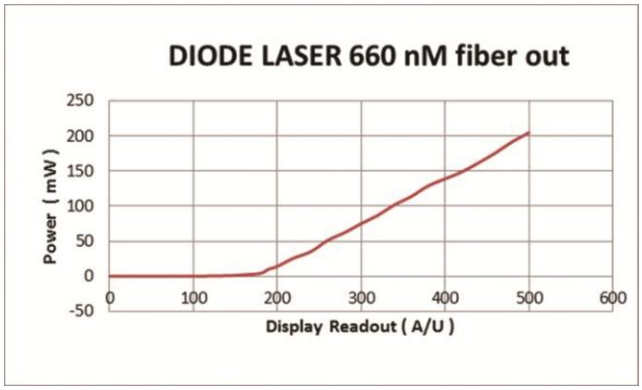

Figure 1: Diode laser 660 nm-fibre out, curve (power mW& current mAMP)

Vaginal smears was then done every morning daily for at least three consecutive successive regular cycles, to gain experience in handling of rats and to be familiar with the technique and morphological features of each phase of estrous cycle and to determine the diestrus phase of the estrous cycle, in which ovulation induction-either by clomiphene citrate or by laser-should be conducted during this phase [26]. Only cyclic rats were used in this study, whereas rats with irregular cycles (Blocked) were excluded.

Oestrous synchronisation was then performed, i.e. the stage of oestrous was determined by vaginal swab microscopically in each animal so that animals in the same oestrous stage were allocated to the same group.

Determination of the phase of oestrous cycle was performed through a collection of vaginal secretions with a plastic pipette filled with 0.5-1 mL of normal saline (NaCl 0.9%) by inserting the tip into the vagina, but not deeply. Vaginal fluid was placed on glass slides. One drop was collected with a clean tip from each rat. This fresh drop was examined (either unstained or stained by methylene blue stain) under a light microscope, without the use of the condenser lens, with 10 and 40 x objective lenses.

A pilot study was conducted using two rats administered clomiphene citrate, at a dose of 20ug/day for 2 consecutive days, starting on the diestrus phase and then sacrificed on the 4th day of the cycle. Serum samples for E_2 and P were collected. Another pilot study was tried using three rats exposed to laser biostimulation at a dose of 150 mj/Cm^2 (5 MW/Cm^2 x 30 sec) for two consecutive days. Starting in the diestrus phase and then sacrificed on the 4th day of the cycle, serum samples for E_2 and P were collected. A 3rd pilot study was done By exposing three rats to laser biostimulation at a dose of 150 mj/Cm^2, for three consecutive days (a cumulative dose of 450 mj/Cm^2) [24] [25], results also recorded.

Blood samples were collected by puncture of the ophthalmic venous plexus for E_2 and P hormonal assay.

Following these pilot studies (Eight rats were used in the pilot studies), the original study was started in January 2014 and continues to February 2016, using a total number of 74 adult female rats. Two died (one from the laser group and one from the clomiphene group and were excluded from the study). The remaining 72 rats were divided into three groups.

- *Group I (Control Group):* Consists of 17 rats used as a control group. Nothing was done for this group.

- *Group II (Clomid Group):* Consists of 19 rats received clomiphene citrate 20 micrograms per rat daily orally-for 2 consecutive days, starting on the diestrus phase of the oestrous cycle [26].

- *Group III (Laser Group):* Consists of 36 rats in which their ovaries were exposed to diode laser 660 nm wavelength (laser vex inc.-DPSSL II) with contact control (fiber-optic introduced through the vagina to reach the vault), using power density of 5 MW/Cm^2, for 30 seconds (total dose of 150 mj/Cm^2), for three consecutive days (a cumulative dose of 450 mj/Cm^2). Starting in the diestrus phase of the oestrous cycle [24] [25].

At the end of the study, at metestrus phase (4th day of starting induction), the phase in which serum Estradiol or progesterone begins to increase [27], blood samples were collected by puncture of the ophthalmic venous plexus & serum were stored at -20ºC until assayed.

The serum Estradiol and Progesterone assay were done by fully automated access system for immunoassay analysis using Electro-Chemi-Luminescence (ECL) detection system.

Data were statistically described regarding range, mean, standard deviation (± SD), and frequencies (number of cases). Comparison between the three study groups was made by one-way analysis of variance (ANOVA) test, and then analysis between every two groups was done by independent-samples t-test. All statistical calculations were done using computer programs, Microsoft Excel Office version 10 (Microsoft Corporation, NY, USA) and SPSS (Statistical Package for the Social Science; SPSS inc., Chicago, IL, USA) for IBM version 22, statistical programs.

A probability value (P value) less than 0.05 was considered statistically significant.

Results

This study was conducted on 72 rats, divided into three groups: 17 rats control, 19 rats' clomiphene & 36 rats' laser group (Table 1).

Table 1: Comparison between the three study groups as regard serum Estradiol level

Estradiol (E2) (pg/ml)	N	Mean	Std. Deviation	Minimum	Maximum	F	P
Clomid Induction	19	32.211	15.0465	16.0	77.0	3.474	0.037
Laser Induction	36	31.222	8.7541	15.0	57.0		
Control	17	24.000	6.8099	13.0	33.0		
Total	72	29.778	10.7824	13.0	77.0		

N: Number; F: ANOVA test; P: significance.

There was the statistically significant difference between the three study groups regarding serum Estradiol (P = 0.037) (Table 2).

Table 2: Comparison between the three study groups as regard serum Progesterone level

Progesterone (ng/ml)	N	Mean	Std. Deviation	Minimum	Maximum	F	P
Clomid Induction	19	30.977	12.2515	13.0	52.0	8.183	0.001
Laser Induction	36	25.299	9.0915	12.0	51.4		
Control	17	18.353	5.2433	7.0	27.0		
Total	72	25.157	10.2544	7.0	52.0		

The cutoff the value of serum Progesterone in ovulation is 3.6ng/ml.

There was the statistically significant difference between the three study groups regarding serum Progesterone (P = 0.001) (Table 3).

Table 3: Comparison between laser & Clomid groups as regard serum Estradiol & serum progesterone levels

	GROUP	N	Mean	Std. Deviation	Std. Error Mean	t	P
Estradiol (E2) (pg/ml)	Clomid Induction	19	32.211	15.0465	3.4519	0.309	0.759
	Laser Induction	36	31.222	8.7541	1.4590		
Progesterone (ng/ml)	Clomid Induction	19	30.977	12.2515	2.8107	1.949	0.057
	Laser Induction	36	25.299	9.0915	1.5152		

N: Number; t: independent test; P: significance.

There was no statistically significant difference between laser-treated group & Clomid induction group regarding serum Estradiol levels (P = 0.759).

There was no statistically significant difference between laser-treated group & Clomid induction group regarding serum Progesterone levels (P = 0.057) (Table 4).

Table 4: Pearson Correlation between serum Estradiol & serum Progesterone levels in control group

GROUP = Control		r
Estradiol (E2) (pg/ml)	Pearson Correlation	1.00
	N	17
Progesterone (ng/ml)	Pearson Correlation	0.585*
	P	0.014
	N	17

There was a significant positive correlation between serum Estradiol & serum Progesterone levels in control group (P = 0.014) (Table 5).

Table 5: Pearson Correlation between serum Estradiol & serum Progesterone levels in clomid group

GROUP = Clomid Induction		r
Estradiol (E2) (pg/ml)	Pearson Correlation	1.00
	N	19
Progesterone (ng/ml)	Pearson Correlation	0.579**
	P	0.009
	N	19

There was a significant positive correlation between serum Estradiol & serum Progesterone levels in clomid group (P = 0.009) (Table 6).

Table 6: Pearson Correlation between serum Estradiol & serum Progesterone levels in the laser group

GROUP = Laser Induction		r
Estradiol (E2) (pg/ml)	Pearson Correlation	1.00
	N	36
Progesterone (ng/ml)	Pearson Correlation	0.206
	P	0.228
	N	36

Non-significant positive correlation was found between serum Estradiol & serum Progesterone levels in laser group (P = 0.228).

Discussion

This new modality of low power laser (LPL) biostimulation was compared with the conventional medical therapy using clomiphene citrate that depends on upregulation of hypothalamic-pituitary-ovarian axis to induce ovulation, and with non-interventional controls.

The purpose of this study is to assess and compare the levels of serum Estradiol and serum Progesterone between the three studied groups, exactly at the same phase of the oestrous cycle (metestrus = diestrus 1) [27].

This is the 1st time that low power laser has been used as a biostimulant to normal (non PCO) ovaries and used to induce ovulation.

The only work done in this field, was carried out by two studies, the first study was conducted by Al-Watban and Andres [24], where they used He-Ne laser (632.8 nm) and solcoseryl in "in vitro" biostimulation on Chinese hamster ovary (CHO) and human skin fibroblast (HSF), using an optimum power density 1.25 mw/cm^2 and cumulative doses of 60-600 mj/cm^2, with average 180 mj/cm^2 given for three consecutive days, resulted in significant increase in cloning efficiency of CHO and HSF cells.

The second work was carried out by Sherein and Hossam Eldein [25] who compared the effect of low power laser biostimulation with clomiphene citrate, in the induction of ovulation with non-interventional controls in female Wistar Albino rats with polycystic ovarian disease (PCO). In this study induction of polycystic ovaries was done by receiving oral letrozole 0.5 mg/kg body weight-daily for 20 days in 60 rats. Rats were divided into three groups, 20 rats in each group.

Clomid group received clomiphene citrate 20 μg/rat orally-daily for 2 consecutive days.

Laser group received general anaesthesia, where their abdomen was opened, and their ovaries were exposed to diode laser 650 nm, using a dose of 150 mj/cm^2 (power density was 30 MW/cm^2), using an apparatus - Intelite R 650-250, daily for 2 consecutive days.

All animals then sacrificed on the 24th day from the 1st day of letrozole administration. Whole blood was collected for serum progesterone assay, and the ovaries were taken for histopathology. This study concluded that low power laser is a new encouraging method for induction of ovulation, and it

is more effective with less complication compared to clomid.

Low power laser irradiation of cells at certain wavelengths can activate some of the native components resulting in alteration of specific biochemical reactions, as well as cell metabolism. This alteration forms the basis for low power laser effects [28].

The mitochondria are sensitive to irradiation with monochromatic visible and near-infrared light. It increases adenosine triphosphate synthesis and consumption of oxygen [29], as well as RNA and protein synthesis in the mitochondria [30].

In our study, we used diode laser 660nm wavelength, since its apparatus forms are small, portable&easily modulated. Also, the depth of penetration of diode laser is suitable to reach the human ovary.

Using a fiber-optic, that was introduced directly through the vagina to deliver laser to the ovaries through the vaginal vault, reduces the number of animal loss (only one rat died in laser group) i.e. 2.7% fatality, when compared to laparotomy and surgical mobilization of ovaries, to be directly exposed to laser beam and re-exposure on the 2nd day, this increases the animal loss to be six rats, i.e. 23% fatality, as done by Sherein and Hossam Eldein [25]. Mechanical manipulation of the ovary during laser application is not physiologic and may have a role in abnormal ovarian response, also using fibre optic can be suitable for application in women by introducing the fibre through an egg-pickup needle socket (used for in vitro fertilisation = IVF) under ultrasound guidance.

Our study shows the statistically significant difference as regard serum Estradiol levels between the three studied groups (P value less than 0.05).

The clomid induction group has higher serum Estradiol values (mean 32.21 ± SD 15.04), compared to the control group (mean 24 ± SD 6.81).

This is in contrast with the study of Kilic-Okman et al., [26] that was carried out to compare the effect of clomiphene citrate and letrozole on ovarian follicles, endometrium and hormone levels in rats. This study found no significant difference between clomiphene citrate treated rats and that given placebo as regard serum Estradiol (P value more than 0.05).

The laser induction group has higher serum Estradiol levels (mean 31.22 ± SD 8.75), compared to the control group (mean 24 ± SD 6.81).

The mean serum Estradiol was not statistically significantly higher in the clomid group compared with laser group (P value more than 0.05).

Also, this study shows the significant difference as regard serum progesterone levels between the three studied groups (P value less than 0.05) statistically.

The clomid induction group has higher serum progesterone values (mean 30.97 ± SD 12.25), compared to the control group (mean 18.35 ± SD 5.24).

The result is in contrast with the study carried out by Sherein & Hossam Eldein [25], which found no significant difference between clomid and control groups as regard serum progesterone (P value more than 0.05)

The laser induction group has higher serum progesterone levels (mean 25.29 ± SD 9.09), compared to the control group (mean 18.35 ± SD 5.24).

This result is by Sherein&Hossam Eldein [25], who found the highly significant difference (P less than 0.001) in serum progesterone towards the laser-treated group.

While in comparing serum progesterone levels between clomid and laser-treated group, the difference was found non-significant (p more than 0.05), this is in contrast with Sherein & Hossam Eldein [25] who found statistically significant difference towards laser stimulated group (p less than 0.05).

Significant positive correlation between serum Estradiol & serum progesterone levels was found in the control group (r = 0.585, P = 0.014). Significant positive correlation between serum Estradiol & serum progesterone levels was found in clomid treated group (r = 0.579, P = 0.009). Non-significant positive correlation between serum Estradiol & serum progesterone levels was found in the laser treated group (r = 0.206, P = 0.228).

In conclusion, this study shows that ovarian laser biostimulation is a new encouraging method for induction of ovulation, at least in animals. This had been proven biochemically by a significant increase in serum Estradiol (that assess the endogenous estrogenic hormonal activity) and serum Progesterone (a good indicator for ovulation and corpus luteum function).

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Basic Science

Genetic Polymorphism of *CYP2A6* and Its Relationship with Nicotine Metabolism in Male Bataknese Smokers Suffered from Lung Cancer in Indonesia

Noni Novisari Soeroso[1*], Rozaimah Zain-Hamid[2], Bintang Y. M. Sinaga[1], Ahmad Hamim Sadewa[3], Tamsil Syafiuddin[1], Elisna Syahruddin[4], Gino Tann[5], Erna Mutiara[6]

[1]Department of Pulmonology and Respiratory Medicine, Faculty of Medicine, University of Sumatera Utara, Jl. Dr Mansyur No.5 Medan 20155, Indonesia; [2]Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Sumatera Utara, Jl. Dr Mansyur No.5 Medan 20155, Indonesia; [3]Department of Biochemistry, Faculty of Medicine, Gadjah Mada University, Jl. Farmako Sekip Utara, Yogyakarta 55281, Indonesia*; [4]Department of Pulmonology and Respiratory Medicine, Faculty of Medicine, University of Indonesia, Jl. Persahabatan Raya No.1, Jakarta 13230, Indonesia; [5]Department of Clinical Pathology, Faculty of Medicine, University of Sumatera Utara, Jl. Dr Mansyur No.5 Medan 20155, Indonesia; [6]Department of Biostatistics, Faculty of Public Health, University of Sumatera Utara, Jl. Dr Mansyur No.5 Medan 20155, Indonesia*

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***Correspondence:** Noni Novisari Soeroso. Department of Pulmonology and Respiratory Medicine, Faculty of Medicine, University of Sumatera Utara, Jl. Dr Mansyur No.5 Medan 20155, Indonesia. E-mail: noni@usu.ac.id

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Abstract

BACKGROUND: Cytochrome P450 2A6 (CYP2A6) is known as an enzyme which is responsible for the metabolism of chemical compounds.

AIM: This study aimed to analyse the relationship between *CYP2A6* gene polymorphism with nicotine metabolism rates and lung cancer incidence among smokers of Batak ethnic group in Indonesia.

METHODS: This study was a case-control study involving 140 research subjects through a purposive sampling technique from three hospitals in Medan, Indonesia. An examination of nicotine metabolism rates was conducted for all subjects using the 3HC/cotinine ratio parameter with LC-MS/MS technique. The examination of the *CYP2A6* gene was performed with PCR-RFLP. Data were analysed with Conditional Logistic Regression test using Epi Info 7.0 software.

RESULTS: The allele frequencies of *CYP2A6*1A, CYP2A6*1B,* and *CYP2A6*4A* found were 44.3%, 48.9%, and 6.8%, respectively. The *1B allele showed the highest metabolism rate. It is found that slow metabolizer individuals were 5.49 times more likely to develop lung cancer (P = 0.01, 95%CI 1.2-24.8).

CONCLUSION: Among the Bataknese smokers studied, the *CYP2A6*1B* allele was found to be the most common allele and showed the highest rate of nicotine metabolism. However, the results show the insignificant relationship among *CYP2A6* genetic polymorphism, nicotine metabolism, and lung cancer incidence.

Introduction

Lung cancer is the largest case of cancer in the world with an increased number of patient at approximately 1.2 million people/year [1], and it also shows an increase in mortality [2]. One cause of lung cancer is from tobacco smoke because of smoking activity with nicotine and nitrosamine as carcinogenic substances contained in it [3]. Carcinogenic substances in cigarettes require metabolic activation which is carried by enzymes in a human body.

Cytochrome P450 (CYP) is a superfamily of heme-containing monooxygenases involved in the metabolism of drugs, environmental pollutants, and food materials which contain chemical compounds and endogenous compounds [4]. Among the family genes of *CYP2A*, only the *CYP2A6* gene encodes active proteins while the other genes are catalytically defective enzymes [5]. *CYP2A6* gene is also

responsible for the metabolism of carcinogenic substances of tobacco [6] [7].

One way to detect the enzyme capacity of CYP2A6 in metabolising tobacco substances, especially nicotine, is by investigating the nicotine metabolite ratio (NMR) between cotinine (COT) and trans-3'-hydroxycotinine (3HC). As known, nicotine in tobacco is metabolized into cotinine (COT) and then into trans-3'-hydroxycotinin (3HC) by CYP2A6 enzymes [8] [9]. The capacity of CYP2A6 enzyme based on NMR can be used to show the levels of body needs in maintaining the nicotine levels. In this case, there are two types of metabolism which occur, namely fast metabolizer and slow metabolizer. The fast metabolizer signifies a high level of need in maintaining the nicotine levels in the body, so it leads to increased consumption of cigarette and a higher risk of lung cancer whereas the slow metabolizer signifies the opposite [10].

Related to the above explanation, several studies found that the genetic polymorphisms of *CYP2A6* have a relationship with nicotine metabolism. Su *et al.,* [11] found that enzyme activity was detected higher in *CYP2A6*1* which indicated a high rate of nicotine metabolism. Nakajima *et al.,* [12] revealed a large difference in the cotinine ratio of nicotine in each. In their follow-up studies, they concluded that the difference between individuals in the formation of cotinine from nicotine has a strong association with the genetic polymorphism of the *CYP2A6* gene, and this result was found in 92 healthy Japanese. Also, another study also detected that there was a difference in the nicotine metabolism rate between Korean population and the Japanese population [13]. Similarly, Caraballo *et al.* [14] and Peârez-Stable *et al.,* [15] found a difference in nicotine metabolism between smokers from the Black race and Caucasian race.

Besides the relationship between the genetic polymorphism of *CYP2A6* and nicotine metabolism, Fujieda *et al.,* [16] found that there was a clear relationship between the genetic polymorphism of *CYP2A6* and cancer risk in the Japanese population. Tanner *et al.,* [17] also reported the same findings in which they examined the relationship between the genetic polymorphism of *CYP2A6* with nicotine metabolism and the risk of lung cancer in the American Indian population.

However, several other studies found that there was no apparent relationship between the genetic polymorphism of *CYP2A6* with nicotine metabolism and the risk of developing lung cancer [16] [18] [19] [20] [21]. Based on this, further research on the relationship between the genetic polymorphisms of *CYP2A6* with nicotine metabolism and the risk of lung cancer in only one ethnic group needs to be conducted to obtain more significant results.

Based on the above explanations, this research was aimed to investigate the relationship between genetic polymorphisms of *CYP2A6* in the population of Batak ethnic group with nicotine metabolism and the risk of developing lung cancer. The three allele investigated were *CYP2A6*1A, CYP2A6*1B,* and *CYP2A6*4A.* This based on the study from Oscarson *et al.,* [22] that found those three alleles as the most frequent allele in the Asian population. Also, the Batak ethnic group was chosen due to their tradition to smoke in several traditional ceremonies and their pure genetic inheritance. Thus, this study would give a more significant model of *CYP2A6* genetic polymorphisms associated with nicotine metabolism and the risk of lung cancer.

Methods

This study involved 140 male Bataknese smokers which were recruited from Haji Adam Malik Hospital, USU Hospital, and Elizabeth Hospital in Medan, North Sumatra, Indonesia. The recruited subjects were pure descendants of the Batak ethnicity whose father, mother, and both grandparents were pure Bataknese. All subjects represented 6 sub-ethnics of Batak (i.e. Karo, Pakpak, Toba, Simalungun, Mandailing, and Angkola) [23]. Out of the 140 subjects, 70 smokers who suffered from lung cancer were specified as case samples, and 70 healthy smokers were specified as control samples. All subjects were asked to answer a questionnaire which consisted of structured information about residence, pedigree chart (three breeds of pure Batak ethnicity), occupational history, smoking status, and history of cancer in the family (parents and siblings). Before research, all subjects signed informed consent.

The inclusion criteria for the case sample were lung cancer patients from the Batak ethnic group who have been diagnosed based on cytology or histopathology examination, male lung cancer patients, and smokers. On the other hand, the inclusion criteria for the control sample were individuals who did not have lung cancer matched with the case group according to age, sex, and Batak people who had a smoking history.

The exclusion criteria for the case sample were lung cancer patients who consumed rifampicin, dexamethasone, phenobarbital, methoxsalen (8-methoxypsoralen), tranylcypromine, tryptamine, coumarin, and neonatal thiol, abnormal function of liver and kidney, and were not undergoing chemotherapy while the exclusion criteria for the control sample were healthy individuals who did not consume drugs.

The methods used in this research were purposive sampling and case-control study. The

research was conducted within a period of one year (November 2016 to April 2017), and it has been accepted by the Ethics Committee of the Faculty of Medicine, University of Sumatera Utara.

Nicotine II chewing gum was obtained from Guardian Pharmacia (Singapore). Nicotine, cotinine and 3-hydroxycotinine were purchased from Sigma (St. Louis, Mo). Pure gene deoxyribonucleic acid (DNA) isolation kit was obtained from Promega (Madison, USA). Restriction enzymes were purchased from New England Biolabs (Beverly, Mass). All other chemicals and solvents which had the highest levels were available commercially.

Blood samples from all subjects were collected as much as 2ml, and then stored at -80°C. Genomic DNA was extracted from peripheral lymphocytes using Puregene DNA Isolation Kit (Promega). The *CYP2A6*1A, CYP2A6*1B,* and *CYP2A6*4A* genotypes used the following primer: 2Aex7F (5'-GRCCAAGATGCCCTACATG-3`) and 2A6R2 (5'-AAAATGGGCATGAACGCCC-3`) [24].

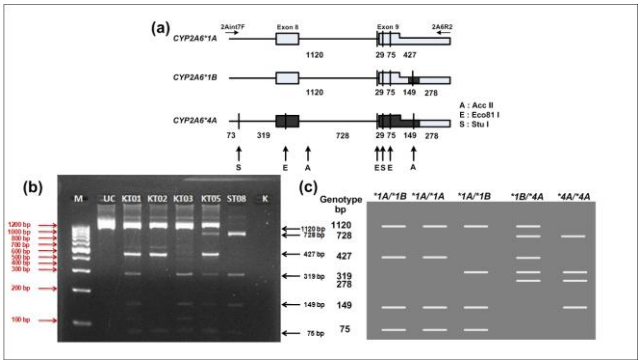

*Figure 1: (a) The CYP2A6*1A, CYP2A6*1B, and CYP2A6*4A allele genotypes by PCR-RFLP. The structure scheme of CYP2A7 and CYP2A6 gene. Dotted boxes and open boxes represented the CYP2A7 and CYP2A6 exon. Lines represented the introns of each gene. The PCR amplification was conducted with a primer pair, shown in horizontal arrows. The amplified DNA was triple-digested by Eco81I, AccII, and StuI. Restriction sites were marked with vertical arrows E, A, and S [33]; (b) Schematic polymerase PCR-RFLP patterns for different of CYP2A6 alleles in the case group 1-5, M = marker, K = control, UC = uncut. The *1A/*1B, 1120, 427, 319, 149, and 75 bp fragments (KT 01). The *1A/*1A, 1120, 427, 149, and 79 bp fragments (KT 02). The *1B/*1B, 1120, 319, 149, and 75 bp fragments (KT 03). The *1A/*4A, 1120, 728, 427, 319, and 278 bp fragments (KT 05). The *4A/*4A, 728, 319, 278, and 129 bp fragments (ST 08). The marker was a 1200 bp ladder marker; (c). Representative photograph of polymerase chain reaction-restriction fragment length polymorphism patterns for different CYP2A6 alleles of CYP2A6*1A, CYP2A6*1B, and CYP2A6*4*

The genomic DNA samples (0.5 µg) was added with PCR mixtures (25 µl) which contained 1 PCR buffer, 1.5 mM $MgCl_2$, 0.4 µM of each primer, 250 µM dNTPs, and 1 U of Taq DNA polymerase. After initial denaturation at a temperature of 95°C for 1 minute, the application was carried out with denaturation at 95°C for 15 seconds, annealing at 60°C for 20 seconds, and extension at 72°C for 3 minutes for 35 cycles, followed by a final extension at 72°C for 7 minutes. The PCR product was triple-digested with restriction enzymes, such as *Eco81I, AccII,* and *StuI*. The product was analysed by electrophoresis at 2% of agarose gel [25]. The *CYP2A6*1A, CYP2A6*1B,* and *CYP2A6*4A* allele genotypes obtained from PCR-RFLP, the schematic polymerase PCR-RFLP patterns for different of *CYP2A6* alleles, and the representative photograph of PCR-RFLP patterns for different *CYP2A6* alleles are shown in Figure 1.

The research subjects should not smoke within 2 weeks to eliminate nicotine and metabolite from cigarettes. On the next day, after a night of fasting, all subjects were asked to chew nicotine gum (Nicotinell gum which contained 4 mg of nicotine) for 30 minutes, and they should chew for 10 per 30 seconds.

Blood samples were collected from the cubital vein just before and two hours after started to chew, and these samples were stored at -20^{o}C before the analysis. Measurement of cotinine and 3'-hydroxycotinine (3HC) was conducted with *Liquid Chromatography Tandem-Mass Spectrometry* (LC-MS/MS). The ratio of 3HC/cotinine was calculated as the nicotine metabolism index. Rubinstein *et al.,* [26] state that the ratio of 3HC/cotinine for slow metabolizers is < 0.5 while the ratio for fast metabolizers is ≥ 0.5. Intraassay and interassay precision tests had been conducted in the LC-MS/MS equipment used.

Data analysis was performed by using Epi Info-7 software. Conditional Logistic Regression test was run to assess the differences between the two groups on demographic factors. Also, conditional logistic regression test and logistic regression test were also run to investigate the relationship between *CYP2A6* genotype and allele with lung cancer incidence and nicotine metabolism rate. The difference between nicotine metabolism rates and *CYP2A6* allele types was tested with the Kruskal Wallis test and Mann Whitney test. Logistic regression test was conducted to assess the effect of the *CYP2A6* allele on nicotine metabolism rate while conditional logistic regression test was conducted to assess the relationship between nicotine metabolism rate with lung cancer incidence. The ratio of nicotine metabolism rate (3HC/cotinine ratio) between the case group and the control group and its relation to *CYP2A6* allele were analysed with the Mann Whitney test. P value < 0.05 was considered significant.

Results

This study involved 70 lung cancer patients in the case group and 70 healthy people in the control group. The demographic characteristics of all subjects, such as 5 sub-ethnics of Batak (i.e. Toba,

Karo, Simalungun, Mandailing, and Pak Pak), types of cigarette, Brinkman Index (multiplication of smoking duration with number of cigarette per day), types of cancer, and Body Mass Index (BMI) can be seen in Table 1.

Table 1 shows that there was no significant difference in age, sub-ethnics of Batak, and the amount of cigarette consumption expressed in the Brinkman Index between the case group and the control group which means that these factors were not potentially biased in this study. The demographic factors which showed significant differences between the two groups were BMI and the types of cigarette.

Table 1: Demographic characteristics of subjects

		Total	Cases (n = 70)	Control (n = 70)	*P-value*
		N (%)	n (%)	n (%)	
Age	<65	106 (75.7)	52 (74.3)	54 (77.1)	0.69
	≥ 65	34 (24.3)	18 (25.7)	16 (22.9)	
Bataknese Sub ethnic	Toba	94 (67.1)	49 (70)	45 (64.3)	0.09
	Karo	34 (24.3)	13 (18.6)	21 (30)	
	Simalungun	4 (2.9)	1 (1.4)	3 (4.3)	
	Mandailing	7 (5)	6 (8.6)	1 (1.4)	
	Pakpak	1 (0.7)	1 (1.4)	0 (0.0)	
BMI	Underweight	16 (11.4)	6 (8.6)	10 (14.3)	0.01[a]
	Normoweight	59 (42.2)	44 (62.9)	15 (21.4)	
	Overweight	29 (20.7)	10 (14.2)	19 (27.1)	
	Obersity	26 (18.6)	10 (14.2)	26 (37.2)	
Type of cigarette	Mild	29 (20.7)	10 (14.3)	19 (27.2)	< 0.001[a]
	Kretek	66 (47.1)	26 (37.1)	40 (57.1)	
	Mix	45 (32.2)	34 (48.6)	11 (15.7)	
Brinkman Index	Mild	13 (9.3)	6 (8.6)	7 (10)	0.63
	Moderate	53 (37.8)	24 (34.3)	29 (41.4)	
	Severe	74 (52.9)	40 (57.1)	34 (48.6)	
Cytology/ histopathology subtype	Squamous cell ca	5 (7.1)	5 (7.1)	0	NA
	Adenocarcinoma	65 (92.9)	65 (92.9)	0	

[a] significant with Conditional Logistic Regression test (matched based on gender and Batak ethnic group); BMI (body mass index), NA (not available).

After the PCR-RFLP was performed on all research subjects, data of *CYP2A6* genotypes and alleles were obtained as shown in Table 2. The frequency of *CYP2A6* genotypes in both groups did not show significant deviations from Hardy-Weinberg Equilibrium ($p > 0.05$ for the case group and the control group). Among 140 male Batak smokers, the frequencies of *CYP2A6*1A, CYP2A6*1B,* and *CYP2A6*4A* alleles were 44.3%, 48.9%, and 6.8%, respectively.

This is quite surprising because *1A allele is assumed as the wildtype in most populations and other ethnic groups. In contrast, this study found that the highest number of allele found was *1B. The highest number of genotypes found was *CYP2A6 *1A/*1B* with 37.1%, and it was followed by *CYP2A6*1A/*1A* with 24.3%.

There was a significant difference in nicotine metabolism rates among the types of the *CYP2A6* allele. Although the average metabolic rate of all allele groups was classified into the slow metabolizer (ratio of 3HC/cotinine ≤ 0.5), the *1B allele showed the highest metabolic rate compared to other alleles, which was 3 times higher than the *4A allele and 1.5 times higher than the *1A allele.

Table 2: Frequency of CYP2A6 genotype and allele and its relation to nicotine metabolism rate

Genotype	Subjects*	
	n	%
*CYP2A6 *1A/*1A*	34	24.3
*CYP2A6 *1A/*1B*	52	37.1
*CYP2A6 *1B/*1B*	42	30
*CYP2A6 *1A/*4A*	4	2.9
*CYP2A6 *4A/*4A*	8	5.7

Allele	Subjects*		*3HC/cotinine Ratio*[d]	
	n	%		
*CYP2A6 *1A*	124	44.3	0.14 ± 0.31	b
*CYP2A6 *1B*	137	48.9	0.21 ± 0,37	a
*CYP2A6 *4A*	19	6.8	0.07 ± 0.21	c

*All frequencies of *CYP2A6* genotypes were in Hardy-Weinberg equilibrium; [a] $p < 0.001$ with Kruskal Wallis test; [b]$p = 0.02$ with Mann Whitney U test; [c] $p < 0.001$ with Mann Whitney U test; [d] data are expressed in mean ± standard deviation.

To investigate the association between *CYP2A6* genotype and allele with lung cancer incidence and nicotine metabolism rate, a cross-tabulation was conducted as shown in Table 3. Nicotine metabolism rate was measured using a 3HC/cotinine ratio parameter in serum, and it was measured 2 hours after the administration of nicotine gum. A ratio value of 3HC/COT < 0.5 was classified as slow metabolizer while a value ≥ 0.5 was classified as fast metabolizer.

Table 3 shows that there was no significant relationship between *CYP2A6* genotype and an allele with lung cancer incidence and nicotine metabolism rate ($P > 0.05$). One interesting point was that almost all subjects were classified as slow metabolizers, and only 13 people (18.6%) were classified as fast metabolizers. Based on Table 3, individuals with *CYP2A6*1B* allele were 2.07 times more likely to be fast metabolizer if compared with the *1A allele (wildtype). In contrast, individuals with *4A allele were more likely to be slow metabolizer although this relationship was not statistically significant. These findings are in line with the data shown in Table 4 which states that the *1B allele has the highest 3HC/cotinine ratio compared to the other alleles.

Table 3: Association of *CYP2A6* genotype and allele with lung cancer incidence and nicotine metabolism rate

		Lung Cancer		*P-value*	Nicotine Metabolism		*P-value*	OR	95% CI
		Case	Control		Fast	Slow			
CYP2A6 Genotype	**1A/*1A*	15 (21.4)	19 (27.1)	0.374[a]	0(0)	34 (26.5)	0.142[b]	NA[c]	NA
	**1A/*1B*	30 (42.8)	22 (31.4)		7(53.8)	45 (35.1)			
	**1B/*1B*	21 (30)	21 (30)		5(38.5)	37 (28.9)			
	**1A/*4A*	2 (2.9)	2 (2.9)		1(7.7)	3(2.3)			
	**4A/*4A*	2 (2.9)	6 (8.6)		0 (0)	8 (6.2)			
	Total	70 (100)	70 (100)		13 (100)	127 (100)			
CYP2A6 Allele	**1A*	62 (44.3)	62 (44.3)	0.187[a]	8(30.8)	116(45.7)	0.19[b]	1[d]	1
	**1B*	72 (51.4)	65 (46.4)		17(65.4)	120(47.2)		2.07	0.8-4.9
	**4A*	6 (4.3)	13 (9.3)		1 (3.8)	18 (7.1)		0.76	0.09-6.45
	Total	140 (100)	140 (100)		26 (100)	254 (100)			

[a] Conditional Logistic Regression test with matched gender and Batak ethnic group; [b] Logistic Regression test; [c] OR not available; [d] Reference value.

Theoretically, individuals who are fast metabolizers will inhale more nicotine in cigarettes, and thereby increase the risk of lung cancer incidence. Surprisingly, this study found the opposite. Individuals who were slow metabolizers were 5.49 times more likely to develop lung cancer than fast metabolizers (95% CI 1.2 – 24.8; $P = 0.01$), as shown in Table 4. The difference in nicotine metabolism rate between the case group and the control group and its

relationship with *CYP2A6* allele can be seen in Figure 2.

Table 4: Association of nicotine metabolism rate with lung cancer incidence

Nicotine Metabolism	Cancer n (%)	Control n (%)	*P - value*	OR
Fast metabolizer	2 (2.9)	11 (15.7)	0.01[a]	1[b]
Slow Metabolizer	68 (97.1)	59 (84.3)		5.49 (1.2-24.8)
Total	70 (100)	70 (100)		

[a] Significant with Conditional Logistic Regression test; [b] Reference value.

Figure 2 showed that there was no significant difference in the level of nicotine, cotinine, and 3HC (OH-cotinine) in plasma based on *CYP2A6* allele types in both groups (the cancer group and the control group). It indicates that the influence of *CYP2A6* allele lies in the nicotine metabolism rate of the subjects (3HC/cotinine ratio), but it does not affect the rate of nicotine absorption in the body.

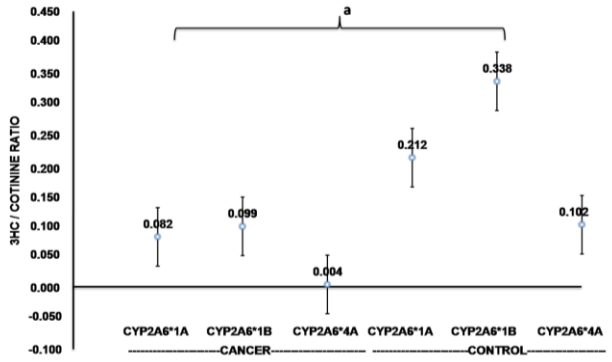

Figure 2: Comparison of nicotine metabolism rate (3HC/cotinine ratio) between the case group and the control group and its relationship with CYP2A6[a] allele. There was a significant difference in the level of 3HC/cotinine ratio between the case group and the control group (P < 0.001 with Mann Whitney test)

Discussion

In this study, the age range of lung cancer patients as the research subjects was 40-64 years. This based on the research conducted by Mong *et al.,* [27] that found that the age of most lung cancer patients was over 60 years (± 51.4%) because exposure to carcinogenic substances took a long time to cause an imbalance between oncogene function and tumor suppressor gene in the growth process of cancer cells, whereas lung cancer found at a young age or below the age of 50 years was associated with genetic factor or started smoking at a young age [28].

The ethnic group studied was Batak as the population of research subjects. In addition to the pure genetic inheritance in Batak, Batak society in North Sumatra has been accustomed to cigarettes, including mild cigarettes, *kretek*, and white cigarettes. Therefore, social environment is highly influential on the smoking behaviour of Batak ethnicity because smoking has an important element in various processes of customary activities. Furthermore, cigarettes must be provided along with food and beverages in every series of traditional event. After the traditional event finishes, cigarettes which are not consumed will be taken back by the event owner. Teenagers and children will usually hide 1-2 cigarettes, and then the cigarettes will be consumed alone furtively or together with their friends. This causes Batak children to have been accustomed to consuming cigarettes since young age [29].

Regarding smoking and different types of cigarette, this study also found that the most common lung cancer found, adenocarcinoma is considered to have a relation to the type of cigarette consumed. This based on the study that mentions that previously the most common type of cytology/histopathology of lung cancer was the squamous cell carcinoma; however, in recent decades, adenocarcinoma has been the most prevalent type of lung cancer. This might occur because of the interest change in the type of cigarette consumed [30]. Thus, in the case of Batak population, *kretek*, which was discovered to be more popular than mild cigarettes contains cloves which make smokers inhale more deeply, so the cigarette smoke will pass through small respiratory tracts which will make them exposed to more carcinogens [31]. Besides, Thun *et al.* [32] state that higher levels of nitrate contained in the tobacco mixture are also associated with the development of adenocarcinoma type of cancer cells. Stellmann *et al.,* [33] also state that extremely high levels of nitrosamines in the peripheral respiratory tract would trigger the occurrence of cell changes into adenocarcinoma. Thus, those results are also by the fact that tobacco-specific N-nitrosamines (TSNAs) induce adenocarcinoma type of lung cancer [34].

Interindividual variation associated with lung cancer risk is determined by uptake variation and metabolism of carcinogenic tobacco substances. The uptake of carcinogenic substances is related to the uptake of nicotine (the addictive component of tobacco). Previous research has found that pulmonary tumorigenesis can be triggered by the anti-apoptotic effect of cotinine through the activation of PI3K/Akt pathway mediated by CYP2A6 [35]. CYP2A6 in smokers is the main enzyme in nicotine metabolism. Nicotine will be metabolized into cotinine (COT), and COT will be metabolized into trans-3-hydroxycotinine (3HC) with the help of CYP2A6 enzymes. If the smoker is a slow metabolizer of nicotine, he only smokes a few cigarettes; thus, the level of exposure to carcinogenic materials will be low. Also, it is also reported that plasma cotinine concentrations, which are the main metabolite of nicotine, play an important role in the development of lung cancer incidence [36]. In the wildtype of *CYP2A6*, the risk of lung cancer incidence will increase. However, if gene deletion *CYP2A6*4* allele is found, it can reduce lung cancer incidence.

The most common *CYP2A6* allele in both groups in this study was *1B. *CYP2A6*1B* gene allele gives a high metabolic index, faster than the effect of *1A allele. Oscarson *et al.,* [22] which discovered the frequency of *CYP2A6*1B* allele was 30.0% in Caucasians (Spaniards) and 40.6% in Asian (Chinese) populations, found that the *CYP2A6* genetic polymorphism might be associated with lung cancer in the American and Asian populations, but it is not the same for the Caucasian population. This suggests that different ethnic will result in a different relationship between the two. In Batak ethnic population, the *1B allele was found about 48.9% higher than some other research population, but no significant relationship was found between *CYP2A6* genotype and allele with lung cancer incidence (P > 0.05). A different result was found by Fujieda *et al.,* [16] which stated that *CYP2A6* is not only one of the major determinants which do affect not only smoking behaviour, but also individual's susceptibility to lung cancer for Japanese Men population.

Several studies have also investigated the relationship between deleted polymorphisms, *CYP2A6*4,* and the risk of lung cancer in different populations. However, the results are inconsistent. One study in Japan reported a 50% reduction in cancer risk related to *CYP2A6*4* statistically while a similar study in China found a twofold increase in lung cancer risk in individuals with *CYP2A6*4.* Several meta-analyses reported a statistically significant 50% lower crude odds ratio in lung cancer with slow metabolizer smokers in the Asian populations. By our results, *CYP2A6*4* was associated with an approximately 30% decreased the risk of lung cancer [18].

The results of this study showed that there was no significant relationship between *CYP2A6* genotype and allele with nicotine metabolism rate (*P* > 0.05). One interesting thing found in this study was that almost all subjects were classified as slow metabolizers, and only 13 people (18.6%) were classified as fast metabolizers. However, there was a significant difference in nicotine metabolism rates among the types of the *CYP2A6* allele. The average metabolic rate of all allele groups was classified as the slow metabolizer (3HC/cotinine ratio ≤ 0.5), but the *1B allele showed the highest metabolic rate compared to other alleles, which was 3 times higher than the *4A allele and 1.5 times higher than the *1A allele. The *1B allele tends to be a fast metabolizer than the *4A allele. Over 20 years ago, individuals who metabolised nicotine poorly would smoke fewer cigarettes a day or less intense per cigarette than smokers who metabolised nicotine more efficiently [18]. This poor metabolism will tend to develop lung cancer because of its lower exposure to carcinogens in nicotine through the smoke. Several studies have reported that smokers who carry a lack of activity or less *CYP2A6* allele would smoke less. However, only in the Asian populations, both Japanese and Chinese, which have a high genetic variant prevalence, have an association between *CYP2A6*, smoking dose, and lung cancer. Theoretically, individuals who are fast metabolizers will inhale more nicotine in cigarettes, and thereby increase the risk of developing lung cancer. Surprisingly, this study found the opposite. Individuals who were slow metabolizers were 5.49 times more likely to develop lung cancer than the fast metabolizers (95% CI 1.2 – 24.8; *P* = 0.01). Based on the above explanation, the relationship between genetic polymorphisms of *CYP2A6* with nicotine metabolism and the risk of developing lung cancer in Batak population was found to have different results from different populations and races. Therefore, further research is important to investigate the differences in other populations and races.

In conclusion, *CYP2A6* activities signified the need for more cigarettes to maintain the levels and the metabolism of nicotine. The most common *CYP2A6* allele in the two groups was *1B. The results showed that no significant relationship was found between *CYP2A6* and nicotine metabolism in lung cancer patients. It was also found that the *1B allele was more likely to be a slow metabolizer than the *1A allele.

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Basic Science

Immunomodulatory, Apoptosis Induction and Antitumor Activities of Aqueous and Methanolic Extract of *Calvatia Craniiformis* in Mice Transfected with Murine Hepatocellular Carcinoma Cells

Ghassan Hamdan Jameel[1], Ali Ibrahim Ali Al-Ezzy[2*], Ibrahim H. Mohammed[3]

[1]Department of Microbiology, College of Veterinary Medicine, Diyala University, Baqubah, Iraq; [2]Department of Pathology, College of Veterinary Medicine, Diyala University, Baqubah, Iraq; [3]Department of Biology, College of Science, Diyala University, Baqubah, Iraq

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Keywords: *C. craniiformis*; H22 cells; Caspase-8; Apoptosis Index

***Correspondence:** Ali Ibrahim Ali Al-Ezzy. Department of Pathology, College of Veterinary Medicine, Diyala University, Baqubah, Iraq. E-mail: alizziibrahim@gmail.com

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Abstract

OBJECTIVES: To evaluate the Immunomodulatory, apoptosis induction and antitumor effects of aqueous and methanolic extracts of *Calvatia craniiformis* regarding the size of tumour mass, caspase-8 expression and apoptotic index (AI%) in mice transfected with murine hepatocellular carcinoma cell line (H22) as an experimental therapeutic system for human hepatocellular carcinoma.

MATERIAL AND METHODS: Forty-eight Balb/C albino mice were transfected in legs with H22 cells. Tumour size was measured twice a week. Caspase-8 protein expression and apoptotic index determination evaluated by Immunohistochemistry.

RESULTS: Tumor size significantly differed between the two groups of mice transfected with H22 cells; the first was treated with *C. craniiformis* aqueous extract (0.3, 0.6, 1.2) mg/kg and the second group was treated with *C. craniiformis* methanolic extract (0.25, 0.5, 1.0) mg/kg compared with control group. The inhibitory activity of aqueous and methanolic extracts was dose and duration dependent. The size of the tumour mass was reduced up to 87.9% when treated with 1.2 mg/kg aqueous extract and 1 mg/kg for methanolic extract. Caspase-8 expression was increased in a dose-dependent manner among H22 bearing mice treated with *C. craniiformis* aqueous extract (0.3, 0.6, 1.2) mg/kg. At 0.3 mg/kg, the intensity of expression was strong in (33.33%) and very strong in (66.67%). While at 0.6 mg/kg and 1.2 mg/kg the intensity of expression was strong in (33.33%) and very strong in (100%) with a significant difference ($P \leq 0.001$). H22 bearing mice treated with (0.25, 0.5, 1.0) mg/kg *C. craniiformis* methanolic extract shows increased caspase-8 expression in a dose-dependent manner. At 0.25 mg/kg, the intensity of expression was strong in (33.33%) and very strong in (66.67%). While at 0.5 mg/kg, the intensity of expression was strong in (33.33%) and very strong in (100%). At 1.0 mg/kg, the intensity of expression was strong in (16.67%) and very strong in (83.33%) with significant difference ($P \leq 0.001$). AI% of H22 bearing mice treated with *C. craniiformis* aqueous and methanolic extracts were significantly increased ($P \leq 0.05$) compared with the untreated control group. No significant difference was reported in AI% between aqueous and methanolic extracts treated groups.

CONCLUSIONS: Extracts of *C. craniiformis* were highly efficient in tumour growth inhibition, causing a reduction in the tumour size clinically and increase the expression of caspase-8 gene product in tumour tissue, causing increase apoptotic index of H22 cells taken from the legs of inoculated mice leading to loss of legs due to bone necrosis. Antitumor activity of *C. craniiformis* aqueous, and the methanolic extract was dose and duration dependent.

Introduction

The first written records on medicinal applications of plants date back to 2600 BC and report the existence of a sophisticated medicinal system in Mesopotamia, comprising about 1000 plant-derived medicines [1]. The Arabs preserved a large amount of the Greco-Roman knowledge during the dark and middle ages (i.e., 5th to 12th centuries), and complemented it with their medicinal expertise, and with herbs from Chinese and Indian traditional medicines [2].

Cellular compounds and the secondary metabolites extracted from edible mushrooms can be used for the treatment of cancer by acting as a

biological response modifier (BRM) [3]. BRM are immunostimulants which can be helpful in treating cancer (where targeted therapy often relies on the immune system being used to attack tumour cells) [4]. One of BRM, Maitake β-glucans called Grifolan, a branched β-1,3-d-glucan extracted from *Grifola frondosa* was found to promote tumour regression and necrosis and was approved to be used as anticancer therapy [5]. Aqueous extracts from Shiitake and Maitake, edible mushrooms showed increased host-mediated antitumor activity against Sarcoma 180 cancer [3]. Lentinan, a protein-free polysaccharide (β-1,3-d-glucans and β-1,6-d-glucans) derived from the fruit body of Shiitake was approved for the treatment of gastric cancer in Japan. Lentinan was found to be instrumental in activating macrophages to stimulate lymphocytes and other immune cell defences like increasing natural Killer cells [3].

The biological characteristics of *Calvatia craniiformis* extracts were studied extensively, as some of their compounds showed medical benefits because they contain active ingredients such as Calvatic acid, which has anti-inflammation and a definite antitumor effect. *C. craniiformis* significantly inhibits the growth of Yoshida sarcoma in cell culture and increase the survival time of mice with leukaemia 1210 [6]. Subsequent investigations have focused on the antitumor properties of calvatic acid, which may represent a model for the synthesis of more specific glutathione transferase-P1-1 inhibitors with possible therapeutic relevance [7].

The CASP8 gene encodes a member of the cysteine-aspartic acid protease (caspase) family. Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis. Caspases exist as inactive proenzymes composed of a prodomain, a large protease subunit and a small protease subunit [8]. Activation of caspases requires proteolytic processing at conserved internal aspartic residues to generate a heterodimeric enzyme consisting of the large and small subunits [9]. This protein is involved in the programmed cell death induced by Fas and various apoptotic stimuli [10]. The N-terminal FADD-like death effector domain of this protein suggests that it may interact with Fas-interacting protein FADD [9] [11].

Biochemically, caspase-8 was found to enter the complex of the inhibitor of NF-KB kinase (IKK) with the upstream Bcl10-MALT1 (mucosa-associated lymphatic tissue) adapter complex which was crucial for the induction of nuclear translocation of NF-κB [12]. Moreover, the biochemical form of caspase-8 differed in the two pathways. For the death pathway, the caspase-8 zymogen is cleaved into subunits that assemble to form the mature, highly active caspase heterotetramer whereas, for the activation pathway, the zymogen appears to remain intact perhaps to limit its proteolytic function but enhance its capability as an adapter protein [13].

The aim of the present study was to evaluate the Immunomodulatory, apoptosis induction and antitumor effects of aqueous and methanolic extracts of Calvatia craniiformis regarding the size of tumor mass, caspase-8 expression and apoptotic index (AI%) in mice transfected with murine hepatocellular carcinoma cell line (H22) as therapeutic experimental system for human hepatocellular carcinoma.

Materials and Methods

C. craniiformis obtained from groves of Al-Khalis region - Diyala province, Iraq. The classification of mushroom achieved in fungi research laboratory, Faculty of Agriculture, University of Baghdad, Iraq by professor Salman Kamel Jabr. *C. craniiformis* belongs to the fungal kingdom Mycota, Class Agaricomycetes, family Lycoperdaceae. Figure 1-A represents the form of fungus discovered in Iraq by our team, and Figure 1-B represents the cross-section with clearly appeared brown colour region filled with spores, which is part of the active ingredients used in the treatment.

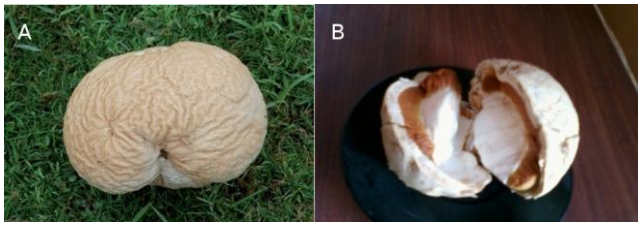

Figure 1: The discovered Calvatia craniiformis forms; A) C. craniiformis in the grove; B) Cross-section of C. craniiformis in laboratory

Fifty gram of soft plant was added to 500 ml of distilled water and then blended for 5-10 minutes until homogeneity. The extract was vibrated for an hour by shaker and then centrifuged for 10 minutes (2000 rpm/min). The sediment was discarded, and the supernatant was distributed in the clean, dry dishes and placed in an incubator for obtaining of the dry extract [14]. *C. craniiformis* crude aqueous extract gave 5 gm out of 75 grams of dry *material*, i.e. the extraction ratio was 6.66 % of crude *C. craniiformis*. The extract was dark brown to black colour, thick and little viscous.

Twenty gram of dry powder was taken and placed in a thimble. The thimble was placed in the Soxhlet device where the solvent, hexane was added to remove fat and chlorophyll. The extraction was conducted for 12 hours at a temperature (40-60^{o}C) for evaporation of solvent used. The obtained powder was transferred to the Reflex device with 70% methanol for three hours. The extract was filtered by a piece of gauze and filter paper then incubated for 24 hours for evaporation of methanol.

Methanolic extract was treated with HCl 1% in a Reflex for 30 minutes and filtrated by Whatman 1 filter paper. Diethyl ether was added to the filtrate in separating funnel and left for 24 hours. Two layers appeared, the top layer is the diethyl ether layer which had been neglected, and the bottom layer is an aqueous layer which picked. PH of the aqueous layer was raise for PH 8 by adding ammonia. Then after the aqueous extract was incubated to remove chloroform, the final form of the extract was obtained [15]. Methanolic extract gave 5 gm of 50 g, i.e. extraction ratio was 10% of the raw material. The resulting extract have yellowish-brown colour, thick and little viscous

To determine any possible toxic effects for *C. craniiformis*, Up-and down method was followed for determination LD50 according to the following equation [16]:

$$LD50 = Xf + Kd$$

Xf: the last dose administered

d: difference between dose levels

k: tabular value calculated from Table (1).

Table 1: the median lethal dose of alcohol and aqueous extract

Type of Extract	Difference between dose levels (d)	Death of the animal or to stay alive after 24 hours	The value of K tabular	The last dose administered (Xf)	Midterm lethal dose (LD50)
Aqueous	25	Ooxo	-439	100	85 mg\kg
Methanolic	50	Oxxx	1.5	200	177 mg\kg

O: the survival animal within 24 hours of injection; X: the death of the animal within 24 hours of injection.

According to acute toxicity study, aqueous extract was administered in the following doses: 0.3 mg/kg, 0.6 mg/kg, 1.2 mg/kg. Methanolic extract was administered in the following doses: 0.25 mg/kg, 0.5 mg/kg, 1 mg/kg.

Forty-eight albino Bclb/C mice (weight 18-20 g) were purchased from Drug investigation department-ministry of health (Baghdad, Iraq). The mice were housed under normal condition and with free access to food and water. Animal experiments and animal care carried out according to protocols approved by the institutional committee for animal care and by the recommendation for the proper use and care of laboratory animals. Mice were divided into four groups, (6) mice for each one. Three groups received extract and one group for control receiving Dimethyl sulphoxide (DMSO).

Murine Hepatocellular carcinoma cells (H22) was received from Tongji Hospital in Tonji University, Joaqon Hughoin (China). The steps for implant tissue carried out under sterile conditions. RPMI (1640) medium used for cultivation of H22 hepatocellular carcinoma cell line and development of full growth [17].

After complete growth, H22 cells were harvested from RPMI 1640 Medium, and 0.1 ml of cells were transfected in the leg of (48) Balb/C albino mice to establish a solid tumour model [18]. The experiment ending with the death of the last mouse from the control group given doses of aqueous and methanolic extract. Tumour size was measured twice a week during the duration of the experiment using special calibre and take the measurement analogy (latitude and longitude), and extracted tumour size [19] [20].

Immunohistochemistry (IHC) used for evaluation of apoptosis. The procedure of IHC was performed according to the manufacturer's instruction, using polyclonal rabbit anti-mouse caspase-8 IgG, ab25901 recognises the p18 form of Caspase-8 [21]. Secondary antibodies, Biotinylated goat anti-rabbit IgG. Final results visualised by using Immunohistochemistry detection kit, Expose Mouse and Rabbit Specific HRP/DAB Detection IHC kit ab80436 [22]

The primary antibody diluted by the common antibody diluent 1:50. Optimal antibodies concentration may vary depending on specimen and preparation method. This optimisation has been done. Both positive and negative controls were included for each run of caspase-8 detection by IHC. The negative control was obtained by replacing the primary antibody with PBS buffer. The positive control was obtained by using tonsil tissue [23].

The expression of the caspase-8 protein was measured by counting the number of positive cells with brown (DAB) nuclear staining under light microscopy X40. For the evaluation of caspase-8 expression, immunostaining was assessed semiquantitatively using a scoring system for both intensity and extent of staining in 10 microscopic fields which were randomly selected and based on the estimated percentage of caspase-8 positive cells, staining results were divided into 5 scores , (0 = no expression, no positive cells; 1 = weak expression, less than 40% positive; 2 = moderate expression, 40-60% positive cells; 3 = strong expression , more than 60% but less than 100% positive cells; 4 = strong ^{high}, 100% positive cells) [24]. Final results for the apoptotic index were expressed as Mean ± SE [25] [26]. Apoptotic index % was determined according to the following equation [27]:

$$Apoptotic\ Index\ \% = \frac{Number\ of\ Apoptotic\ cells}{Total\ number\ of\ cells\ per\ field} \times 100$$

Statistical analysis was performed using SPSS version 16 software. One-way Analysis of Variance (ANOVA), used to find out the significance of differences in caspase-8 expression; AI % between groups that composed of continuous variables. Mann-Whitney test used to find out the difference between aqueous and methanolic extract AI% activity. The level of Significance at ($P < 0.05$) and ($P < 0.01$).

Results

As shown in Figure 2, Administration of aqueous extract of *C. craniiformis* in 0.3 mg/kg, 0.6 mg/kg, 1.2 mg/kg to H_{22} tumour-bearing mice shows significant differences (P ≤ 0.01) in tumour size compared with control group. Among given doses, 1.2 mg/kg was effective dose causing a reduction in tumour size in last day of the experiment (36th), in which tumour size was 960 mm^3 compared with 1564.57 mm^3 for 0.6 mg/kg and 3559.20 mm^3 for 0.3 mg/kg while in the control group the tumour size was 5747.05 mm^3.

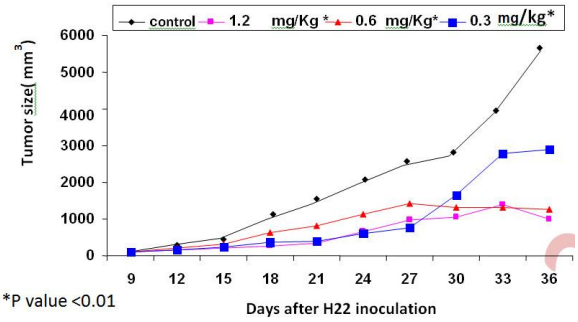

Figure 2: Tumor size follow up in mice transfected with H22 murine tumor cells treated with aqueous extract of C craniiformis; Significant difference in tumor size (p-value < 0.01) in the leg of inoculated mouse compared with H22 bearing mouse in control group that loss the legs inoculated with H22 Cells due to necrosis in the bones

Administration of methanolic extract of *C. craniiformis* in 0.25 mg/kg, 0.5 mg/kg, 1 mg/kg to H_{22} tumour-bearing mice shows a significant difference (P ≤ 0.01) in tumour size compared with control group. A significant difference in tumour size throughout experiment was reported. Tumour size was increased slightly in the treated group compared with control. In last day of the experiment (36th), tumour size was 1167.20 mm^3 in the group treated with 1 mg/kg compared with 1332.64 mm^3 for 0.5 mg/kg and 2076.33 mm^3 for 0.25 mg/kg while in control group the tumour size was 7747.04 mm^3 as shown Figure 3.

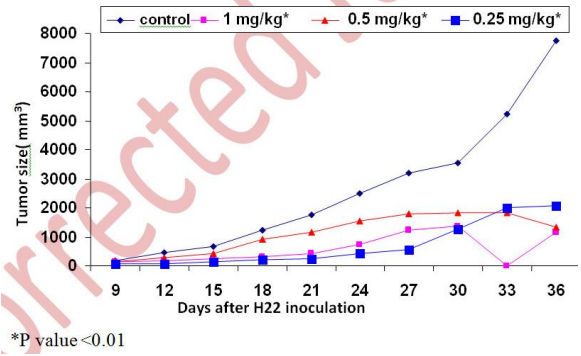

Figure 3: Tumor size follow up in mice transfected with H22 cells treated with a methanolic extract of C. craniiformis; the Significant difference in tumour size (p-value < 0.01) of mouse inoculated legs compared with the H22 bearing mouse in control group that loss the legs inoculated with H22 Cells due to necrosis in the bones

Among given doses, 1 mg/kg was effective dose causing reduction in tumor size in last day (36th), in which tumor size was 1167.28 mm^3 compared with 1332.64 mm^3 for 0.5 mg/kg and 2076.33 mm^3 for 0.25 mg/kg while in the control group the tumor size was 7747.05 mm^3 as shown in Figure 3. Inhibitory activity of the aqueous and methanolic extract was dose and duration dependent. The extract of *C. craniiformis* was effective in reduction of H22 tumour size at dose 1.2 mg/kg for aqueous extract and 1 mg/kg for methanolic in which H22 tumour mass was reduced in size for up to 87.9% mg/kg as shown in Figure 2 and 3.

Figure 4-B shown that H22 bearing mice treated with *C. craniiformis* aqueous extract using three consecutive doses (0.3, 0.6, 1.2) mg/kg shows significant inhibition of a tumour in the leg of the mouse compared with the H22 bearing mouse in control group that loss the legs inoculated with H22 Cells due to necrosis in the bones.

Table 2: The effect of *C. craniiformis* extracts on the apoptotic index of H22 bearing mice

Type of Extract	Dose (mg/kg)	Apoptosis index %	ANOVA (P-value)
Aqueous Extract	Untreated Control group	19.32	P ≤ 0.05
	0.3	25.34	
	0.6	26.70	
	1.2	27.21	
Methanolic Extract	Untreated Control group	18.30	P ≤ 0.05
	0.25	24.53	
	0.5	25.06	
	1	28.16	
Mann-Whitney test (p-value)	P > 0.05		

As shown in Table 2 and Figure 5-A, H22 bearing mice treated with *C. craniiformis* aqueous extract using three consecutive doses (0.3, 0.6 ,1.2) mg/kg shows a significant increase (P ≤ 0.05) in caspase-8 expression and hence in apoptotic index % (27.21%, 26.70%, 25.34%) compared with untreated control group (19.32%).

Figure 4: Effect of aqueous and Methanolic extracts of C carniiformis on H22 tumor development in the legs of inoculated mice; A) control group inoculated with H22 cells (Left), losing of leg due to bone necrosis and increased tumor size, compared with normal uninoculated mouse (right), increased tumor size, leading to loss of leg due to bone necrosis and destruction; B) H22 cells bearing mouse treated with aqueous extract of C carniiformis, shows inhibition of the tumor growth (right arrow) compared with untreated control group inoculated with H22, increased tumor size, (left) leading to loss of leg due to bone necrosis and destruction; C) H22 cells bearing mouse treated with methanolic extract of C carniiformis, mouse shows inhibition of tumor size (left arrow) compared with untreated H22 inoculated mouse (right)

Figure 4-C shown that H22 bearing mice treated with *C. craniiformis* methanolic extract using

three consecutive doses (0.25, 0.5, 1.0) mg/kg shows significant inhibition of a tumour in the leg of the mouse compared with the H22 bearing mouse in control group that loss the leg inoculated with H22 Cells due to necrosis in the bones. As shown in Table 2 and Figure 5-B H22 bearing mice treated with *C. craniiformis* methanolic extract (0.25, 0.5, 1.0) mg/kg shows significant increase (P ≤ 0.05) in the caspase-8 expression and AI% (28.16%, 25.06%, 24.53%) compared with control group (18.30 %). No significant difference was reported in AI% between aqueous and methanolic extracts treated groups.

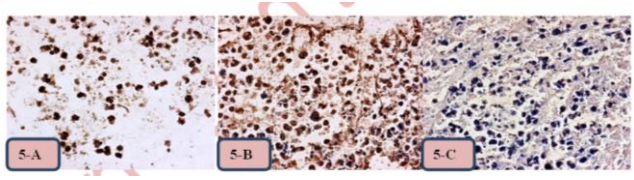

Figure 5: Immunohistochemical staining tissue sections from the leg of H22 murine hepatocellular carcinoma bearing mouse show cellular expression of caspase-8. 5-A: Tissue sections from the leg of an H22 bearing mouse treated with a crude aqueous extract of C. craniiformis. Up to 40% Cells with positive staining of caspase-8 expression stained with DAB chromogen (dark brown) counterstained with Mayer's hematoxylin (400X). 5-B: Tissue sections from the leg of an H22 bearing mouse treated with a crude methanolic extract of C craniiformis. Up to 100% of Cells with positive caspase-8 expression stained with DAB chromogen (dark brown) counterstained with Mayer's hematoxylin (400X). 5-C: untreated H22 hepatocellular carcinoma (control group).

As shown in Table 3, H22 bearing mice treated with *C. craniiformis* aqueous extract using three consecutive doses (0.3, 0.6, 1.2) mg/kg show increase in caspase-8 expression in a dose-dependent manner. At 0.3 mg, the intensity of expression was strong in (33.33%) and very strong in (66.67%). While at 0.6 mg and 1.2 mg the intensity of expression was strong in (33.33%) and very strong in (100%).

Table 3: Effect of *C. craniiformis* extracts on the caspase-8 expression of H22 bearing mice

Caspase-8 mRNA Expression Score	Control group	H22 Bearing mice treated with *C. Craniiformis* aqueous extract			H22 Bearing mice treated with *C. Craniiformis* methanolic extract		
		0.3mg	0.6mg	1.2mg	0.25 mg	0.5 mg	1 mg
0	1 (16.67%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
1	3 (50%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
2	2 (33.33%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
3	0 (0%)	2 (33.33%)	0 (0%)	0 (0%)	2 (33.33%)	4 (66.67%)	1 (16.67%)
4	0 (0%)	4 (66.67%)	6 (100%)	6 (100%)	4 (66.67%)	2 (33.33%)	5 (83.33%)
Total No.(%) of Mice	6 (100%)	6 (100%)	6 (100%)	6 (100%)	6 (100%)	6 (100%)	6 (100%)
P value			< 0.001			< 0.001	

A significant difference (P ≤ 0.001) in caspase-8 expression was reported. In H22 bearing mice treated with *C. craniiformis* methanolic extract using three consecutive doses (0.25, 0.5, 1.0) mg/kg show increase in caspase-8 expression in a dose-dependent manner. At 0.25 mg, the intensity of expression was strong in (33.33%) and very strong in (66.67%). While at 0.5 mg, the intensity of expression was strong in (33.33%) and very strong in (100%). At 1 mg, the intensity of expression was strong in (16.67%) and very strong in (83.33%). A significant difference (P ≤ 0.001) in caspase-8 expression was reported.

Discussion

The intake of mushrooms proved to be effective in cancer prevention, tumour growth inhibition and has high anti-tumour activity and a prevent tumour metastasis due to the high content of antioxidants thus, several mushrooms derived compounds are now increasingly used as an adjuvant to standard radio and chemotherapy [28] [29].

In current study, administration of aqueous (0.3 mg/kg, 0.6 mg/kg, 1.2 mg/kg) and methanolic extracts (0.25 mg/kg, 0.5 mg/kg, 1 mg/kg) of *C. craniiformis* to H22 tumor-bearing mice show significant differences (P ≤ 0.01) in tumor size compared with control group in a dose and duration-dependent manner. Among given doses, 1 mg/kg of methanolic extract was effective dose causing a reduction in tumour size in the last day (36^{th}), tumour size was 1167.28 mm^3 compared with 7747.05 mm^3 among the control group. This comes in line with [28], stated that as mushrooms contain different bioactive polyphenolic compounds, it effective in tumour growth inhibition and tumour metastasis. These compounds act as effective antioxidants based on their excellent ability to scavenge free radicals and act as reducing agents based on their high polyphenolic and ergosterol contents [30]. It was claimed that the puffball antioxidant capacity attributed to the presence of various chemicals such as ascorbic acid, carotenoids, esterified phenolics, and free and nonflavonoid phenolics and flavonoids [30] as well as ergosterol such as ergosterol ester, gallic, homogentisic, protocatechuic, p-hydroxybenzoic, and o- and p-coumaric acids, and other phenolic derivatives such as 3,4-dihydroxybenzaldehyde, ergothioneine, alkaloids, steroids, terpenoids [31] and selenium which protect cells from damage that might lead to chronic diseases and help to strengthen the immune system, as well [32] [33].

The aqueous extract of *C. craniiformis* was effective in reduction of H22 tumour size at dose 1.2 mg/kg and 1 mg/kg for methanolic in which H22 tumour mass was reduced in size for up to 87.9% mg/kg. The reduction in tumour size proves the presence of a restriction in the tumour growth, angiogenesis inhibition and apoptosis induction as well as an increase in activity of the Immune system for fighting against cancerous cells. This comes in line with the fact that β-D-glucans which is one of

important constituent of *C. craniiformis* can inhibit tumour growth through inhibition of DNA polymerase and have the ability to modify Oncoprotein gene expression [34]. β-D-glucans, a protein-bound polysaccharide compound binds via specific receptors expressed as surface markers on phagocytic cells also play a vital role in stimulation and activation of phagocytic cells to invade tumour mass and stating destructive effects [35]. β-glucan caused direct enhancement of the colony-forming units granulocytes/macrophages (CFU-GM) response of bone marrow cells progenitors. Mushrooms are containing more than one polysaccharide with antitumor activity. The responses to different polysaccharides are likely to be mediated by different cell surface receptors, which may be present only on specific subsets of cells and may trigger distinct downstream responses. A combination of such responses involving different cell subsets could conceivably provide greater tumour inhibition that could be induced by a single polysaccharide [36]. A protein bound polysaccharide stimulates the functional maturation of macrophages and can scavenge active oxygen species which is widely prescribed for cancers of digestive organs like stomach, oesophagus colon etc. [37].

H22 bearing mice treated with *C craniiformis* aqueous extract (0.3, 0.6, 1.2) mg/kg shows significant increase in caspase-8 expression and hence in apoptotic index % (27.21%, 26.70%, 25.34%) compared with untreated control group (19.32%). H22 bearing mice treated with *C. craniiformis* methanolic extract (0.25, 0.5, 1.0) mg/kg shows significant increase in the caspase-8 expression and apoptotic index % (28.16%, 25.06, 24.53%) compared with control group (18.30%).

The increase of AI% in H22 bearing mice indicate cytotoxic effects of *C. craniiformis* on tumour cells. This cytotoxic effects started by induction of the apoptotic process. Apoptosis can be induced *via* two different pathways. The extrinsic pathway is triggered by the ligation of death receptors such as CD95 and recruiting of caspase-8 to the death-inducing signalling complex [24].

The intrinsic pathway is initiated by the release of cytochrome *c* from the mitochondria, which interacts with apoptosis protease activating factor-1 (APAF-1), caspase-9 and deoxyadenosine triphosphate to form the apoptosome complex. Links between the death receptor and the mitochondrial pathway exist at different levels [38].

One of the possible pathways in H22 tumour inhibition is the activation of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) which is a cytokine that is produced and secreted by most normal tissue cells. TRAIL plays a critical role in the NK cell-mediated and IFN-γ-dependent suppression of subcutaneous growth of TRAIL-sensitive tumours [10] [39]. TRAIL selectively induces apoptosis in cancer cells while normal cells are refractory [40]. TRAIL binds to Death Receptor (DR)-4 or -5 expressed on the plasma membrane of tumor cells ,resulting in recruitment of adapter molecules, Fas-Associated Death Domain (FADD), Procaspase-8 and 10 to the intracellular death domain (DD) of both receptors DR4 and DR5 [41] forming Death Inducing Signal Complex (DISC) [42]. This recruitment and clustering results in procaspase-8 dimerisation, activation, processing and release of active caspase-8 from the complex. Caspase-8, then, activates downstream the effector caspases including procaspase-3, -6, and -7, leading to activation of specific kinases resulting in classical apoptotic cell death [43]. DR4 and DR5 expression on cancer cells have been suggested be one reason for TRAIL's selective anti-tumour properties [44].

C. craniiformis lectins play a vital role in the inhibition of tumour growth. β-D-glucans which is one of the important constituents of *C. craniiformis* can inhibit tumour growth through inhibition of DNA polymerase and have the ability to modify Oncoprotein gene expression [34]. β-D-glucans, a protein-bound polysaccharide compound binds via specific receptors expressed as surface markers on phagocytic cells also play a vital role in stimulation and activation of phagocytic cells to invade tumour mass and stating destructive effects [35]. β-glucan caused direct enhancement of the colony-forming units granulocytes/macrophages (CFU-GM) response of bone marrow cells progenitors and activated the alternative complement pathway [45]. Antitumor β-glucan induced the release of IL-1, IL-6 and TNF-α from macrophages [46].

Mushrooms are containing more than one polysaccharide with antitumor activity. The responses to different polysaccharides are mediated by different cell surface receptors, which may be present only on specific subsets of cells and may trigger distinct downstream responses. A combination of such responses involving different cell subsets could conceivably provide greater tumour inhibition that could be induced by a single polysaccharide [36]. A protein bound polysaccharide extracted from the mushroom displays various unique biological activities including the stimulation of functional maturation of macrophages and have an ability to scavenge active oxygen species which is widely prescribed for cancers of digestive organs like stomach, oesophagus colon and others [37]. The puffball *Calvatia candida* contains alkaloids, steroids and terpenoids, and have potent antioxidant activities [31]. *C. craniiformis* contain different bioactive polyphenolic contents and ergosterol compounds, ascorbic acid, carotenoids, esterified phenolics, and nonflavonoid phenolics and flavonoids [30]. These compounds act as effective antioxidants based on their excellent ability to scavenge free radicals and act as reducing agents. Different types of bioactive organic compounds showing antioxidant activities were isolated

from Tuber spp. These include ergosterol such as ergosterol ester, a wide range of phenolic acids such as gallic, homogentisic, protocatechuic, p-hydroxybenzoic, and o- and p-coumaric acids, and other phenolic derivatives such as 3,4-dihydroxybenzaldehyde. Mushrooms are the leading source of the essential antioxidant selenium, which protects cells from damage that might lead to chronic diseases and help to strengthen the immune system, as well [32]. Ergothioneine which is one of important constituent of *C. craniiformis* can protect cells from distraction via antioxidant activity [47], in contrast to lectins which have inhibitory effects on the mitotic activity of tumour cells [14] [34]. Gallic acid, containing *C. craniiformis* which is one of the types of phenols, as well as a longer parts tannin, appears to works as an antioxidant that helps in protecting human cells from damage caused by oxidative stress processes [48]. Glucooligosacharide present in *C. craniiformis* have anticancer therapeutic effects due to its activation for T lymphocytes, NK cells and phagocytic cells leading to increase in production of TNF-α and increase macrophages cytotoxicity against tumour cells via perforin-granzyme system [15] [34] [49] [50].

Although, no significant difference was reported in AI% between aqueous and methanolic extracts treated groups, the increased expression of caspase-8 leads to increased AI% and reduction in tumour size in dose and duration-dependent manner. This primarily due to the effect of antioxidant and inhibitory compounds found in *C. craniiformis* which triggered the apoptotic signals in tumour cells after binding with tumour cells such as Lectins, Ergothioneine, β-glucan, Glucooligosacharide which are main chemicals appeared after conducting chemical analysis of the components of the head fruiting of *C. craniiformis* by our team. Some of these compounds have hematopoietic and immunomodulatory activities which bringing the importance of its use in vivo, particularly in experimental animals as the use of these component led to inhibition of cancerous cells. Gallic acid which is one of *C. craniiformis* components have cytoprotective action and can maintain the cells from damage [48]. Several major substances with immunomodulatory and/or antitumor activity have been isolated from *C. craniiformis*. These include mainly polysaccharides (in particular β-D-glucans), polysaccharopeptides (PSP), polysaccharide proteins, and proteins. Furthermore, other bioactive substances, including triterpenes, lipids, and phenols, have been identified and characterised in mushrooms with proven medicinal properties.

Polysaccharopeptides (PSP) present in *C. craniiformis* influence cancer metastasis in a number of ways: 1) by suppression of intravasation through the inhibition of tumor cells infiltration, 2) by suppression of tumor cell attachment to endothelial cells through the inhibition of tumor cell-induced platelet aggregation, 3) by suppression of tumor cell migration after extravasation through the inhibition of tumor cell mobility, and 4) by suppression of tumor growth after extravasation through the inhabitation of angiogenesis, the modulation of cytokine production and the augmentation of effector cell function [51] [52] and activation of alternative complement pathway [45].

As in another study on *C. versicolor* mushroom, the possible anti-tumor activity of *C craniiformis* may achieve due to various mechanisms mainly by Inhibition of DNA of tumor cells, Enhancement of cytokine production, antitumour activity in wide range of animal systems, Tumor cell killing effect, Inhibition of carcinogenesis, antioxidant effects, induction of apoptosis and antiproliferative effect; anti-invasion effects and anti-angiogenesis effects; tumouricidal and cytotoxicity effects; antimetastatic activity; Immunoprotective effects during radiation and chemotherapy [51].

The major immunomodulating effects of active substances derived from mushrooms include mitogenicity and activation of immune cells, such as hematopoietic stem cells, lymphocytes, macrophages, dendritic cells (DCs) and natural killer (NK) cells, resulting in the increased production of cytokines, including interleukins (ILs) IL12, TNF-α; INF-γ and the ability to modulate the differentiation capacity of CD4+ T cells to mature into Th1 and/or Th2 subsets. Evidence indicates that mushrooms active substances induced TH differentiation toward Th1 more than Th2 subset and induced most Th1-specific cytokines (IL-2, IFN-γ, and LT) and Th2-specific cytokine (IL-4) in tumour-bearing animals [45]. Thus regulated cytokine production and possessed both anti-tumour and immunopotentiating activities. The main mechanism might be an anti-teratogenic effect attributed to free radical trapping and prevention of chromosome injury, coupled to an immunomodulating effect linked to the modulation of cytokines production and effect cell function.

The various experimental evidence demonstrated that the anti-tumour action of mushroom polysaccharides is due to the enhancement and potentiation of the cell-mediated immune system through the regulation of immunomodulatory cytokines and activation of the complement system and NK cells [53]. However, the mechanism of anti-tumour actions of PSP from most fungi is still not clear. It is accepted that anti-tumour polysaccharides enhance various immune responses, and act as biological response modifiers [53]. PSP is nonspecific immunopotentiators and exerts immunomodulatory actions by promoting the proliferation of T-lymphocytes, the activation of macrophages, NK cells, and Th cells, thereby inducing the production of antibody and interleukins [54] also has a favourable effect on the activation of leukocyte chemotactic locomotion and phagocytic activity [51].

The obvious effect of *C. craniiformis* is to stop the process of T lymphocytes apoptosis, a cells which responsible for fighting against viral infections who can dodge the immune system and also urged the liver cells to kill lymphocytes T effective, It was noted that the liver cells infected with HCV could urge or speed up the process of getting rid of activated T lymphocytes via apoptosis. Murine hepatocytes expressing a transgene encoding the HCV structural proteins core, envelope 1 (E1) and envelope 2 (E2) enhance the apoptosis of activated T cells. Unlike normal liver, which appears to remove only activated CD8+ T cells selectively, enhanced apoptosis determine for both $CD4^+$ and $CD8^+$ T cells via Fas–FasL-dependent pathway [55] [56].

In conclusion, extracts of *C. craniiformis* were efficiently inhibited H22 tumour growth, leading to a reduction in the tumour size clinically and increase the expression of caspase-8 gene product in tumour tissue. This effect is causing an increase in the apoptotic index of H22 tumour cells taken from legs of inoculated animals, causing protection of H22 inoculated legs from losing compared with an untreated control group which lost their legs due to necrosis and destruction in the bones. Antitumor activity of *C. craniiformis* aqueous and the methanolic extract was dose and duration dependent. These findings indicate the usefulness of *C. craniiformis* extracts as a novel antitumor agent for hepatocellular carcinoma, with its proved apoptosis induction through caspase-8 activation pathway.

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Basic Science

Ameliorative Effect of Silymarin on Scopolamine-induced Dementia in Rats

Salma A. El-Marasy[1*], Reham M. Abd-Elsalam[2], Omar A. Ahmed-Farid[3]

[1]Pharmacology Department, National Research Centre, Giza, Egypt; [2]Pathology Department, Faculty of Veterinary Medicine, Giza, Cairo University, Cairo, Egypt; [3]Physiology Department, National Organization for Drug Control and Research, Giza, Egypt

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Keywords: Silymarin; Scopolamine; Object recognition test; Dementia; Donepezil; Rats

***Correspondence:** Salma A. El-Marasy. Pharmacology Department, National Research Centre, Giza, Egypt. E-mail: salma_el_marasy@hotmail.com

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Abstract

AIM: This study aims to elucidate the possible ameliorative effect of silymarin on scopolamine-induced dementia using the object recognition test (ORT) in rats.

METHODS: The study was extended to demonstrate the role of cholinergic activity, oxidative stress, neuroinflammation, brain neurotransmitters and histopathological changes in the anti-amnestic effect of silymarin in demented rats. Wistar rats were pre-treated with silymarin (200, 400, 800 mg/kg) or donepezil (10 mg/kg) orally for 14 consecutive days. Dementia was induced after the last drug administration by a single intraperitoneal dose of scopolamine (16 mg/kg). Then behavioural, biochemical, histopathological, and immunohistochemical analyses were then performed.

RESULTS: Rats pre-treated with silymarin counteracted scopolamine-induced non-spatial working memory impairment in the ORT and decreased acetylcholinesterase (AChE) activity, reduced malondialdehyde (MDA), elevated reduced glutathione (GSH), restored gamma-aminobutyric acid (GABA) and dopamine (DA) contents in the cortical and hippocampal brain homogenates. Silymarin reversed scopolamine-induced histopathological changes. Immunohistochemical analysis showed that silymarin mitigated protein expression of the glial fibrillary acidic protein (GFAP) and nuclear factor kappa-B (NF-κB) in the brain cortex and hippocampus. All these effects of silymarin were similar to that of the standard anti-amnestic drug, donepezil.

CONCLUSION: This study reveals that the ameliorative effect of silymarin on scopolamine-induced dementia in rats using the ORT maybe in part mediated by, enhancement of cholinergic activity, anti-oxidant and anti-inflammatory activities as well as mitigation in brain neurotransmitters and histopathological changes.

Introduction

Dementia is characterised by impairments in memory and other cognitive abilities [1]. Dementia of Alzheimer's type (DAT) is the most common type of dementia. Alzheimer's disease (AD) is characterised by the accumulation of amyloid plaques, hyperphosphorylation of tau protein, oxidative stress, neuroinflammation leading to neuronal death [2]. Deficiency in cholinergic neurotransmission in the cerebral cortex and hippocampus accounts for dementia in AD patients [3].

Object recognition test (ORT), measures non-spatial working memory in rodents, which is severely impaired in patients suffering from DAT [4] [5]. The test depends on the spontaneous exploratory behaviour of rodents, exposed to the novel environment. In comparison to other cognitive parameters, ORT does not involve reinforcement/response interaction and therefore resembles procedures used in humans and have a predictive validity [6] [7].

Scopolamine, a muscarinic receptor blocker that disrupts cholinergic neurotransmission leading to memory impairment associated with DAT [8]. Scopolamine-induced dementia is a widely used animal model for investigating cognitive enhancing drugs [9] [10].

Acetylcholinesterase inhibitors (AChEIs) are

the most common drugs used for DAT. However, these drugs may cause peripheral cholinergic side effects that may restrict their use [11]. Therefore, efforts have been directed towards the use of alternative anti-amnestic therapies with lower side effects [12].

Silymarin is a flavonoid isolated from the seeds and fruits of Milk Thistle plant. Therapeutically, silymarin is used for the treatment of liver diseases [13] [14]. It possess anti-cancer [15] [16] anti-apoptotic effects [17], in addition to its renoprotective [18] and cardioprotective [19] activities.

Silymarin is a potent anti-oxidant agent, being able to cross the blood-brain barrier and exerts a neuroprotective effect in various neurodegenerative disorders like Parkinson's disease, stroke, and ageing [20] [21] [22]. Although it has been demonstrated that silymarin suppresses the accumulation of amyloid plaques in an animal model of AD [23], according to the author's knowledge its possible memory-enhancing the effect on scopolamine-induced dementia has not been investigated.

Therefore, the present study aims to elucidate the possible ameliorative effect of silymarin on scopolamine-induced dementia using the ORT. The study was extended to demonstrate the role of cholinergic activity, oxidative stress, neuroinflammation, neurotransmitters and histopathological changes in the anti-amnestic effect of silymarin in demented rats. Donepezil, an AChEI, was used as an anti-amnestic standard drug for comparison.

Material and Methods

Animals

Male albino Wistar rats weighing 120–150 g were used throughout the experiment. They were obtained from the animal house colony of the National Research Centre (Dokki, Cairo, Egypt) and were housed for at least one week in the laboratory room before testing under a 12 h alternating light/dark cycle. Animals were fed standard laboratory pellets with water ad libitum. All animal procedures were performed by the Ethics Committee of the National Research Centre, Egypt (registration number 17/004) which is by the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and complied with the guidelines from the Canadian Council on Animal Care.

Drugs and chemicals

Scopolamine hydrobromide was purchased from Sigma–Aldrich (MO, USA) and was dissolved in saline (0.9% NaCl). Donepezil hydrochloride was purchased from Pfizer (Giza, Egypt) and was freshly prepared in 1% tween 80 in water. Silymarin was supplied by CID pharmaceutical company (Giza, Egypt) and was freshly prepared in 1% tween 80 in water.

Treatments

Rats were randomly allocated into 6 groups (8 rats/group in the object recognition test) as follows: group I received saline (0.9% NaCl solution) and served as normal while group II received scopolamine (16 mg/kg, i.p.) [24] and served as control. Both groups received saline for 14 days. Groups III–V rats orally received Silymarin (200, 400, 800 mg/kg/day), respectively; Group VI rats orally received Donepezil (10 mg/kg/day) for 14 consecutive days. Scopolamine was administered as a single dose 30 min after the last administration in groups II-VI.

The selection of the doses of silymarin was based on the previously published data of Galhardi, Mesquita [25]. The dose of donepezil was selected according to the previously published data of Schreiber, Vivian [26].

Behavioral test

Object recognition test

The test apparatus was designed as described by Ennaceur and Delacour [27]. Three days before testing, each rat was allowed to explore the apparatus for 2 min, while on the testing day, 30 min following scopolamine injection, a session of two trials, 2-min each was allowed. In the ‘‘sample’’ trial (T1), two identical objects were placed in two opposite corners of the apparatus. A rat was placed in the apparatus and was left to explore these two identical objects. After T1, the rat was placed back in its home cage, and an inter-trial interval of 1h was given. Subsequently, the ‘‘choice’’ trial (T2) was performed. In T2, a new object (N) replaced one of the objects that were presented in T1; then rats have exposed again to two different objects: the familiar (F) and the new one (N).

Exploration was defined as follows: directing the nose toward the object at a distance of no more than 2 cm and/or touching the object with the nose.

From this measure, a series of variables were then calculated: the total time spent in exploring the two identical objects in T1, and that spent in exploring the two different objects, F and N in T2. The discrimination between F and N in T2 was measured by comparing the time spent in exploring the F with that spent in exploring the N. DI is the discrimination index and represents the difference in exploration time expressed as a proportion of the total time spent exploring the two objects in T2. DI was then

calculated; DI = N-F/N+F.

Brain homogenate preparation for biochemical analysis

Rats were euthanised by decapitation following the ORT. The whole brain was carefully excised, cortical and hippocampal brain tissues were isolated, immediately weighed to avoid any effects from drying, and stored at −80°C.

Cortical and hippocampal brain tissues were homogenised (MPW-120; Medical Instruments) in 10% (*w*/*v*) ice-cold phosphate buffer. Then, the homogenate was centrifuged using a cooling centrifuge (2k15; Sigma/Laborzentrifugen) at 4000 rpm for 5 min, and the resulting supernatant was used for determining the brain contents of Acetylcholinesterase activity (AChE), malondialdehyde (MDA), and reduced glutathione (GSH) in addition to brain neurotransmitters namely norepinephrine (NE), dopamine (DA), serotonin (5-HT), and gamma-aminobutyric acid (GABA) were also assessed.

Determination of Acetylcholinesterase (AChE) activity

Acetylcholinesterase activity was determined in cortex and hippocampus according to the method described [28] [29], the developed colour was read spectrophotometrically immediately at 412 nm, and the AChE activity was determined in μM per SH group (μMSH) from a standard curve.

Determination of lipid peroxidation content

Lipid peroxidation was assayed by measuring cortical and hippocampal MDA content according to the method described before [30]. The supernatant was read spectrophotometrically at 532 nm, and the MDA content is expressed in nanomoles of MDA per milligram tissue.

Determination of reduced glutathione (GSH) content

The cortical and hippocampal GSH contents were determined according to the method described before by Ellman [31]. Calculation of GSH was based on a standard glutathione curve and is expressed in micromoles of GSH per gram tissue.

Determination of norepinephrine (NE), dopamine (DA), and serotonin (5-HT) contents

Brain monoamines, namely, NE, DA, and, 5-HT, were estimated using HPLC (Agilent 1200 series; Agilent Technologies, California, USA) as described previously [32]. Cortical and hippocampal contents of monoamines are expressed in micrograms of monoamine per gram tissue, and were calculated as follows:

$$\text{Monoamine content (μg/g tissue)} = AT/AS \times CS \times \text{dilution factor}$$

Where: AT = area under the curve for the sample, AS = area under the curve for the standard, CS = concentration of the standard (μg/mL), and the dilution factor = 10.

Determination of Gamma-aminobutyric acid (GABA) content

Cortical and hippocampal GABA content (μmol/g tissue) was estimated using HPLC (Agilent 1200 series; Agilent Technologies, California, USA) according to the precolumn phenylisothiocyanate derivatisation technique described by Heinrikson and Meredith [33]. Cortical and hippocampal contents of GABA are expressed in micrograms per gram tissue.

Histopathological examination

The brain tissues were collected from different groups and fixed in 10% neutral buffered formalin then processed for obtaining 4 μm paraffin-embedded sections. The sections were stained with hematoxylin, and eosin stain then examined under the microscope [34].

Immunohistochemistry analysis of GFAP and NF-KB p65

The immunohistochemistry was performed according to methods described before [35]. Brain tissue sections were deparaffinized in xylene and rehydrated in graded alcohol. Hydrogen Peroxide Block (Thermo scientific, USA) was added to block the endogenous peroxidase activity. Antigen retrieval was done by pretreated tissue sections with 10 mM citrate in a microwave oven for 10 minutes. Sections were incubated for 2 hours with one of the following primary antibodies: rabbit anti-GFAP polyclonal antibody (ab7260; Abcam, Cambridge, UK) at dilution 1:2000 and rabbit anti-NF-KB P65 polyclonal antibody (ab16502; Abcam, Cambridge, UK) diluted 1 Ug/ml. The sections were rinsed with PBS then incubated with Goat anti-rabbit IgG H&L (HRP) (ab205718; Abcam, Cambridge, UK) for 10 min. The sections were rinsed again with PBS. Finally, sections were incubated 3, 3'-diaminobenzidine tetrahydrochloride (DAB, Sigma). The slides were counterstained with haematoxylin then mounted. Primary antibodies were replaced by PBS for negative controls.

Evaluation of GFAP and NF-κB p65 immunostaining

The quantitative immunoreactivity of GFAP and NF-κB was evaluated in the brain cortex and hippocampus region. In each group, five brain sections were examined. The GFAP immunoreactivity was analysed in 10 microscopical fields per each section under high-power microscopical field (x 400) and represented as a percentage of the positively stained area. NF-κB positive cells and total cell number were counted in 10 microscopical fields per each section under high-power microscopical field (x 400), and the percentage of positively stained cells (%) was calculated. The image analysis was performed by Leica Qwin 500 Image Analyzer (Leica, Cambridge, England).

Statistical Analysis

Data concerning the ORT, biochemical analysis are presented as the mean±SEM for 8 rats per group in the behavioural tests and 6 rats per group in the biochemical tests. Comparisons between more than 2 groups in the ORT and biochemical were carried out using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test, except for the comparison of total exploration time in T1 and T2 in the ORT which was carried out using 2-way ANOVA followed by Tukey's multiple comparisons test and when comparing the exploration times of the F and N objects in T2, Student's t-test was used. Immunohistochemical analysis was carried out by one-way analysis of variance (ANOVA) followed by Newman Keuls multiple comparison tests. All analyses utilised GraphPad Prism 6.0 statistical package for Windows (GraphPad, San Diego, Calif.). Statistical significance was set at $P < 0.05$.

Results

Figure 1a reveals that dementia induced by a single i.p. dose of scopolamine (16 mg/kg) 30 min before starting T1 in the ORT did not significantly affect the total exploration time in T1 and T2. Oral administration of silymarin (200, 400, 800 mg/kg), and donepezil (10 mg/kg) for 14 consecutive days, before scopolamine, did not also show any difference in the total exploration time in T1 and T2. During T2, Scopolamine-induced demented rats did not reveal any significant difference in the exploration time of N as compared to their exploration time of F. Scopolamine-induced demented rats explored N and F objects similarly. Rats pre-treated with silymarin (200, 400, 800 mg/kg) were similar to donepezil and explored the N object significantly more than F (Figure 1b). DI indicated that all rats, except scopolamine treated rats, discriminated N significantly better than F (Figure 1c).

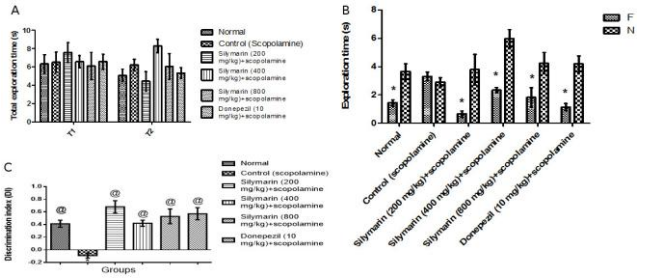

*Figure 1: Effect of Silymarin on scopolamine-induced dementia in rats using the object recognition test; A) Total exploratory time in T1 and T2; b) Exploration time of familiar (F) vs the novel object (N) in T2; c) Discrimination index (DI). Results are expressed as mean ± SEM (n = 8). Statistical analyses were carried out in a) by two-way ANOVA followed by Tukey's multiple comparison tests, in b) by using Student's t-test, while in c) by one-way ANOVA followed by Tukey's multiple comparison tests. *Significant difference versus correspondent N group at P < 0.05. *Significant difference from the normal group at P < 0.05. @Significant difference from control (scopolamine) group at P < 0.05*

As depicted in Figure 2a and b, demented rats showed a significant increase in cortical and hippocampal AChE activity to be 14.19 and 11.39 µmol SH/g/min as compared to normal rats. Pre-treatment with silymarin (200, 400, 800 mg/kg) significantly decreased AChE to be 75.83, 52.36, 50.74%, respectively in cortical tissue and 52.94, 43.2, 35.21%, respectively in hippocampal tissue as compared to control group. Similarly, donepezil administration resulted in significant decrease in AChE activity to be 47.71 and 45.23% respectively in cortical and hippocampal tissue as compared to control group.

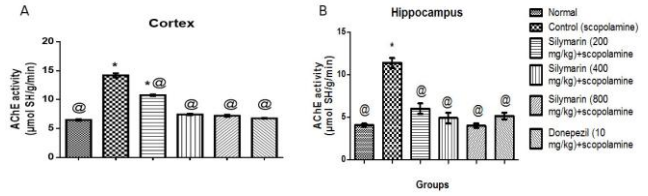

*Figure 2: Effect of Silymarin on A) cortical and B) hippocampal acetylcholinesterase (AChE) activity in scopolamine-induced dementia in rats. Results are expressed as mean ± SEM (n = 6). Statistical analysis was carried out by one-way ANOVA followed by Tukey's multiple comparison tests. *Significant difference from the normal group at P < 0.05. @Significant difference from control (scopolamine) group at P < 0.05*

Results demonstrated in Figure 3 a, b reveals that demented rats increased significantly cortical and hippocampal MDA content to be 163.22% and 149.48% of normal rats, respectively. Preventive treatment with silymarin in doses of 200 and 400 and 800 mg/kg restored cortical MDA content to be 69.62%, 64.70% and 67.06% of demented rats, respectively. Oral administration of silymarin (200 and 400 mg/kg) restored hippocampal MDA content to be 62.30% and 63.96% of demented rats, respectively.

Silymarin in a dose of 800 mg/kg significantly reduced hippocampal MDA content to be 53.68% and 80.25% of the scopolamine control group and normal group, respectively. Also, Donepezil restored cortical and hippocampal MDA content to be 67.37% and 65.02% of control rats, respectively.

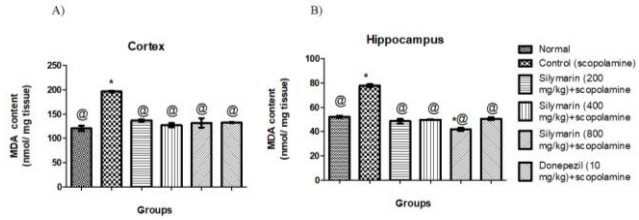

*Figure 3: Effect of Silymarin on A) cortical and B) hippocampal reduced malondialdehyde (MDA) activity in scopolamine-induced dementia in rats. Results are expressed as mean ± SEM (n = 6). Statistical analysis was carried out by one-way ANOVA followed by Tukey's multiple comparison tests. *Significant difference from the normal group at P < 0.05. @Significant difference from control (scopolamine) group at P < 0.05*

Regarding GSH content, Scopolamine administration significantly reduced cortical and hippocampal GSH contents to be 83.28% and 88.64%, respectively as compared to normal rats. Silymarin administration in doses of 200, 400 and 800 mg/kg significantly restored cortical GSH contents to be 111.69%, 121.53% and 123.77% respectively. Preventive administration of silymarin (200 and 400 mg/kg) restored hippocampal GSH contents to be 113.83% and 111.17% respectively. The results were similar to donepezil as it significantly restored cortical GSH contents to be 124.16% and hippocampal GSH contents to be 111.55% of the control group (Figure 4 a, b).

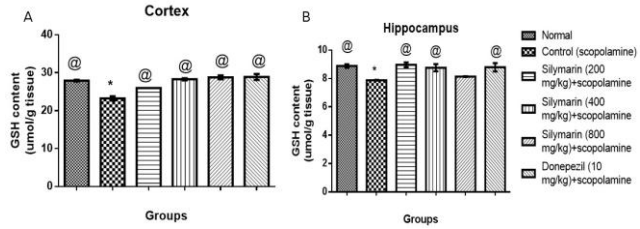

*Figure 4: Effect of Silymarin on A) cortical and B) hippocampal reduced glutathione (GSH) activity in scopolamine-induced dementia in rats. Results are expressed as mean ± SEM (n = 6). Statistical analysis was carried out by one-way ANOVA followed by Tukey's multiple comparison tests. *Significant difference from the normal group at P < 0.05. @Significant difference from control (scopolamine) group at P < 0.05.*

The effect of silymarin on cortical and hippocampal neurotransmitters in scopolamine-induced demented rats was summarised in Tables 1 & 2. Demented and treated rats did not show any significant change in cortical and hippocampal NE and 5-HT contents. Scopolamine-induced demented rats showed a significant decrease in cortical and hippocampal DA contents to be 58.36% and 22.92% of normal rats, respectively. Demented rats also significantly reduced cortical and hippocampal GABA contents to be 62.69% and 48.41% of normal rats, respectively. Silymarin in doses of 400 and 800 mg/kg significantly elevated cortical DA content to be 142.04% and 145.86% of control rats, respectively and cortical GABA content to be 145.86% and 144.52% of control rats, respectively.

Table 1: Effect of silymarin on cortical neurotransmitters content in scopolamine-induced dementia in rats

Parameters / Treatment	Cortical neurotransmitters (µg/g tissue)			
	Brain Monoamines			
	NE	DA	5-HT	GABA
Normal	0.95 ± 0.07	2.69 ± 0.07@	0.57 ± 0.03	7.13 ± 0.29@
Control (scopolamine)	0.65 ± 0.05	1.57± 0.09*	0.59 ± 0.05	4.47 ± 0.46*
Silymarin (200 mg/kg) +scopolamine	0.91 ± 0.18	2.11 ± 0.22	0.65 ± 0.03	5.90 ± 0.40
Silymarin (400 mg/kg) +scopolamine	0.87 ± 0.09	2.23 ± 0.11@	0.64 ± 0.07	6.52 ± 0.20@
Silymarin (800 mg/kg) +scopolamine	0.91 ± 0.05	2.29 ± 0.18@	0.65 ± 0.04	6.46± 0.31@
Donepezil (10 mg/kg) + scopolamine	0.97 ± 0.01	2.35 ± 0.13@	0.64 ± 0.05	6.18± 0.58@

Results are expressed as mean ± SEM (n = 6). Statistical analysis was carried out by one-way ANOVA followed by Tukey's multiple comparison tests.*Significant difference from normal group at *P* < 0.05. @Significant difference from control (scopolamine) group at *P* < 0.05.

Silymarin in doses of 200, 400, and 800 mg/kg restored hippocampal DA contents to be 433.33%, 396.97%, and 472.73% of control rats, respectively and restored hippocampal GABA contents to be 288.47%, 192.52%, and 217.88% of control rats, respectively. Similarly, donepezil restored cortical and hippocampal DA contents to be 149.68% and 433.33% of control scopolamine group, respectively in addition to cortical and hippocampal GABA contents to be 138.26% and 177.92% of control scopolamine group, respectively.

Table 2: Effect of silymarin on hippocampal neurotransmitters content in scopolamine-induced dementia in rats

Parameters / Treatment	Hippocampal Neurotransmitters (µg/g tissue)			
	Brain Monoamines			
	NE	DA	5-HT	GABA
Normal	0.67±0.04	1.44*±0.06	0.64±0.03	11.32@±0.32
Control (scopolamine)	0.65±0.06	0.33@±0.02	0.71±0.04	5.48*±0.15
Silymarin (200 mg/kg) +scopolamine	0.63±0.05	1.43@±0.06	0.70±0.06	12.52@±1.11
Silymarin (400 mg/kg) +scopolamine	0.65±0.05	1.31@±0.07	0.65±0.06	10.55@±0.85
Silymarin (800 mg/kg) +scopolamine	0.63±0.04	1.56*@±0.06	0.69±0.04	11.94@±0.52
Donepezil (10 mg/kg) + scopolamine	0.63±0.04	1.43@±0.06	0.73±0.01	9.75@±0.55

Results are expressed as mean ± SEM (n = 6). Statistical analysis was carried out by one-way ANOVA followed by Tukey's multiple comparison tests. *Significant difference from the normal group at *P* < 0.05. @Significant difference from control (scopolamine) group at *P* <0.05.

The control group revealed normal histological finding (Figure 5A, 6A). The scopolamine treated group showed marked thickening of the endothelium lining blood vessels with clear perivascular oedema, degenerative neuronal changes in the form of central chromatolysis and neuronal swelling in the brain cortex. Moreover, some neuron was shrunken with pyknotic or lytic nuclei (Fig. 5B)

and neuronophagia. In the hippocampus region, the scopolamine treated group showed thickening of the endothelium lining blood vessels with perivascular and pericellular oedema; some pyramidal cells degenerate with neuronophagia and gliosis (Fig. 6B).

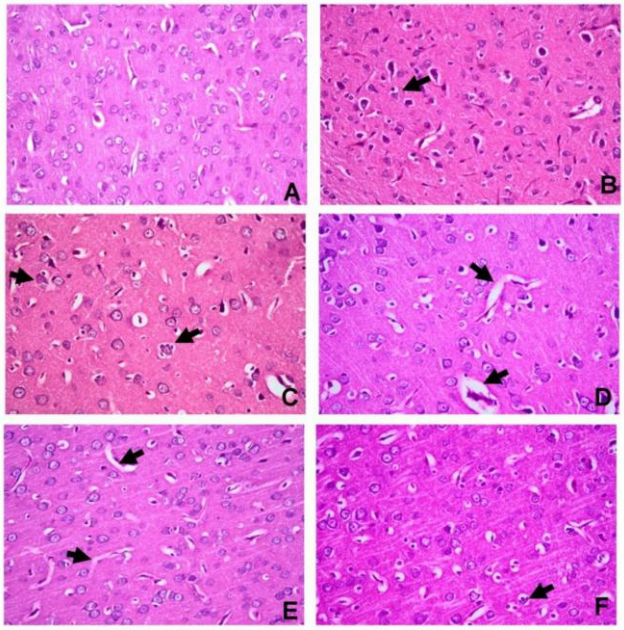

Figure 5: Histopathological changes in the brain cortex of the different treated groups (H&E x400). A. Normal control group showing normal histological finding; B. Scopolamine treated group showing marked neuronal degeneration (arrow) with neurophagia and gliosis; C. Silymarin (200 mg/kg) + scopolamine showing moderate neuronal degeneration with neurophagia (arrow); D. Silymarin (400 mg/kg) + scopolamine showing moderate perivascular edema (arrow) with mild neuronal degeneration; E. Silymarin (800 mg/kg) + scopolamine showing slight perivascular edema (arrow); F. Donepezil (10 mg/kg)+ scopolamine group showing mild neuronal degeneration (arrow) and neurophagia

Swelling of the pyramidal cells was also observed. The previous described histopathological changes were markedly attenuated in all treated group with donepezil (Fig. 5F, 6F) and silymarin in a dose-dependent manner (Fig. 5 & 6 C, D, E) compared to the scopolamine treated group.

Figure 9 summarised the results of immunohistochemical evaluation of GFAP and NF-kB expression in the different experimental groups. In GFAP immunostaining, the astroglia cell in the control group appeared with thin processes and lightly stained cell body (Figure 7A). While the astroglia cell in the scopolamine treated group appeared with thick, dense processes and darkly stained cell body (Figure 7B). The scopolamine treated group showed a significant increase in GFAP immunostaining comparing to control group (Figure 7B). The Donepezil (Figure 7F) and silymarin-treated groups (Figure 7C, D, E) showed a significant reduction in GFAP immunostaining compared to the scopolamine treated group (Figure 9A).

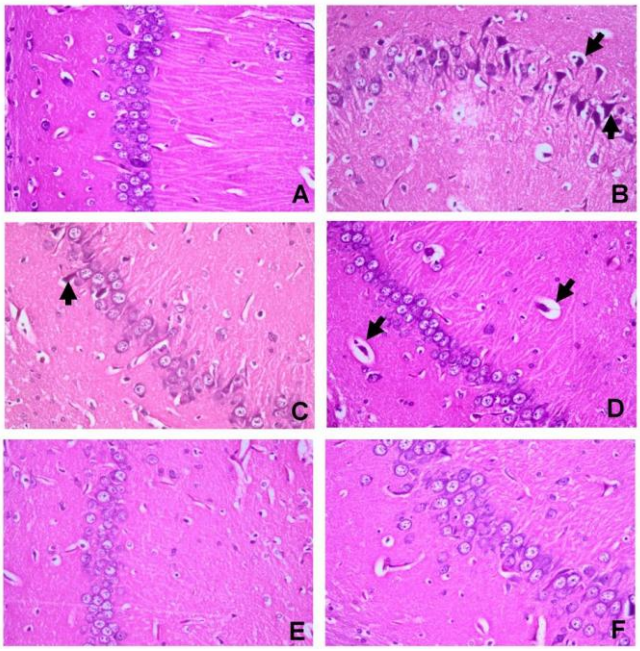

Figure 6: Histopathological changes in the hippocampus of the different treated groups (H&E X400). A. Normal control group showing normal histological picture; B. Scopolamine treated group showing marked degeneration of pyramidal cells (arrow) with neuronophagia and pericellular edema; C. Silymarin (200 mg/kg) + scopolamine showing mild degeneration of pyramidal cells (arrow); D. Silymarin (400 mg/kg) + scopolamine showing thickening of the endothelial lining blood vessels with perivascular edema (arrow); E. Silymarin (800 mg/kg) + scopolamine showing normal histological finding.; F. Donepezil (10 mg/kg) + scopolamine showing mild pyramidal cells swelling

NF-kB immunostaining was detected in cytoplasm and or nucleus of the neuron and glial cells. The cytoplasmic staining of the cells represented in the active form of NF-kB. While nuclear staining of the cells represented the active form of the NF-kB. So, the nuclear-stained cells were only counted as the immunopositive cells. The percentage of immunopositive cells for NF-kB immunostaining of the scopolamine treated group (Figure 8B) was significantly elevated than the control group (Figure 8A). The donepezil (Figure 8F) and silymarin-treated groups (Figure 8 C, D, E) showed a significant reduction in the percentage of NF-kB immunopositive stained cells in dose-dependent manner compared to the scopolamine treated group (Fig. 9B).

Discussion

This study adds new information on the memory-enhancing the effect of silymarin (200, 400, 800 mg/kg), which was similar to donepezil in scopolamine-induced demented rats using the ORT. To the best of the authors' knowledge, this is the first report that highlighted the involvement of cholinergic activity, oxidative stress biomarkers namely (MDA and

GSH), inflammatory biomarkers such as NF-KB and GFAP, brain neurotransmitters as well as histopathological changes in the anti-amnestic effect of silymarin in scopolamine-induced demented rats using the ORT.

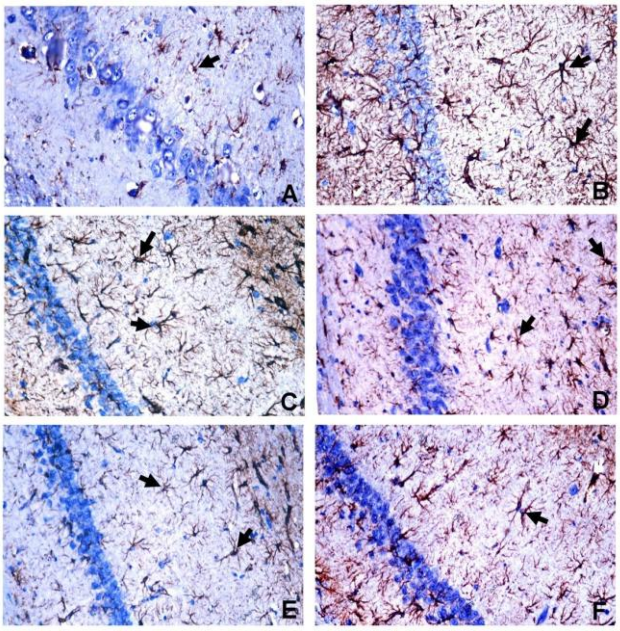

Figure 7: Representative GFAP immunohistochemistry in the hippocampus region of different treated groups (X400). A. Control group showing weak immunostaining of astroglia cells (arrow); B. Scopolamine treated group showing strong immunostaining of astroglia cells with thick, dense processes and darkly stained cell body (arrow); C. Silymarin (200 mg/kg) + scopolamine and D. Silymarin (400 mg/kg) + scopolamine showing moderate immunostaining of astroglia cells (arrow); E. Silymarin (800 mg/kg) + scopolamine showing thin processes and lightly stained cell body of astroglia cells (arrow); F. Donepezil (10 mg/kg) + scopolamine group showing reduction in the immunostaining of astroglia cells (arrow)

In this study, scopolamine-induced demented rats impaired recognition memory in the ORT, as demented rats explored both F and N objects similarly and were not able to discriminate between both objects. These results are in harmony with prior studies [36] [37].

Silymarin-treated rats reversed scopolamine-induced non-spatial working memory impairment in the ORT as they were able to discriminate between the F and N objects and increased the time spent in identifying the N object concerning the F object. These effects are similar to the standard drug, donepezil.

Since the total exploration time within T1 and T2 were similar in demented, silymarin and donepezil-treated rats, therefore it can be deduced that attentional and sensorimotor activities did not influence rats' performance in the ORT. This indicates that memory enhancing effects of silymarin and donepezil was independent on non-specific factors of rats.

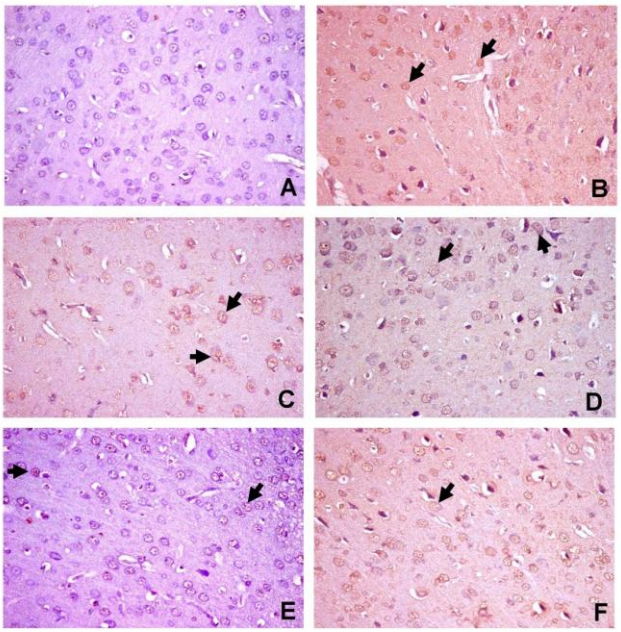

Figure 8: Representative NF-kB immunohistochemistry in the brain cortex of different treated groups (x 400). A. Control group showing very weak nuclear reaction; B. Scopolamine treated group showing strong and numerous nuclear staining (arrow); C. Silymarin (200 mg/kg) + scopolamine and D. Scopolamine + S400 showing moderate nuclear immunoreaction (arrow); E. Scopolamine + S800 showing weak nuclear immunostaining (arrow); F. Donepezil treated group showing reduction in the immunopositive cells (arrow)

In this work, donepezil, which is a potent AChEI, administered orally at 10mg/kg/day for 14 consecutive days before scopolamine ameliorated cholinergic deficits, oxidative stress, inflammation, and histopathological changes in the brain of scopolamine-induced demented rats. These findings are in concordance with prior studies [38][39] and emphasize the effectiveness of donepezil as standard anti-amnestic agent for screening novel therapeutics for treating cognitive deficit.

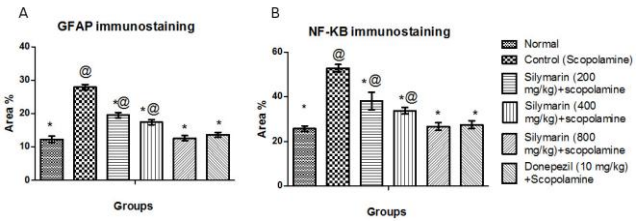

*Figure 9: Immunohistochemical evaluation of GFAP and NF-kB expression in the different treated groups. A. The bar chart of GFAP immunostaining expressed as area %. B. The bar chart of NF-kB immunostaining expressed as the percentage of positively stained cells. Statistical analysis was carried out by one-way ANOVA followed by Newman Keuls multiple comparison tests. *Significant difference from the normal group at P < 0.05. @Significant difference from control (scopolamine) group at P < 0.05*

In this study, Scopolamine-induced dementia resulted in cholinergic system dysfunction as evidenced by elevation in AChE activity, an important enzyme which hydrolyses ACh, an essential neurotransmitter involved in learning and memory.

This finding is in line with prior studies [40] [41].

Preventive treatment with silymarin and donepezil decreased cortical and hippocampal AChE activity, indicating that silymarin might have ameliorated cognitive, cognitive deficit in the ORT partly via enhancing cholinergic neurotransmission. Prior studies have reported that silymarin has also protected against high fat diet-induced dementia and manganese-induced neurotoxicity via restoration of AChE activity [42] [43].

Oxidative stress has been implicated in the pathogenesis of DAT. This is apparent in the current investigation as scopolamine-induced dementia resulted in the elevation of cortical and hippocampal MDA content, the final product of lipid peroxidation and subsequent reduction of the endogenous anti-oxidant namely GSH, due to elevated Reactive Oxygen Species (ROS). This finding is inconsistent with prior studies [44] [45] and implies that scopolamine associated oxidative stress accounts for memory impairment in the study.

Preventive treatment with silymarin reversed scopolamine associated oxidative stress to be similar to that of donepezil as it reduced cortical and hippocampal MDA content, lipid peroxides formation by elevating the ROS scavenging activity of cortical and hippocampal GSH content. This reveals that ameliorative effect of silymarin on scopolamine-induced dementia in rats may be partly due to its antioxidant activity. Several studies have also demonstrated the neuroprotective of silymarin against oxidative stress associated with experimentally-induced Parkinson's disease and cerebral ischemia [46] [47].

Neurotransmitters such as GABA and dopamine have a greater impact on memory retrieval and consolidation than 5-HT and NE [48]. GABAergic and DAergic deficits contribute to memory impairment in patients suffering from AD [49] [50].

In line with this notion, scopolamine-induced pathological changes were coupled with deficits in cortical and hippocampal GABA and dopamine contents. This finding is inconsistent with that of [51] [52]

Pretreatment with silymarin restored GABA and DA contents to be similar to donepezil. This implies that anti-oxidant effect of silymarin may contribute to the preservation of neurotransmitters, and thereby memory-enhancing effect in amnestic rats. Previous studies reported that silymarin restored DA content in 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinson's disease and stress-induced depression in mice [53] [54].

As mentioned before, elevated AChE activity in DAT aggravates the formation of Aβ plaques which in turn activates astrocytes and upregulate GFAP, an indicator of neuroinflammation [55] [56].

Scopolamine-induced dementia in this work showed an elevation in hippocampal and cortical protein expression of GFAP when compared to normal rats. This finding is in harmony with a prior study [57] and implies that scopolamine upregulated inflammatory cascade via astrocytic activation.

Silymarin administration counteracted scopolamine-induced elevation in protein expression of GFAP, by suppressing astrocyte activation. This reveals that inhibition of astrocytic activation might have resulted from suppression of inflammatory cascade and oxidative stress.

Oxidative stress contributes to the elevation of ROS which results in activation of NF-*κB*, a transcription factor responsible for regulation of pro-inflammatory genes such as, cytokines and chemokines [58] [59] [60].

In this work, scopolamine-induced dementia increased protein expression of NF-*κB* in the brain cortex and hippocampus, indicating that elevated inflammatory response resulting from oxidative stress might account for cognitive deficit in amnestic rats.

Prior treatment with silymarin combated the elevated protein expression of NF-*κB* in the brain cortex and hippocampus of demented rats; this suggests that silymarin ameliorated cognitive deficit via suppression of inflammatory cascade probably through its anti-oxidant effect in demented rats. This finding is inconsistent with other studies [61] [62] [63] [64].

It can be concluded that the memory-enhancing the effect of silymarin in scopolamine-induced demented rats using the ORT may be partly mediated by, attenuating cholinergic deficits, anti-oxidant and anti-inflammatory effects as well as amelioration in DAergic, GABAergic neurotransmission and histopathological changes. Further pre-clinical studies are warranted to investigate other mechanisms that may underlie the anti-amnestic effect of silymarin. Clinical studies are needed to address the validity of silymarin to prevent or slow down the progression of DAT.

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Basic Science

A Bayesian Analysis With Informative Prior on Disease Prevalence for Predicting Missing Values Due To Verification Bias

Abdollah Hajivandi[1], Hamid Reza Ghafarian Shirazi[2*], Seyed Hassan Saadat[3], Mohammad Chehrazi[4]

[1]Bushehr University of Medical Sciences, Bushehr, Iran; [2]Social Determinant of Health Research Center, Yasuj University of Medical Sciences, Yasuj, Iran; [3]Behavioral Sciences Research Center, Lifestyle Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran; [4]Department of Epidemiology and Reproductive Health, Reproductive Epidemiology Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran

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***Correspondence:** Hamid Reza Ghafarian Shirazi. Social Determinant of Health Research Center, Yasuj University of Medical Sciences, Yasuj, Iran. E-mail: gshr3@yahoo.com

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Abstract

AIM: Verification bias is one of the major problems encountered in diagnostic accuracy studies. It occurs when a standard test performed on a non-representative subsample of subjects which have undergone the diagnostic test. In this study we extend a Bayesian model to correct this bias.

METHODS: The study population is patients that have undergone at least two repeated failed IVF/ICSI (in vitro fertilization/intra cytoplasmic sperm injection) cycles. Patients were screened using ultrasonography and those with polyps were recommended for hysteroscopy. A Bayesian modeling was applied on mechanism of missing data using an informative prior on disease prevalence. The parameters of the model were estimated through Markov Chain Monte Carlo methods.

RESULTS: A total of 238 patients were screened, 47 of which had polyps. Those with polyps were strongly recommended to undergo hysteroscopy, 47/47 decide to have a hysteroscopy and in 37/47 polyps confirmed. None of the 191 patients with no polyps detected in ultrasonography underwent a hysteroscopy. A model using Bayesian approach was applied with informative prior on polyp prevalence. False and true negatives were estimated in the Bayesian framework. The false negative was obtained 14 and 177 true negatives were obtained, so sensitivity and specificity was estimated easily after estimating the missing data. Sensitivity and specificity were equal to 74% and 94% respectively.

CONCLUSION: Bayesian analyses with informative prior seem to be powerful tools in the simulation of experimental space.

Introduction

Advances in medical technology have provided doctors with the various ways of diagnostic methods to identify patients. Diagnostic accuracy studies help physicians in selecting the most appropriate test to evaluate the patient's clinical situation and decision making about treatment by examining the characteristics of these tests. The sensitivity and specificity of diagnostic tests are assessed by comparing the results of evaluated tests with result of the standard test that is performed on the same patients. Generally the most accurate test for diagnosis is considered as the gold standard. For evaluation of the diagnostic test in best situation both tests are performed for all study subjects. One of the problems often encountered in these studies is that due to it being costly or invasive. The standard test is not performed on all the study subjects. This seems reasonable in the clinic setting but in a diagnostic test study it creates verification bias [1]. Verification bias is a selection bias and occurs when the standard test performed on a non-representative subsample of study subjects which have already undergone the diagnostic test. This occurs, for example, when inclusion probabilities for the subsample are dependent on the initial stage results and/or on a

covariate related to the disease status. This bias is usually divided into two types: differential bias and partial bias. Partial verification bias occurs when standard test is only performed on patients who have a positive diagnostic test result. Differential verification bias occurs when standard test which perform on patients with positive test result, is different to the standard test that is performed on patients who have a negative result [1] [2]. In most studies, there is no result for gold standard test for some people and any kind of verification bias are considered from the viewpoint of missing data. So there is no result for gold standard test for some people. Models that are built based on this view are dependent on the process of missing data. Begg and Greenes were the first researchers who tried to correct the partial verification bias [3]. They used the diseased proportion of the sample which had been used to perform the gold standard test on. Their models were based on the conditional independency assumption; this assumption means that missing process or sample selection to done gold standard only dependent on the test result and not on the true disease status. On the other hand, missing event of a sample given the test result is independent of true disease status. Some researchers think this assumption is unrealistic and in some cases this can be misleading [4] [5]. But Kosinski [6] tried to overcome this problem by considering that in addition to the test result, the missing process was dependent on the true disease status and unobserved information about the disease [5] [7]. Obviously, having the full data we could compare the different models in predicting the results of the gold standard test. Estimation of the models was based on Frequentist and maximum likelihood approach. Problems in these methods are described in Martinez and Buzoianu [5] [7]. It seems that problems of these methods are due to a new variable called v which is defined as the missing process. In this article we have covered the missing process and problems associated with it which have not been studied before. We eliminated the variable V using a new definition and fitted a new Bayesian model that is not dependent on V. In section 2, we proposed the detailed model in the Bayesian framework to estimate sensitivity and specificity. In section 3, a real data example is described and the Bayesian model is applied and the results are presented. Finally, In Section 4 we discuss the model and its results.

Let that missing process occurs simultaneously with the result of the diagnostic test [7]. On the other hand, if the test result was positive, the gold standard test should be performed but if the result was negative the gold standard test is not performed. The test process is depicted below:

$$Test\ Result \begin{cases} positive \{ Gold\ standard \\ negative \{ Miss \end{cases}$$

Therefore, the probability of missing is zero for a positive test result and 1 for a person with a negative result. If T represents the diagnostic test result and V represents the missing process, the following probability exists.

$$P(V|T) = \begin{cases} 1 & V \neq T \\ 0 & V = T \end{cases}$$

$$P(V|T) = \frac{P(V,T)}{P(T)} = 1 \xrightarrow{yields} P(V,T) = P(T) \quad (1)$$

If G represents the gold standard result then the aim is to find a function to predict the gold standard status for individuals with a negative diagnostic test result. Thus, sensitivity and specificity could be estimated. The model is started with following probability:

$$P(G|T,V) = \frac{P(G,T,V)}{P(T,V)} = \frac{P(T)P(G|T)P(V|G,T)}{P(T,V)} \quad (2)$$

Where according to the definition 1, probability [2] exists in the following form:

$$P(G|T,V) = \frac{P(T)P(G|T)P(V|G,T)}{P(T)} = P(G|T)P(V|G,T) \quad (3)$$

As we considered (1) T and V to occur simultaneously, information about the events T and G gives full information about V. therefore the relationship [3] can be rewritten as bellow:

$$P(G|T,V) = P(G|T)$$

So, it can be concluded that the missing process is independent of the disease status given the diagnostic test status (it can be assumed that the disease status will be clarified by the gold standard test), but the disease status has direct impact on the missing process. For example, in someone who is suffering from a disease, the probability of a positive diagnostic and the missing probability are low. Table 1 is a cross table which illustrates the diagnostic test and gold standard frequencies.

Table 1: Diagnostic test and gold standard result

	Positive Gold standard	Negative Gold standard
Positive test	y_{11}	y_{12}
Negative test	y_{21}	y_{22}

Probability $P(G|T)$could be obtained by the following:

$$P(G^+|T^+) = \frac{P(T^+|G^+)P(G^+)}{P(T^+|G^+)P(G^+) + (1 - P(T^-|G^-))P(G^-)} = P_{11}$$

$$P(G^+|T^-) = \frac{(1 - P(T^+|G^+))P(G^+)}{P(T^-|G^-)P(G^-) + (1 - P(T^+|G^+))P(G^+)} = P_{21}$$

$$P(G^-|T^+) = \frac{(1-P(T^-|G^-))P(G^-)}{P(T^+|G^+)P(G^+) + (1-P(T^-|G^-))P(G^-)} = P_{12}$$

$$P(G^-|T^-) = \frac{P(T^-|G^-)P(G^-)}{(1-P(T^+|G^+))P(G^+) + (P(T^-|G^-))P(G^-)} = P_{22}$$

Where, $P(T^+|G^+)$ and $P(T^-|G^-)$ are the sensitivity (SN) and specificity (SP) of the diagnostic test respectively. A reasonable initial model is to assume that the number of each cell $\{y_{ij}\}_{i,j=1,2}$ has the following distribution

$$y_{ij} \sim Binomial(P_{ij}, n_i), \sum_i n_i = N \quad i,j = 1,2$$

$$P_{11} + P_{12} = 1$$

$$P_{21} + P_{22} = 1$$

$$SN = \frac{y_{11}}{P(G^+)N} \quad (4)$$

$$SP = \frac{[P(G^-)N] - y_{12}}{P(G^-)N} \quad (5)$$

$$P(G=0) = 1 - P(G=1)$$

$$P(G=1) \sim beta(a,b)$$

Therefore, the posterior probability function for disease prevalence is:

$$P(P(G^+)|y_{ij}) \propto \binom{n_i}{y_{ij}} P_{ij}^{y_{ij}} (1-P_{ij})^{(n_i - y_{ij})} \frac{1}{B(a,b)} P(G^+)^{a-1} (1-P(G^+))^{b-1} \quad (6)$$

Proof of the formulas is given in Appendix A.

Repeated IVF failures are one of the problems of infertility centers. Structural abnormalities in uterine cavity such as fibroids and mullerian anomalies can play an important role in the failure of embryo implantation during IVF cycles [8]. Repair of uterine cavity pathologies has been suggested as a therapeutic action to improve the results of ART cycles in these individuals. Hysteroscopy is considered as a gold standard test for evaluating the uterine cavity in infertile patients. This method allows direct observation of the uterus and cervix, Thus Increasing the accuracy of diagnosis. This can also highlight uncertain results of other diagnostic methods [9]. Uterine anomalies are usually well diagnosed with hysteroscopy [10]. A number of studies reported that sensitivity, specificity, positive predictive value and negative predictive value of transvaginal sonography is similar to the results of hysteroscopy [8]. Some studies also found no strong correlation between the result of transvaginal sonography and hysteroscopy [11]. It is recommended that all women undergoing IVF candidates, before doing IVF placed under hysteroscopy [12]. But given that hysteroscopy is an invasive procedure, maybe it is not performed on all patients who assessed using transvaginal sonography, and done only for patients who have had positive results of transvaginal sonography. Consequently verification bias occurs.

At First we have analyzed the data obtained from 140 patients admitted to the Royan Institute, and transvaginal sonography and hysteroscopy was performed for every one of them, these patients have at least two Repeated IVF/ICSI cycles which have failed. With regard to Hysteroscopy as the gold standard, we calculated the sensitivity and specificity of vaginal sonography in detecting polyps with the frequentist method, then with assuming that Hysteroscopy has been performed only on patients who have positive results of vaginal sonography. We estimated the sensitivity and specificity of vaginal sonoghraphy with Bayesian approach and compared these estimations with sensitivity and spasticity of the actual data.

Since there was expert belief about disease prevalence but not about sensitivity and specificity, we therefore formulated those in terms of disease prevalence. Furthermore, to account for the uncertainty of prevalence, we used the Beta distributions as informative. Hyper-parameters $(a \text{ and } b)$ were determined by subjective percentiles technique [13]. The prior information was provided by a gynecologists and a midwife independently. Each expert provided the best guess and a 90% prior credible interval for the prevalence. In particular we wanted upper boundaries and lower bound, without consulting the Literature, of the true value of the polyp prevalence in the population. The across-expert average of these quantities is listed below:

$$P(p < 0.33) = 0.95$$

$$P(p < 0.001) = 0.05$$

The averaged credible interval for prevalence is used to determine the hyperparameters a and b.

$$p \sim beta(0.5981227, 5.610896)$$

In the present setting, for instance, a Monte Carlo sample of 100000 draws from the prior which gives $P(0.001 < p < 0.33) \approx 0.9$, so the prior information is being represented as desired.

Prior distribution of prevalence must be truncated from 0.15 because generated numbers below this cut point cause sensitivity and specificity to become negative. Therefore, the truncated beta distribution has been truncated between 0.15 and 1. There is no truncated distribution in OpenBugs; the I (.,.) operator is used only to denote censored observation [14]. However, when all parameters in a prior distribution are observed, the I (.,.) operator can be used for modeling truncated prior distributions. If there are unknown parameters the inferences will be wrong [15]. OpenBugs remove this ambiguity between truncation and censoring by introducing the truncation operator T (.,.) [16].

Therefore, the prior density for $\{p\}_{T(0.15,1)}$ is

$$P(p) = \frac{1}{B(a,b)} p^{a-1}(1-p)^{b-1}$$

We apply the model to real data that was introduced previously. In total 238 patients were undergoing ultrasound tests for the diagnosis of polyps. 191 people had negative test results and 47 had positive results. The gold standard test was then done for two groups. The results are showed in Table 2.

Table 2: Results of diagnostic and gold standard tests

	G^+	G^-
T^+	**37**	**10**
T^-	**13**	**178**

Thus, the sensitivity, specificity, positive and negative predictive value is easily calculated as follows:

$$SN = \frac{37}{50} = 0.74$$

$$SP = \frac{178}{188} = 0.946$$

$$PPV = \frac{37}{47} = 0.787$$

$$NPV = \frac{178}{191} = 0.932$$

But the problem will be started when the gold standard test is not performed on people with negative diagnostic test results. So Table 2 comes in the form below:

Table 2: A contingency table that depict missing mechanism

	G^+	G^-
T^+	**37**	**10**
T^-	***NA***	**NA**

NA: Not Available.

In Bayesian approach, model [6] is posterior density function of disease prevalence. The parameter of the model [1] was estimated using simulation from the posterior distribution. The Markov Chain Monte Carlo (MCMC) method using Adaptive Metropolis Block was applied [13]. Algorithm was run in OpenBugs 3.1.2 environment [14] [15]. After 10000 times iteration the chain was converged to posterior distribution and 5000 initial samples were discarded as Burn-in period. Graphical methods such as autocorrelation function, posterior density and trace plot were used for checking convergence of chains [16] (not shown). The program was run in OpenBugs given in Appendix 2. Bayesian Results after sampling (simulation) were shown in Table 3.

Table 3: Estimation of parameters simulated by MCMC algorithm in Bayesian framework

Parameters	Posterior mean	Standard deviation	2.5 credible interval	97.5 credible interval	Start	Sample
Sensitivity	0.7417	**0.1092**	0.5139	0.925	**5001**	**10000**
Specificity	0.9272	0.0411	0.8289	0.9842	**5001**	**10000**
PPV	**0.7858**	0.0586	0.6578	0.8868	**5001**	**10000**
NPV	0.9269	0.03828	0.83	0.9783	**5001**	**10000**

Discussion

Verification bias is a type of selection bias that occurs when the standard test performed on a non-representative subsample of study subjects which have already undergone the diagnostic test. In most studies there is no result for gold standard test for some people. There are several ways to solve this problem. For example we assume that results of the gold standard test are negative for all these people. This approach is clinically inappropriate. Since there is a possibility of error in diagnostic test and consequently negative predictive value may not be 1. This method greatly increased the sensitivity and specificity values and therefore overestimation can occur. Another way is results of previous tests for people who have had the gold standard test to be replaced. The estimates from this strategy could be subject to bias, because the conditions governing the present situation may be different from any of the previous experiments. In this paper a new statistical model was presented based on conditional probabilities where the model parameters, sensitivity and specificity, was estimated from the Bayesian approach [17]. The conditional probabilities are the same positive and negative predictive values which provide information about the model. The data missing process is different from other corresponding studies, meaning the gold standard results do not exist for individuals with a negative test. The assumption of conditional independence that was used by Begg and Greenes is quite applicable in case of missing process of current data. When a patient's diagnostic test is specified then the missing state is determined automatically. Thus the disease status is independent of the missing process given knowing the diagnostic test result. On the other hand, the actual status of the disease increases or decreases the chances of missing. So the model can be defined based on conditional probabilities $P(G \mid T)$. To construct better models and predict the missing values more accurately, information must enter the study according to an expert's opinion because the probability distribution does not give enough information about the missing values. This could be done with considering the disease prevalence in the population as uncertain and attributing the probability distribution to it. This can give useful information to

the model about the overall distribution of the gold standard. The proposed model is for diagnostic test ultrasound against the gold standard hysteroscopy in detection of polyps. But whether or not it can be applied to other tests, should be studied to allow the estimation of the parameters, after the probability of disease combined with the distribution of diagnostic tests. Finally, we hope stronger and more powerful Models in the development of diagnostic tests can be developed in the future.

Appendix A:

Proofs of formula (4) and (5):

$$\frac{y_{11}+y_{21}}{N} = P(G=1)$$

$$SN = \frac{y_{11}}{y_{11}+y_{21}} = \frac{y_{11}}{N.P(G=1)}$$

$$\frac{y_{12}+y_{22}}{N} = P(G=0)$$

$$SP = \frac{y_{22}}{y_{12}+y_{22}} = \frac{(N.P(G=0)-y_{12})}{N.P(G=0)}$$

Appendix B: Openbugs codes

In order to run the model with informative beta distribution for prevalence of disease the following code was used:

```
model{
PG1~dbeta(2,12) T(0.1541400874,1)
SN<-(0.7858*10)/(0.2142*238*PG1)
SP<-((238*(1-PG1))-10)/(238*(1-PG1))
for(i in 1:a){
for(j in 1:b){
y[i,j]~dbin(p[i,j],n[i])
}}
p[1,1]<-(SN*PG1)/((SN*PG1)+((1-SP)*(1-PG1)))
p[2,2]<-(SP*(1-PG1))/((SP*(1-PG1))+((1-SN)*PG1))
p[2,1]<-((1-SN)*PG1)/(((1-SN)*PG1)+(SP*(1-PG1)))
p[1,2]<-((1-SP)*(1-PG1))/(((1-SP)*(1-PG1))+(SN*PG1))
sensitivity<-y[1,1]/(y[1,1]+y[2,1])
specificity<-y[2,2]/(y[2,1]+y[2,2])
}
```

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Post-Surgical Repair of Cleft Scar Using Fractional CO_2 Laser

Aida Mossaad[1], Amr Kotb[1], Moustapha Abdelrahaman[1], Hatem Al Ahmady[2]

[1]National Research Center, Cairo, Egypt; [2]Al Azhar University, Cairo, Egypt

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***Correspondence:** Aida Mossaad. National Research Center, Cairo, Egypt. E-mail: aida109@hotmail.com

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Abstract

BACKGROUND: Postoperative scarring is a common cause of patients dissatisfaction. Several modalities have been developed to overcome such a problem following surgical repair. Despite precise surgical technique, still, some scars would remain over the time, mostly due to the weak formation or inadequately replaced collagen fibres in the underneath dermis especially those following unilateral or bilateral cleft lip repair surgery.

AIM: of this study is to evaluate whether a 10,600 nm fractional ablative carbon dioxide (CO_2) used early during the healing period would result in better postoperative scars.

METHODS: In the present study six patients complained from cleft lip scars resulting from lip revision surgery. Each patient had six fractional ablative CO_2 laser sessions for treatment along six months to obtain a complete collagen cycle. Vancouver Scar Scale VSS was used as a method of evaluation of the scar using 4 points scale evaluating vascularity, pliability, thickness & colour of the skin and Visual Analogue Scale VAS from (0-10) was used to assess the severity of pain as well as a survey questionnaire for the rate of patient's satisfaction. Also, digital clinical photos assessment before&after were compared.

RESULTS: Patients expressed a significantly greater degree of satisfaction with the treatment using a subjective 4-point scale. All patients observed dramatic improvement in their lip scars after FCO_2 laser sessions following their surgeries with the better psychological state. The assessment was done by clinical observation according to VSS before (9.17 ± 2.2) while after (3.33 ± 1.9) with a highly significant P value <0.001 and VAS for the rate of pain & satisfaction that ranged from (8.0 ± 0.9) as well as series of photos taken before and after the procedure. No long-term complications were noted however patients complained of annoying pain during the session as well as crust formation that lasted up to 5 days after surgery. In the present study, we introduce the effectiveness of ablative fractional 10,600 nm CO_2 laser for treatment of postoperative cleft lip scar after secondary surgical cleft repair rather than ablative CO_2 due to its reported complications such as postoperative infection, erythema and pigmentary changes along with prolonged downtime healing. In the current study, we chose early laser treatment within the first six months before complete collagen organisation which will be easier to manage the older scars. Patients mostly complained about the pain during the session as well as dark-coloured crust formation post session that stayed from 3-5 days however they all observed a massive improvement of their scars following treatment protocol.

CONCLUSION: Facial wounds sutured in layers heal in a good manner. Patients prefer early treatment with a fractional CO_2 ablative laser for postoperative surgical scars. The use of a CO_2 fractional laser is safe and effective also causes high patients satisfaction.

Introduction

Postoperative scarring is a common cause of patients dissatisfaction. Several modalities have been developed to overcome such a problem following surgical repair. Despite precise surgical technique, still, some scars would remain over the time, mostly due to the weak formation or inadequately replaced collagen fibres in the underneath dermis especially those following unilateral or bilateral cleft lip repair surgery.

This study aims to evaluate whether a 10,600 nm fractional ablative carbon dioxide (CO_2) used early during the healing period would result in better postoperative scars [1] [2]. Carbon dioxide (CO_2) resurfacing ablative laser has been the main treatment of facial scar since its introduction in the mid-1990's. Recently, a new generation of fractional micro ablative CO_2 lasers has been introduced. According to the concept of fractional photothermolysis, theses layers ablate only a fraction of the epidermal and dermal layers of the skin in the target area instead of full ablation with less aggressive impact on the tissues. A microscopic array of thermal wounds is created within very tiny zones, adjacent to these areas; the epidermis and dermis are spared.

That procedure employs fractional thermolysis to occur, so healing becomes more rapid compared to fully ablative CO_2 laser and downtime healing is relatively reduced. This micro ablative process of laser skin resurfacing has been proven to be safe and effective with less damage to skin [3] [4]. Intralesional Corticosteroids also have long been used in the treatment of hypertrophic and restricted scars. Fractional ablative lasers create zones of ablation at variable depths of the skin with subsequent induction of wound healing and collagen remodelling. Recent studies suggest that ablative zones may also be used for in immediate postoperative period to enhance delivery of drugs and other substances such as platelet-rich plasma injection [5] [6].

Carbon dioxide CO_2 laser ablative fractional resurfacing produces skin damage with the removal of the epidermal layer and variable portions of the dermal layer as well as associated residual heating, resulting in new collagen formation and skin tightening. The non-resurfaced epidermis helps tissues to heal rapidly with short-term postoperative erythema [7] [8]. Despite the effectiveness of ablative CO_2 resurfacing laser for the face, its application has been limited due to its undesirable side effects as pigmentary changes and prolonged hyperemia.

New fractional CO_2 laser skin resurfacing technology is associated with shorter periods of hyperemia, resulting in shorter recovery time compared to older ablative technology. Also, the side-effects are minor and infrequent. This new technology of fractional photothermolysis (FP) provides significant clinical improvement resulting in higher patient's satisfaction [9] [10].

Assess safety and efficacy of post-surgical cleft scar with **an** ablative fractional CO_2 laser**.**

Material and Methods

This study included six patients (3 males & 3 females) age ranging from 15 to 20 years old that had post-surgical secondary repair of cleft lip scar. Patients were being treated with fractional Carbon dioxide laser 10,600 nm. Patients were being selected randomly from the outpatient clinic of Al Azhar University, Faculty or Oral & Dental Medicine, Oral & Maxillofacial Surgery Clinic and National Research Center Cairo, Orodental Genetics Clinic. Patients started the laser sessions one month after the secondary cleft revision surgery took place. Protocol of sessions: Six sessions every four weeks for six months to have a complete collagen cycle. Laser parameters: Power 22.5 mJ, pulse width 500 microsecond, stack 3 and density 0.8. Using Bison Medical Fire-xel* laser device. Vancouver Scar Scale VSS was used as a method of evaluation of the scar using 4 points scale evaluating vascularity, pliability, thickness and colour of the skin and Visual Analogue Scale VAS from (0-10) was used to assess the severity of pain as well as a survey questionnaire for the rate of patient's satisfaction. Also, digital clinical photos assessment before&after were compared. The patients agreed to their enrollment in the study by signing a written informed consent. Patients were aware of the nature of the laser treatment and understood that this phenomenon did not influence their systemic or oral health. Safety measures were being considered as wearing protective eye shields for patients&all operating staff in the room.

Results

In the present study, six patients complained from cleft lip scars resulting from lip revision surgery. Each patient had six fractional CO_2 laser sessions for treatment along six months to obtain a complete collagen cycle. A greater decrease in VSS score was noted in the treated scars, especially regarding texture and thickness. Patients also expressed a significantly greater degree of satisfaction with the treatment as assessed using a subjective 4-point scale.

All patients observed dramatic improvement in their lip scars after FCO_2 laser sessions following their surgeries with the better psychological state. Assessment was done by clinical observation according to Vancouver scar scales VSS before (9.17 ± 2.2) while after (3.33 ± 1.9) with highly significant P value < 0.001 and Visual Analogue Scale VAS for rate of pain and satisfaction that ranged from (8.0 ± 0.9), Table 1 as well as series of photos taken before and after the procedure (Figure 1).

No long-term complications were noted however patients complained from annoying pain during the session as well as crust formation that lasted up to 5 days after surgery.

Table 1: Showing VAS and VSS before and after treatment

Variable		MEAN ± SD	RANGE (MIN-MAX)
Sex (no. (%))	Male	3 (50.0%)	
	Female	3 (50.0%)	
Age (years)		17.8 ± 2.7	7 (15-22)
Visual analogue scale (vas)		8.0 ± 0.9	2 (7-9)
Vancouver scar scale (vss) before			
	Vascularity	1.83 ± 0.4	1 (1-2)
	Pigmentation	1.83 ± 0.4	1 (1-2)
	Pliability	3.83 ± 1.3	3 (2-5)
	Height	1.67 ± 0.5	1 (1-2)
	Total VSS Score	9.17 ± 2.2	6 (5-11)
Vancouver scar scale (vss) after			
	Vascularity	0.67 ± 0.5	1 (0-1)
	Pigmentation	0.67 ± 0.5	1 (0-1)
	Pliability	1.33 ± 0.5	1 (1-2)
	Height	0.67 ± 0.5	1 (0-1)
	Total VSS Score	3.33 ± 1.9	4 (1-5)

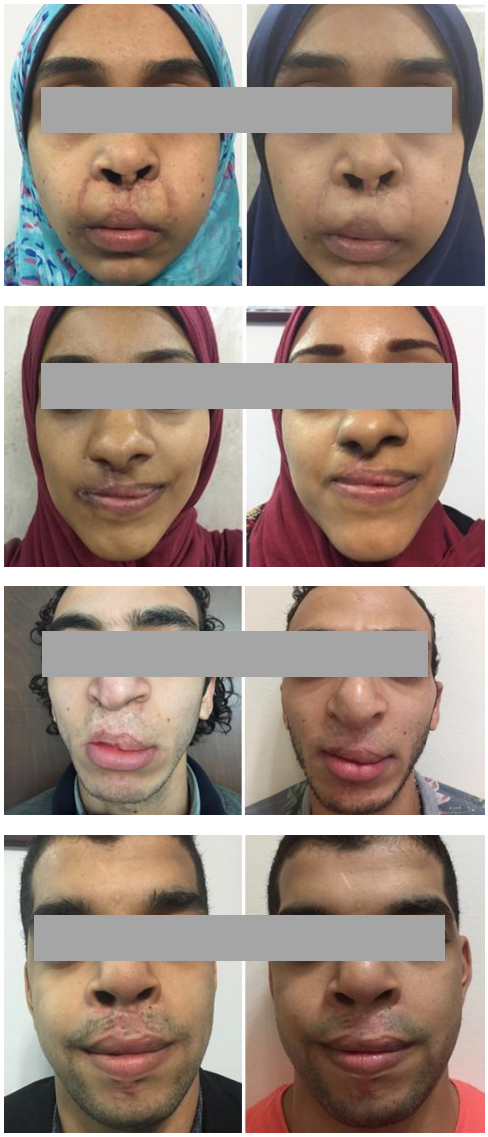

Figure 1: Cases 1-4 before and after laser

Discussion

Cleft lip scars following lip revision surgeries are the most annoying to the appearance of most patients especially with a bilateral cleft lip. In the present study, we introduce the effectiveness of ablative fractional 10,600 nm CO_2 laser for treatment of postoperative cleft lip scar after secondary surgical cleft repair. This study included only six patients according to a few numbers of patients who have agreed to go for the second revision of their lip surgery followed by such a painful procedure using laser treatment. Postoperative scarring occurs because of the impaired resolution or healing. In the current study, we chose early laser treatment within the first six months before complete collagen organisation which will be easier to manage the older scars. Laser therapy is still considered a challenge. The ablative resurfacing CO_2 laser is effective in the treatment of postoperative surgical scars; however, it has many complications such as postoperative infection, erythema and pigmentary changes along with prolonged downtime healing.

On the other hand, non-ablative CO_2 lasers (e.g. the 1064 Nd: YAG laser, and the 1,450 nm diode laser) are known to improve scar appearance by stimulating collagen production and remodelling [10] [11]. Choi JE et al., [12] compared treatment of hypertrophic scars between Er: YAG fractional laser EYFL and CO_2 fractional lasers CO_2FL using Vancouver scar scale VSS&5 point grading scale. Patients were questioned about their rate of satisfaction and treatment outcomes.

After the final treatment, average percentage changes of VSS were 28.2% for EYFL and 49.8% for CO_2FL. The improvement was evident regarding pliability, while insignificant regarding vascularity and pigmentation. Based on physician's global assessment, mean grade of 1.8 for EYFL and 2.7 for CO_2FL was achieved. Eiler RE et al., [13] compared using different modalities in postoperative scar treatment including ablative fractional CO_2, non-ablative micro needling fractional radiofrequency&intralesional corticosteroids and observed different outcomes as regard healing and suggested a combination therapy with such modalities. In the current study, we chose fractional CO_2 laser rather than ablative CO_2 or intralesional injection. Patient's subjective satisfaction scores matched the physician's objective evaluation. Patients mostly complained about the pain during the session as well as dark-coloured crust formation post session that stayed from 3-5 days however they all observed a massive improvement of their scars following treatment protocol. No scar ointments were used after surgery or laser sessions, just soothing post laser creams to lessen the burning sensation felt by patients.

In conclusion, facial wounds sutured in layers heal in a good manner. Patients prefer early treatment with a fractional CO_2 ablative laser for postoperative surgical scars. The use of a fractional CO_2 laser is safe and effective also causes high patients satisfaction.

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Diagnostic Efficacy of 24-hr Esophageal pH Monitoring in Patients with Refractory Gastroesophageal Reflux Disease

Atoosa Gharib[1], Mojgan Forootan[2*], Marjan Sharifzadeh[3], Saied Abdi[4], Mohammad Darvishi[5], Ahmad Eghbali[6]

[1]Shahid Modarres Clinical Research and Development Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran; [2]Department of Gastroenterology, Gastrointestinal and Liver Diseases Research Center (RCGLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran; [3]Shahid Beheshti University of Medical Sciences, Tehran, Iran; [4]Department of Optometry, Shahid Beheshti University of Medical Sciences, Tehran, Iran; [5]Infectious Diseases and Tropical Medicine Research Center (IDTMRC), Department of Aerospace and Subaquatic Medicine, AJA University of Medical Sciences, Tehran, Iran; [6]Modares Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran

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***Correspondence:** Mojgan Forootan. Department of Gastroenterology, Gastrointestinal and Liver Diseases Research Center (RCGLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran. E-mail: mforootan2003@yahoo.com

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Abstract

BACKGROUND: Gastric reflux is one of the most important causes of the referral of patients to the internal clinic, which in some cases causes problems for patients due to resistance to common treatments. Therefore, timely diagnosis and treatment of this group of patients are very important.

AIM: The purpose of the present study was to determine the off-proton pump inhibitor (off-PPI) 24 h pH-impedance analyses in patients with refractory gastroesophageal reflux disease (GERD) attending to Taleghani Hospital since 2009 to 2017.

METHODS: In this observational descriptive-comparative off-PPI study, 572 patients with refractory GERD who were referred to Taleghani Hospital in Tehran from 2009 to 2017 were selected, and the results of 24 h pH Impedance analysis were then assessed.

RESULTS: The results of 24h pH-impedance indicated that 7% of cases belonged to Pure Acid Reflux followed by weakly Acid (1%), non-acid (0.3%), mixed & gas (5.2%), functional (58.4%) and oesophagal hypersensitivity (28%). Furthermore, weakly acid plus acid was also found to be 8% and Weakly Acid + Acid + Non-Acid were determined as 8.3%.

CONCLUSIONS: Our findings suggested that nearly more than half of the patients with refractory GERD would have a functional disorder in the 24h pH-impedance analysis.

Introduction

Reflux or gastroesophageal reflux disease (GERD) is one of the common gastrointestinal disorders, with many risk factors such as diabetes and hypertension [1]. This disorder is present in 16% of the general population and can be associated with common clinical symptoms, such as heartburn and chest pain [2]. However, clinical symptoms in GERD patients are not limited to gastrointestinal symptoms and can also be manifested as non-gastro-intestinal symptoms, including respiratory disorders, sleep disturbances, and atorvastinuratory symptoms [3]. The disease causes a 2.5-hour absence from the workplace, a 23 per cent reduction in efficiency, and a 30 per cent reduction in the normal performance of the individual. In general, there is a significant reduction in the quality of life in patients suffering from GERD [4]. It also imposes huge costs on individuals and health systems [5]. Therefore, treatment for GERD patients is important for improving their quality of life. Treatment in this area is divided into two categories of therapeutic and surgical treatments, both of which not only reduce the severity of the symptoms of the patients but also significantly improve their quality of life [6] [7]. It is worth noting that, in both short and long-term, the effectiveness of surgical treatments is far more than pharmaceutical treatments, and drug therapies are particularly

effective on clinical symptoms such as dysphagia [6][8]. However, 40% of patients do not show any proper therapeutic response, and they refer to refractory GERD, which requires the adoption of other therapies [9]. The causes of GERD Refractory include Acid Reflux, Non-Acid Reflux, Esophageal Hypersensitivity, and Functional Heart Burn [10]. Moreover, only a few studies have been done in Iranian patients [11] [12] [13].

On the other hand, GERD is a long-term condition in which stomach contents enter the oesophagus and cause symptoms or complications. Complications include esophagitis, oesophagal strictures, and Barrett's oesophagus. There are some risk factors involved in the disease, including obesity, pregnancy, smoking, hiatus hernia, and taking some special medications. Drugs that affect gastric reflux are described to be as follow: antihistamines, calcium channel blockers, antidepressants and sleep medications. Diagnosis among people who do not respond in simple ways may be made in other ways, such as gastroscopy, oesophagal pH monitoring, and or impedance-pH monitoring [13] [14] [15] [16].

Therefore, the goal of this study was to evaluate the causes of refractory GERD in patients who referred to Taleghani Hospital from 2009 to 2017 as off- proton pump inhibitor (off-PPI).

Methods

This study was conducted by a descriptive cross-sectional study of off-PPI. A total of 572 patients with refractory GERD who referred to Tehran Taleghani Hospital from 2009 to 2017 were evaluated. Endoscopy results and response to medical treatment were controlled as interventional factors. In the manometer, the absence of motion disorders, such as achalasia and diffuse oesophagal spasm (DES), was confirmed.

The required data were extracted from patients' files, including the age, sex, duration of GERD symptoms, pH and Impedance parameters, and symptom association probability (SAP), as well as proximal extension and bolus clearance time (BCT). Then, the prevalence of different parameters of the 24h PH Impedance was extracted from them using file contents. Finally, data analysis was performed using SPSS software version 24. The mean and standard deviations were used to evaluate quantitative variables, where qualitative variables were presented as absolute and relative frequency. The tests used in this field included chi-square and analysis of variance. The significance level for the relationships between variables was considered 0.5.

Inclusion criteria

Failure of medical treatment with protein pumps inhibitors (PPIs) for at least one month, once or twice daily [12].

Exclusion criteria

1: Patients who had anti-reflux surgery, either PPI or H2-blocker.

2. Patients with atypical GERD symptoms.

3. Motion disorders, such as achalasia and diffuse oesophagal spasm.

4. Non-Iranian patients.

5. Patients with abnormal manometry.

6. Age younger than 18 or over 80 years old. 7. Systemic disease.

Results

In this study, 572 subjects were studied. Their mean age was 38.2 years (range 18-80 years), and mean duration of clinical symptoms was 5.1 years (from 1 to 16 years). Also, 48.3% were males, and 51.7% were females. DeMeester Score was abnormal in 44.2% of patients and Total Reflux Time in 45.5% of patients. The frequency of reflux and the frequency of long-term reflux was 40% and 24.1%, respectively. In 2.6% of patients, BCT was abnormal and proximal extension was observed in 41.8% of subjects. Attenuation correction (AC) findings in the upright and supine positions were attributed to frequencies of 20.5% and 25.2%, respectively. Weakly Acid (WA) findings in the upright and supine positions were abnormal in 50.9% and 49.5% respectively. Abnormal Non-Acid (NA) findings in the upright and supine positions were observed at 6.3 and 3.3 per cent. Mixed findings were abnormal in 61.9% and 65.4%, based on the upright and supine positions (Table 1).

Table 1: Frequency distribution of data based on various findings in patients

		Count	Layer N%
DeMeester Score	Abnormal	253	44.2%
Total Reflux time	Abnormal	260	45.5%
Number of Reflux	Abnormal	229	40%
Number of Long	Abnormal	138	24.1%
Longest Reflux	Abnormal	242	42.3%
BCT	Abnormal	15	2.6%
Proximal Extension	Pos	239	41.8%
Upright AC	Abnormal	117	20.5%
Supine AC	Abnormal	144	25.2%
Upright WA	Abnormal	291	50.9%
Supine WA	Abnormal	283	49.5%
Upright NA	Abnormal	36	6.3%
Supine NA	Abnormal	19	3.3%
Upright Mixed	Abnormal	354	61.9%
Supine Mixed	Abnormal	374	65.4%

SAP findings were related to the symptoms of the patient in 55.8% of the patients, while 30.2% of the patients had SAP findings without any association with the symptoms of the patient. They were also Results in 7% of cases were Pure Acid Reflux followed by Weakly Acid (1%), Non-Acid (0.3%), Mixed & Gas (5.2%), Functional (58.4%) and Esophageal Hypersensitivity (28%). Furthermore, Weakly Acid plus Acid was also found to be 8%, and Weakly Acid + Acid + Non-Acid were determined as 8.3% (Table 2).

Table 2: Frequency of diagnosis in patients

		Frequency	Per cent
Valid	Pure Acid Reflux	40	7
	Weakly Acid	6	1
	Non-Acid	2	0.3
	Mixed&Gas	30	5.2
	Functional	334	58.4
	Oesophagal Hypersensitivity	160	28
	Total	572	100

Analysis of variance (ANOVA) did not show a significant difference in the frequency distribution of diagnosis based on the age of the patients (P = 0.216). The frequency distribution of diagnosis did not show a significant statistical relationship regarding gender-based on chi-square test (P = 0.721). The prevalence of functional conditions in men and women was revealed to be 59.1 and 57.6%, respectively while the cases of hypersensitivity were reported in 27.4% of men and 28.6% of women (Table 3).

Table 3: Distribution of diagnosis based on gender

		Diagnosis						Total
		Pure Acid Reflux	Weakly Acid	Non-Acid	Mixed&Gas	Functional	Oesophagal Hypersensitivity	
Gender	Female	19 (6.9%)	2 (0.7%)	2 (0.7%)	15 (5.4%)	159 (57.6%)	79 (28.6%)	276 (100%)
	Male	21 (7.1%)	4 (1.4%)	0	15 (5.1%)	175 (59.1%)	81 (27.4%)	296 (100%)
Total		40 (7%)	6 (1%)	2 (0.3%)	30 (5.2%)	334 (58.4%)	160 (28%)	572 (100%)

The frequency of diagnosis showed that the duration of symptoms was not statistically significant (P = 0.429) based on the ANOVA test.

Discussion

In this study, we investigated the various causes of GERD refractory in patients who were referred to Taleghani patient in Tehran from 2009 to 2017. All subjects in this study were Off PPI, meaning that patients did not take PPI and anti-acid for 2 weeks before testing. The test results showed that 7% of the cases belonged to Pure Acid Reflux based on 24 h pH-impedance, followed by Weakly Acid (1%), Non-Acid (0.3%), Mixed & Gas (5.2%), Functional (58.4%) and Esophageal Hypersensitivity (28%). Also, Weakly Acid + Acid + Non-Acid cases were found to be 8.3%, where is the most common cause of refractory cases followed by hypersensitivity, which is consistent with other studies in this area [17]. These results are consistent with other studies in this area [17]. Penagini et al., (2015) evaluated 50 patients with refractory GERD in Italy. They determined that 15 of the patients (30%) had functional heartburn [18], while this rate was about 2 times higher in our research. In a cross-sectional study, Frazzoni et al., examined 80 patients with refractory GERD, 35% of them had functional heartburn [19], which was lower than the result of our study. In another study, Savarino et al. performed an analytical cross-sectional study in Italy with 219 patients suffering from refractory GERD that 39% had functional heartburn [20]. In the present study, this was higher which could be due to the 3-fold sample size.

Jung et al., (2007) in the United States, assessed 2298 patients with refractory GERD and found that 3% of men and 4% of women had suffered from functional disorders such as functional heartburn [21]. The results of the study are in agreement with the results of the current study. In another study by Savarino et al., 2009 found that 27% of patients with refractory GERD suffered from functional heartburn [22], which was half the amount, obtained in our study. The higher number of the present study can be because of the examination centre as a referral hospital. A cross-sectional study by Mohammed Khan et al. in 2014 found that almost 60% of patients with refractory PPIs NERD and SAP (+) had no acid reflux, and about half of nonerosive gastroesophageal reflux disease (NERD) patients on PPI had normal multichannel intraluminal impedance-pH (MII-pH) monitoring, which was equally divided into two groups: Functional Heart Burn and hyper-sensitive esophagus [23]. We did not find this equal ratio in our study, and the frequency of functional cases was higher. Herregods et al., reported in an analytical cross-sectional report that roughly one-third of patients referring to GERD symptoms have problems other than reflux, the most common of which is Functional Heart Burn. This justifies why these patients do not benefit from anti-acid therapy [24], and in our study, this is proven. Moreover, different studies on various subjects have published the regarding the above results [25] [26] [27] [28] [29].

In conclusion, our data suggest that more than half of GERD patients in the 24h pH-impedance analysis have functional disorders. Therefore, due to the high incidence of functional and hypersensitivity cases, we can treat the remaining cases according to the prevalence before making expensive and inaccessible tests. Taken together, it is recommended to use a treatment period for functional and hypersensitivity, such as selective serotonin reuptake inhibitors (SSRIs) (fluoxetine, etc.).

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An Investigation of the Prevalence of Subclinical Brain Lesions in MRI Images of Migraine Patients

Khadijeh Haji Naghi Tehrani*

Department Neurology, Islamic Azad University, Tehran Medical Sciences Branch, Tehran, Iran

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***Correspondence:** Khadijeh Haji Naghi Tehrani. Department of Neurology, Islamic Azad University, Tehran Medical Sciences Branch, Tehran, Iran. E-mail: dr_tehrani10@yahoo.com

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Abstract

BACKGROUND: The use of the MRI method has opened up a new perspective on pathogenesis, diagnosis and treatment of brain lesions.

AIM: Therefore, this study aimed to evaluate the prevalence of brain subclinical lesions in MRI images of migraine patients.

METHODS: This cross-sectional study was conducted on 300 patients with a migraine referred to Baqiyatallah and Amir Al-Momenin Hospitals from 2005 to 2006. We measured the relationship between the results of MRI and the type of brain subclinical lesion by indices such as age, gender, type of a migraine, the number of migraine attacks, blood pressure and heart diseases, cholesterol, diabetes and thyroid diseases. Finally, data were analysed by IBM SPSS statistics software version 23. The significance level in this study was considered as $P > 0.05$.

RESULTS: From among 300 patients, 87.7% were women in the age range of 13-72 years. Moreover, the results indicated that with increasing age, blood pressure and some migraine attacks, the frequency of abnormal MRI also is increased significantly as well as the ratio of a migraine with aura was significantly higher than a migraine without aura in individuals with abnormal MRI. Also, the ratio of white matter lesions (WML) is higher in a classical migraine (a Migraine with aura). Statistical analyses did not reveal any significant relationship between MRI results on age, diabetes, cholesterol, heart and thyroid diseases.

CONCLUSION: The prevalence of abnormal MRI in older people and those with high blood pressure and migraine with aura is higher, and the ratio of subclinical lesions in the population of a migraine with aura is more common than a migraine without aura.

Introduction

A migraine is a common and chronic neuro-vascular disease that occurs as a result of numerous and severe attacks of headaches and disturbances in the nervous system [1]. The prevalence of a migraine in the United States and Europe is higher than in other parts of the world [2]. Furthermore, the migraine outbreak in men is between 2% to 15%, and in women between 4% and 35%. Symptoms include nausea, photophobia, vomiting, dizziness, diarrhoea and consciousness disorder [3] [4] [5] [6] [7].

The definitive and main pathophysiology of migraine has been remained uncertain. A migraine with aura is a type of a migraine, which exposed to visual and sensory disturbances before the start of the attack it affects about one-fifth of patients with a migraine [8]. Studies have shown that the most common cause of a headache is vascular difficulties [9]. Hypothalamic interactions, as well as the lack of balance between the sympathetic and parasympathetic nervous system, also cause autonomic brain symptoms in some types of migraine [10].

A migraine can increase the risk of developing brain subclinical lesions such as white matter lesions (WML). Some MRI-based studies have reported an increase in WML prevalence in migraine patients [11] [12] [13] [14]. Over the past three decades, there have been many different and controversial studies on the correlation between a migraine and abnormal cerebral paraclinical changes such as WML lesions, quasi-stroke lesions (ILLs), and volume changes of grey matter (GM) and white matter (WM) identified by MRI [15].

Therefore, the present study was designed to investigate the prevalence of brain subclinical lesions in MRI images of patients with a migraine.

Methods

This cross-sectional research was a descriptive and analytical study. The population under study was migraine patients referring to Baqiyatallah and Amir Al-Momenin Hospitals during the years 2005 to 2006. Initially, based on International Criteria of Headache International Society and using the related questionnaires, it was established whether the patient referred to the clinic had a migraine and then if it was positive, the type of migraine was identified. Accordingly, the number of patients with a migraine participating in the research was 300 people. In this study, the frequency of brain subclinical lesions in MRI was determined by people with a migraine. The questionnaire included questions about duration of the disease, the frequency of attacks per week, the duration of each attack, location of pain and quality of pain, presence or absence of aura, exacerbating factors, symptoms, history of previous diseases (patient inclusion criteria including high blood pressure, high cholesterol, diabetes, Cardiovascular and asthmatic diseases) and family history. To avoid any mistakes, the questionnaire was completed by the researcher himself. Patients were of different age groups without any specific age limitation. The simple non-random sampling method was applied. In all patients, the prevalence of brain subclinical lesions was evaluated in MRI images of patients with a migraine referred to Baqiyatollah and Amir Al-Momenin hospitals. Patient information was analysed using a questionnaire and radiologist's reports of patients' MRI using SPSS version 23 software, with the help of appropriate statistical tests; the probability value less than 5% was considered significant.

Results

Our population was in the age range between 13 and 72 years. The highest percentage of subjects with normal MRI (26.3%) were at the age range of 21-40 years, while the lowest percentage of subjects with normal MRI (4.3%) were in the age group of 51-60 years. On the other hand, the highest percentage of patients with abnormal MRI (4.3%) was in the age range of 41 to 50 years old and the lowest (0.3%) in the range of 13 to 20 years old. The results of statistical Chi-square test showed that the frequency distribution difference of MRI results in different age groups was significant (P-value = 0.019) (Table 1).

Table 1: Relationship between MRI results and different age groups

Variables		Year						DF	P-value
	Age ranges	13-20	21-30	31-40	41-50	51-60	61-72		
MRI	Normal	9.7%	26.3%	26.3%	17.3%	4.3%	4.7%	5	0.019
	Abnormal	0.3%	2.3%	2.0%	4.3%	0.7%	1.7%		

Based on the estimation of the regression coefficient (0.044) it can be concluded that the frequency of abnormal MRI is directly related to the age. The correlation between the type of MRI and the number of migraine attacks per month by Chi-square test showed that with an increase in the number of migraine attacks per month, the relative frequency of abnormal MRI has also increased significantly (P-value < 0.05) (Table 2).

Table 2: Relationship between MRI results and the frequency of migraine attacks per month

Variables		The frequency of attack in a month		P-value
	Attack in month	1 time in a month	More than 1 time in a month	0.001
MRI	Normal	36.3	52.3	
	Abnormal	2.3	9.0	

From between 300 patients, 12.3% of them were male and 87.7% male. 12.2% of women and 5.6% of men were of abnormal MRI, but Chi-square test revealed that MRI status does not depend on gender (P-value > 0.176). The statistics also showed that the status of MRI results is significantly related to high blood pressure, so that with the increase in blood pressure the ratio of people with abnormal MRI to people with normal MRI is significantly increased (P-value < 0.05) (Table 3).

Table 3: Relationship between MRI Results and Patients' Blood Pressure

Variables		Blood pressure		P-value
		Normal	High	
MRI	Normal	84.7%	4.0%	0.001
	Abnormal	8.0%	3.3%	

However, no association was found between the status of MRI results with cholesterol (P-value > 0.454), diabetes (P-value > 0.226), heart diseases (P-Value>0.316) and thyroid diseases (P-value > 0.454). Statistical analysis revealed that there is a statistically significant relationship between the type of a headache (a common migraine and classical migraine) and MRI status. This means that the ratio of a classic migraine (a migraine accompanied by aura) to a common migraine (a migraine without aura) is increased significantly in people with abnormal MRI (P-value = 0.007) (Table 4).

Table 4: Relationship between MRI results and migraine headache type

Variables		Headache category		P-value
		Common migraine	Classic migraine	
MRI	Normal	80.3%	8.3%	0.007
	Abnormal	8.3%	3.0%	

The highest incidence of lacunar infarct (2.3%) was in the age range of 41 to 50 years and the lowest (0%) in the range of 13 to 30 years. Also, the highest prevalence of WML was in the range of 21-30 years old (2.3%), and the lowest (0%) was between the ages of 51 and 60 years. From the perspective of the Chi-square test, these differences are significant (P-value = 0.003), or, in other words, the type of brain subclinical lesion depends on the age of the patients, as the age increases the incidence of lacunar infarct increases and the prevalence of WML decreases (Table 5).

Table 5: Relationship between the type of brain subclinical lesion and patient's age

		Subclinical cerebral lesion			P-value
		Lacunar infarct N (%)	WML N (%)	Normal N (%)	
Age Ranges	13-20	0 (0%)	1 (0.3%)	29 (9.7%)	0.003
	21-30	0 (0%)	7 (2.3%)	79 (26.3%)	
	31-40	1 (0.3%)	5 (1.7%)	79 (26.3%)	
	41-50	7 (2.3%)	6 (2.0%)	52 (17.3%)	
	51-60	2 (0.7%)	0 (0%)	13 (4.3%)	
	61-72	4 (1.3%)	1 (0.3%)	14 (4.7%)	

The incidence of Lacunar infarct and WML in patients with high blood pressure was equal to 1.7% and 1% and in other individuals 3% and 5.7%, respectively. Statistical analyses revealed a correlation between the blood pressure variable and the prevalence of lacunar infarct and WML lesions so that the ratio of these lesions was significantly higher in those with hypertension. The results also showed that in patients with high blood pressure, the incidence of lacunar infarct is higher and in those with normal blood pressure the incidence of WML lesion is higher (P-value = 0.001) (Table 6).

Table 6: the relationship between the type of subclinical brain lesion and patients' blood pressure

		Blood pressure		P-value
		Normal	High	
Subclinical cerebral lesion	Lacunar infarct	9 (3%)	5 (1.7%)	0.001
	WML	17 (5.7%)	3 (1%)	
	Normal	252 (84%)	14 (4.7%)	

The incidence of lacunar infarct and WML complication in a common migraine was 3.7% and 5%, respectively, and in a classical migraine, it was 1% and 1.7% respectively. Based on the results of proportional statistical tests, there was a significant relationship between the type of subclinical brain lesions and migraine type.

However, in both types of a migraine, the percentage of WML is higher, but the proportion of WML in a classical migraine (a migraine with aura) is greater than that of a common migraine (a migraine without aura). It is also evident in the data that the ratio of the presence of subclinical lesions in the classical migraine population is more than a common migraine (P-Value = 0.034) (Table 7).

Table 7: the relationship between the type of brain subclinical lesion and type of a migraine headache

		Migraine category		P-value
		Common	Classic	
Subclinical cerebral lesion	Lacunar infarct	11 (3.7%)	3 (1.0%)	0.034
	Wml	15 (5.0%)	5 (1.7%)	
	Normal	240 (80.0%)	26 (8.7%)	

Statistics did not reveal any significant relationship between the type of subclinical brain lesions and gender variables (P-value = 0.341), the duration of the disease (P-value = 0.712), and the number of migraine attacks per month (P-value = 0.072).

Discussion

Migraine is a cerebrovascular disorder; according to studies, relatively high rate of migraine disabling characteristics have led researchers to explore different ways to detect intracranial lesions in these patients. A large proportion of these known lesions can be considered as a risk factor for stroke. The incidence of migraine-related stroke, or in other words, every stroke that occurs in migraine conditions ranges from 1.44 to 1.7 in every 100,000 per year [1]. The present study also was designed and conducted aimed at measuring and finding the relationships between the status of MRI results and the type of brain subclinical lesion on the one hand and the indices like age, gender, and type of migraine, the number of migraine attacks, blood pressure and heart diseases, cholesterol, diabetes and thyroid diseases on the other. The results of this study showed that with increasing age, increased blood pressure and increased number of migraine attacks in the month, the abnormal MRI frequency also increased significantly. The statistics also showed that the ratio of classic migraine to common migraine in patients with abnormal MRI increased significantly (P-value < 0.05). Meanwhile, there was no significant relationship between MRI results and age, diabetes, cholesterol, heart and thyroid diseases. On the other hand, it was found that the relative frequency of Lacunar infarct was higher in people with hypertension and older age. Regarding the non-experimental study, which was done in a cross-sectional fashion, and considering that this study cannot determine the causal relationship and only can explain the accompaniment of two phenomena, it can not necessarily be concluded that the presence of brain subclinical lesions in the MRI of patients with Migraine represents the history of ischemic stroke or demyelination disease in the past or its occurrence in the future, but the existence of a relationship can lead us to a wider and more prospective research. The researches whose reports have been published have definitely not declared the causal relationship between these

variables, but despite numerical differences, there is a correlation between these factors. For example, based on data resulting from a comprehensive meta-analysis conducted by Swartz et al., the WML lesion is found in migraine neuronal imaging of individuals with migraine more than non-migraine individuals and it has a high odds ratio equal to 3.9 (95% CI = 2.26-6.72) [14]. While in their meta-analysis study in 2013 Bashir et al., reported less probability than Swartz et al., (CI 1.07-2.65%OR 1.68, 95); it showed that it only was associated with migraine with aura not with Migraine without aura [15]. The results of the study conducted by Bashir et al., are in line with the results of the present study. Because our results also proved that the proportion of WML lesion in the classical migraine (Migraine with Aura) is greater than common migraine (migraine without aura). Kruit et al., & Trauninger et al., reported in separate studies that an increased risk of subclinical infarction and WML lesion is associated with an increased incidence of headache [16] [17]. At a higher level and compared with the study of these two research groups, it was also evident in our data that the ratio of the presence of subclinical lesions in the classical migraine population is more than common migraine. Erdelyi-Botor et al, after tracking the results of MRI in migraine sufferers, showed that WMH increases with the increase in migraine duration [18]. Le Pira et al also reported in their study that there was no relationship between the number of migraine attacks and the progression of brain subclinical lesions [19]. However, our study proved that there is no significant relationship between the type of brain subclinical lesion (Lacunar infarct and WML) and the number of migraine attacks and the duration of the disease. Headache associated with arterial pressure is one of the issues discussed in migraine specialist assemblies. Some people believe that a headache may be a sign of arterial pressure, but sometimes people with high blood pressure may not have the risk of migraine and other types of headaches. Conversely, in some studies this relationship was inverse [20]. The results of this study showed that the status of MRI results is significantly associated with high blood pressure and with the increase in blood pressure, the proportion of people with abnormal MRI to normal MRI increases significantly. Despite the widespread variation in the reports of researchers and despite some controversial and ambiguous cases, there is a comprehensive conformity between this research and the other similar studies. The main reasons for these differences can be attributed to factors such as sample size and target population, age and gender composition, clinical characteristics, technical factors, and technological differences and the like.

In conclusion, the prevalence of abnormal MRI in older people and those with high blood pressure, as well as those who are involved in migraine with Aura (a classical migraine), is significantly higher. Also, about the prevalence of various subclinical lesions in the population under study, it can be concluded that the ratio of the presence of subclinical lesions in the classical migraine population (a Migraine with aura) is more common than a migraine. Ultimately, it's important to note that although clinical studies have shown an increase in the prevalence of brain infarction and white matter lesions of WML in migraine patients, but the increase in the prevalence of these lesions in these patients has not any clear explanation.

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Assessment of the Role of the Anti-Mullerian Hormone, Luteinizing Hormone/Follicle Stimulating Hormone Ratio in the Diagnosis of Polycystic Ovary Syndrome in Sudanese Women

Hafza Tola[1], Mohammed Abbas[2], Elsir Abu Alhassan[3], Nassr Eldin Shrif[1], Mohammed Rida[3*]

[1]Faculty of Medical Laboratory Sciences, Alzaim Alazhari University, Sudan; [2]Medical Laboratory Sciences Program, Allied Health Department, College of Health Sciences, University of Bahrain, Kingdom of Bahrain; [3]Dr. Elsir Abu Alhassan Fertility Center, Khartoum, Sudan

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***Correspondence:** Mohammed Rida. Dr Elsir Abu Alhassan Fertility Center, Khartoum, Sudan. E-mail: mohedalirida@gmail.com

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Abstract

BACKGROUND: The diagnosis of polycystic ovary syndrome (PCOS) is not an easy procedure, as the signs and symptoms are heterogeneous and of undefined aetiology.

AIM: This study is aimed to evaluate serum anti-Mullerian hormone (AMH) level and luteinizing hormone (LH)/folic stimulating hormone (FSH) ratio in women with PCOS in Sudan and to assess the diagnostic efficiency for the diagnosis of PCOS.

METHODS: In a case-control study, Serum AMH, LH, FSH was measured in the early follicular phase from Sudanese patients (N = 230) with PCOS and100 controls. The LH/FSH ratio was calculated, and its diagnostic power was evaluated by receiver operating characteristic curves.

RESULTS: The means of serum AMH, serum LH level and LH/FSH ratio of the test, were significantly increased in the test group compared to the control group (P-value < 0.000). The AMH sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) were found to be 83%, 99%, 99%, and 72% respectively. Serum AMH was considered adequate measures for the diagnosis of PCOS; its level showed an area under the ROC curve of 0.98 (95% confidence, P-value < 0.000). The best compromise between 98% specificity and 90% sensitivity was obtained with a cut-off value of 3.3 ng/mL for PCOS diagnosis. There was no correlation between age, body mass index (BMI) and AMH level in the test group.

CONCLUSIONS: The Serum AMH level and LH/FSH ratio were higher in patients than in control. However AMH level has better discriminative power and good diagnostic potency for the diagnosis of the PCOS among Sudanese women.

Introduction

Polycystic ovary syndrome (PCOS) is one of the most common female endocrine disorders that affects up to 10% of women worldwide. PCOS is accompanied by an imbalance of female sex hormones and increased androgen production leading to infrequent or prolonged menstrual periods, excess hair growth, acne and obesity [1]. The exact aetiology is not yet known. However many risk factors have been demonstrated such as genetic and epigenetic or environmental factor leading to intraovarian hyperandrogenism [1]. Lack of ovulation in PCOS results in continuous high levels of estrogen and insufficient progesterone which lead to increased serum luteinizing hormone (LH) levels as well as changes in Anti-Mullerian Hormone (AMH) secretion. The higher the antral follicles count, the higher AMH levels, and women with PCOS typically have high numbers of antral follicles [2].

The diagnosis of PCOS is not easy as signs and symptoms are heterogeneous, the lack of well-defined diagnostic criteria makes identification of this common disease confusing to many clinicians. The guideline made by the American Society of Reproductive Medicine (ASRM) and European Society for Human Reproduction Medicine (ESHR) delivered in 2003 (Rotterdam criteria) determined the diagnostic criteria for PCOS which include: irregular menstrual cycle, androgen excess symptoms, and ovary ultrasound. A diagnosis of PCOS requires the

presence of at least two of the three features after other androgen-excess disorders have been excluded [3].

There are a considerable number of reasons to believe that many women with PCOS have been missed diagnosed, because of the extensive heterogeneity in the clinical presentation of PCOS. Furthermore, with the rising epidemic of obesity, the prevalence of PCOS may increase, as obesity potentially worsens the endocrine and metabolic profile of PCOS [4]. To the best of our knowledge, this is the first study to assess the diagnostic efficiency of AMH level and LH/FSH ratio for the diagnosis of PCOS among Sudanese women.

Material and Methods

A total of 230 Sudanese women with PCOS diagnosed based on Rotterdam criteria were enrolled in this study. The control subjects were 100 women. The study was conducted at Dr Elsir Abu Alhassan Fertility Center, Khartoum, Sudan. Patients with a history of menstrual disturbances (hypothyroidism, congenital adrenal hyperplasia, Cushing's syndrome, hyperprolactinemia and hirsutism were excluded. Both groups were matched regarding age and BMI (BMI was given by = Weight(kg)/Height(m^2)). The study was ethically approved by the ethical approval committee for medical research of Alzaim Alazhari University, and informed consent was collected before the beginning of the study.

Blood sampling was performed in the early follicular phase, between day 2 and 5 after the last menstrual period both in PCOS patients and controls. Serum AMH levels were assessed by ELISA (Enzyme-Linked Immune Sorbet Assay) using BECKMAN COULTER Kit reagents. Serum LH and FSH were measured by mini VIDAS technology using BIOMERIEUX Kits reagent. The assay principle combines an enzyme immunoassay sandwich method with final fluorescent detection (ELFA). The method and the steps were followed as per company instructions.

Data were analysed using the statistical package for social sciences (SPSS ver.17) (IBM Corp., Armonk, NY, USA). Comparison of means of AMH, FSH, LH hormone and LH/FSH ratio was conducted using a t-test. Associations between hormonal levels between control and study groups were measured by using the Chi-square test. Correlation between age, BMI and AMH were tested using person correlation. The test was considered significant when the *P* value is less than 0.05. Receiver operating characteristic (ROC) curves were constructed to examine the diagnostic test performance, *i.e.* its capacity to discriminate between controls and patients with PCOS. Sensitivity (y-axis) against [1-specificity (x-axis)] was plotted at each threshold level, and the area under the curve (AUC) was computed by the nonparametric Wilcoxon test. AUC represents the probability of correctly identifying controls and patients with PCOS. A value of 0.5 means that the test result is no better than chance.

Results

The mean age of the test group and control group was (28.17 ± 5.12), (28.98 ± 5.52) respectively. BMI means of test and control was (25.72 ± 4.86), (25.16 ± 5.57) respectively.

The mean of serum AMH, LH level and serum LH/FSH ratio of study subjects was significantly increased ($P < 0.000$) compared to the control group. There was the insignificant difference ($P = 0.06$) between the mean of serum FSH level of test group and control group (6.30 ± 3.64) compared to (7.23 ± 5.24) respectively, as shown in Table1.

Table 1: Comparison between mean of AMH, LH, FSH, LH/FSH ratio for test and control group

Variable	Test group (n = 230)	Control group (n = 100)	*P*-value
AMH ng/ml	9.61 ± 5.82 (3.79-15.43)	1.80 ± 0.66 (1.14-2.46)	0.000
LH mIU/L	10.55 ± 7.82 (2.73-18.37)	7.77 ± 6.79 (0.98-14.56)	0.000
FSH mIU/L	6.30 ± 3.64 (2.66-9.94)	7.23 ± 5.24 (1.99-12.47)	0.06
LH/FSH ratio	1.80 ± 1.16	1.12 ± 1.22	0.000

The table shows the mean±standard deviation, range in brackets (Min-Max) and p-value. A t-test was used for comparison; *P* value less than 0.05 considered significant.

Table 2 showed the diagnostic power of AMH about sensitivity and specificity when 4.0 ng/ml was used as cut off point. The sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) were observed at 83%, 99%, 99% and 72% respectively. The diagnostic power of the LH/FSH ratio about sensitivity and specificity when 1:1 was used as cut off point were observed at 72%, 76%, 84% and 62% respectively.

Table 2: Shows the sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of AMH and LH/FSH ratio to diagnose of PCOS

	AMH	LH/FSH
Sensitivity	0.834	0.729
Specificity	0.990	0.765
Positive Predictive Value (PPV)	0.995	0.843
Negative Predictive Value (NPV)	0.727	0.620
Likelihood Ratio+ (LR+)	85.07	2.399
Likelihood Ratio- (LR-)	5.967	2.571

The AUC for the different hormone serum measurements is shown in Table 3. The AUC for AMH was 0.98 (95% confidence interval, $P < 0.000$), the compromise between specificity and sensitivity was (96% and 92%), (96% and 91%), (98% and 90%) was

obtained with threshold values of AMH 3.1, 3.2, 3.3ng/ml respectively.

Table 3: AUC of serum hormone concentrations for PCOS detection in women

Hormone	AUC (95% CI) , *P* < 0.000	Compromise between specificity and sensitivity	Cut-off values
AMH	0.98	96%, 92% 96% and 91% 98% and 90%	3.1 ng/ml 3.2 ng/ml 3.3 ng/ml
LH/FSH ratio	0.74	76%, 72%	1:2

The AUC for LH/FSH ratio was 0.74 (95% confidence interval *P* < 0.000) and the best compromise between specificity and sensitivity (76%, 72%), was obtained with threshold values of LH/FSH ratio 1:1 as shown in Figure 1.

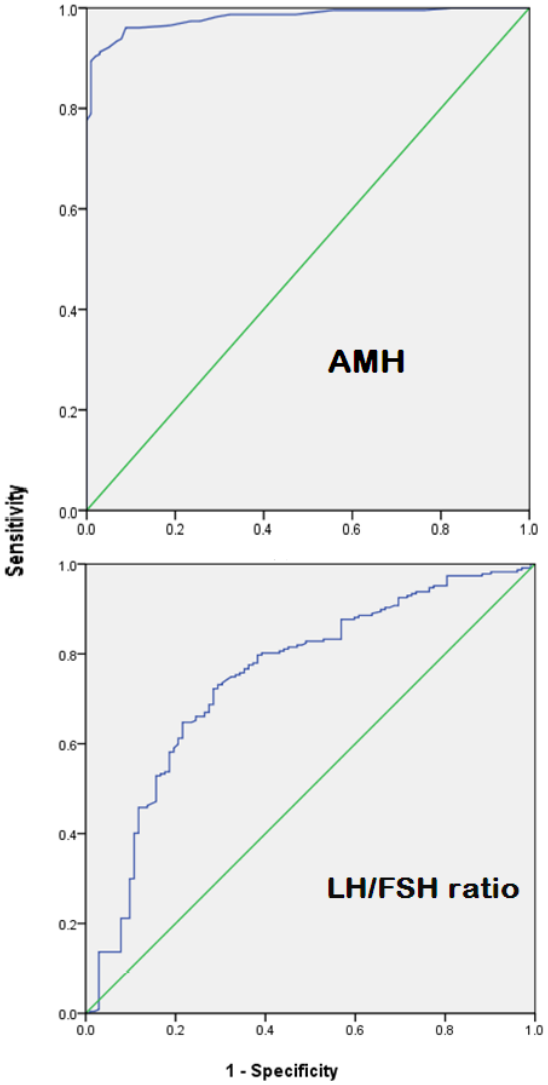

Figure 1: ROC curves for the detection of PCOS AMH and LH/FSH ratio

The results also showed that there was no correlation (*P* = 0.488) between age and AMH level in the test group (r = -0.046). Furthermore, there was no correlation (*P* = 0.492) between BMI and AMH level in the test group (r = 0.039), Figure 2 and 3 respectively.

Discussion

The present study is the first study to our knowledge to assess the role of the AMH level and LH/FSH ratio in the diagnosis of PCOS among Sudanese women.

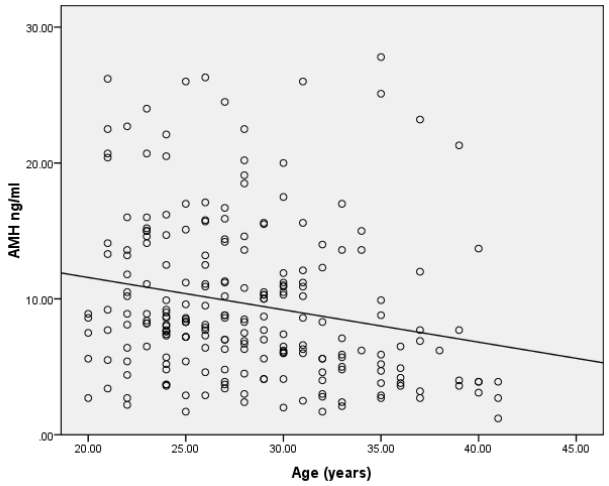

Figure 2: A scatter plot shows the correlation between the level of AMH and age in test (P-value = 0.488)

The significant increase of AMH level in PCOS women compared to healthy women in this study is by the study by Bungum L *et al.,* [5], in Sweden, they found a significant difference in mean AMH levels between the groups, the highest values being seen in the PCOS group. The result also agrees with results obtained by Pawelczak M *et al.,* [6], they noticed a positive relationship between serum AMH and ovarian volume as well as peripheral follicular distribution in adolescents with PCOS.

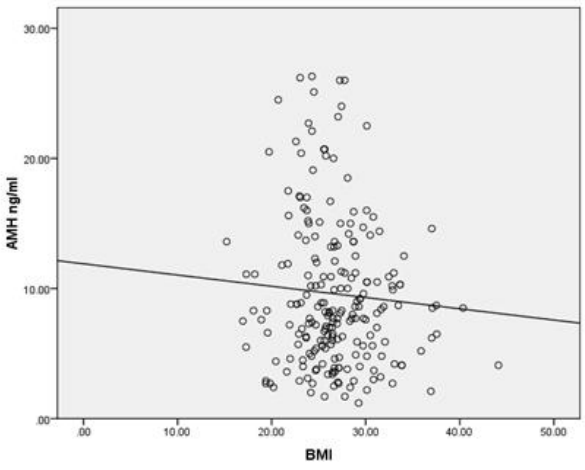

Figure 3: A scatter plot shows the correlation between the level of AMH and BMI in the test group (P-value = 0.492)

In the present study, the level of the LH and LH/FSH ratio was significantly higher in patients than in control group. In contrast, there was no significant

difference level of FSH level. This may be due to increased androgen level in PCOS women, which leads to abnormalities of ovaries hormone that contributing to a relative suppression of FSH production. Our finding is in harmony with the results reported by Siebert TI *et al.,* [7]. Sahmay S *et al.* also found the significant differences between the mean serum, FSH, LH and LH/FSH ratio of PCOS women and control groups in their study [8]. The specificity and NPV of AMH and LH/FSH ratio to diagnosis PCOS in the current study agrees with the results reported by Wetzka *et al.,* [9], AMH showed 90% specificity with 71.2% sensitivity for the diagnosis of PCOS. In the existing study Serum AMH, and LH/FSH ratio was considered adequate measures for the diagnosis of PCOS, they AURC levels showed 0.98 and 0.74 (95% confidence, *P*=0.000) respectively. Similarly, Pigny P *et al.* conveyed that AMH measurement has been found to offer a relatively high specificity and sensitivity (92 and 67%, respectively) for PCOS [10]. In contrast to our result, recently Cengiz H *et al.* did not find AMH to be a reliable predictor for the presence of PCOS [11]. Furthermore, Li et *a.,l* reported that the serum AMH measurements presented a relatively poor diagnostic power, with a sensitivity of 61.7% and a specificity of 70%. They attributed the causes to the lower prevalence of hyperandrogenism, obesity, and insulin resistance to racial differences in their study [12].

The cut-off value of AMH in this study (3.3 ng/ml) is compatible with the previous result obtained by Sahmay S *et al.,* [8] the large sample size was the common denominator between both studies, unlike cut-off values from other studies with small sample size [12] [13] [14]. The lack of correlation between age, BMI and AMH level observed in the current study is agreed again with result gotten by Sahmay S *et al.*, they concluded there was no relation of age and BMI between PCOS and non-PCOS subjects in their study [8].

In conclusion, AMH was shown to be a useful parameter for the diagnosis of PCOS in this study. Our results suggested that AMH is primarily a marker of ovarian function and not associated with other organ pathologies such as adrenal gland dysfunction or metabolic disturbances.

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Clinical Science

Immunotherapy in Allergic Rhinitis: It's Effect on the Immune System and Clinical Symptoms

Samaneh Kouzegaran[1], Mohammad Ali Zamani[2], Reza Faridhosseini[1], Houshang Rafatpanah[3], Abdolrahim Rezaee[3], Hadis Yousefzadeh[4], Rahman Movahed[5], Farahzad Jabbari Azad[1], Hooman Tehrani[1*]

[1]Allergy Research Center, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran; [2]Pediatric Department, Shahrekord University of Medical Sciences, Shahrekord, Iran; [3]Immunology Research Center, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran; [4]Immunology Research Center, Student Research Committee, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran; [5]Otorhinolaryngology-Head & Neck Surgery, Sinus and Surgical Endoscopic Research Center, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

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Keywords: Allergic rhinitis; Subcutaneous Immunotherapy; Cytokine

***Correspondence:** Farahzad Jabbari Azad and Hooman Tehrani, Allergy Research Center, Ghaem Hospital, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran. Tel- Fax: +985118458769. E-mail: Jabbarif@mums.ac.ir, TehraniH911@mums.ac.ir

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Abstract

BACKGROUND: Allergic rhinitis is one of the most common allergic diseases and characterised by sneezing, rhinorrhea, nasal congestion and nasopharyngeal itching. Subcutaneous immunotherapy (SCIT) for specific allergens is an effective treatment and induces the inhibitory effect of T regulatory lymphocytes and decreases clinical symptoms in allergic rhinitis.

AIM: In this study effect of subcutaneous immunotherapy with specific allergens on clinical symptoms and T regulatory and T Helper cells cytokines, in patients with allergic rhinitis are evaluated.

METHODS: In this study, 30 patients with moderate to severe allergic rhinitis according to clinical criteria and positive skin prick test for aeroallergens were selected and treated by SCIT. Clinical symptoms and T cells cytokines IL4, IL17, IFN gamma, TGF beta, GITR, FOXP3 and IL-10 (by RT-PCR) were evaluated before and one year after initiation of treatment.

RESULTS: Thirty (30) patients with allergic rhinitis at age range 15-45 years old were treated by SCIT, and 23 (14 female, 9 male) patients continued the study, and 7 patients did not continue treatment. After immunotherapy, clinical symptoms decreased significantly. The specific cytokines TGF beta and IL10 levels increased and changes were statistically significant. (Respectively $P = 0.013$ and $P = 0.05$) The IL17 level was also increased, but not statistically significant. ($P = 0.8$) IFN gamma, IL4, GITR, FOXP3, all decreased, but the changes were not statistically significant ($P > 0.05$).

CONCLUSION: Subcutaneous Immunotherapy for specific allergens decreases clinical symptoms in patients with allergic rhinitis and induces tolerance in T lymphocytes, especially by increasing T regulatory cells cytokines, TGF beta and IL10.

Introduction

Allergic rhinitis (AR) is one of the most common types of allergy worldwide. Recent studies have shown an increase in its prevalence during recent decades ranging from 1.4 to 45%. Allergic rhinitis has direct and indirect effects on the quality of life and is accompanied by a group of another disease including asthma, middle ear inflammation, nasal polyps, sinusitis and lower respiratory tract infections [1] [2].

The diagnosis of AR is made based on clinical symptoms such as sneezing, rhinorrhea, itchy nose and nasal congestion when there is no sign of lower respiratory tract infections or anatomic abnormalities of the nose. Also when lab findings including a positive prick test and IgE specific antibody are in

favour of allergy, regarding the patients' history and clinical examination [3] [4].

The treatment of allergic rhinitis initially includes the avoidance of allergens especially common inhaled allergens. Indoor allergens such as house dust mites (HDM) especially in bed and house fungi which grow in damp places besides pets, indoor plants, grass, trees and grass pollen and other allergenic plants should also be avoided [1] [3] [4] [5]. Also, certain medications such as oral antihistamines, topical decongestants, inhaled corticosteroids and in certain cases oral corticosteroids are prescribed [1] [3] [5]. Immunotherapy with allergens is a therapeutic method in which the allergen is gradually and with an incremental dose administered resulting in the alleviation of clinical symptoms and reduced disease severity while preventing disease progression [1] [5] [6].

Immunotherapy with allergens has proven efficacy in the treatment of allergic rhinitis/ asthma and allergy to insect sting [1]. Today, subcutaneous injection of an allergen (SCIT) is the most common type of immunotherapy. Several studies have shown desirable clinical efficacy in the single and combined administration of allergens [1] [7] [8]. In different studies immunotherapy has resulted in a decrease in the number of principal cells in allergic responses (eosinophils/basophils and mast cells) and an increase in IgG4; moreover changes in lymphocytes including a rise in CD8+ and Treg cells and a decrease in IL4 and IL5 levels has been observed [1] [4] [6].

It seems that immunotherapy has a major role in the induction of specific Treg cells and that the induction of tolerance in T lymphocytes is the base of immunotherapy. The tolerance of peripheral T lymphocytes is recognised by the production of allergen-specific Treg cells. In addition to tolerance induction, immunotherapy prevents sensitisation towards new allergens and allergy progression [3][9][10]. CD4+ Treg-cells are divided into two categories: Natural Treg (nTreg) and Inducible Treg (iTreg) cells. Each of these subsets has a specific marker and express their specific receptors which for nTreg cells include CCR4/GITR/CTLA4/CD62L and for iTreg cells include IL10/TGF-beta. Natural Treg cells react to autoantibodies which are expressed in the thymus whereas Inducable Treg cells react towards peripheral antigens which are expressed by dendritic cells [1] [11] [12].

The nTreg cells originating from the thymus express an intracellular marker named FOXP3. It has been proved that following immunotherapy, the presence of FOXP3+ cells is increased in the nasal mucosa and this increase is consistent with the improvement of allergic rhinitis [4] [12].

The present study aimed at evaluating the effect of immunotherapy on the clinical symptoms and cytokine changes related to T lymphocytes in moderate to severe AR patients.

Methods

The present study is an experimental interventional trial which investigates the effect of immunotherapy on the clinical symptoms and the immune system of patients with moderate to severe perennial AR in Ghaem Hospital, Mashhad, IRAN during October 2008 to October 2009. A full medical history was initially taken from patients with perennial AR, and a thorough physical examination was performed. Prick test with the standard method and by using the common regional aeroallergens was performed with 6 extracts (Hollister, USA) to confirm the basis of allergy. In the mentioned test more than 3mm induration from the negative control was regarded as a positive test result.

The used tools were the following:

1. A structured questionnaire according to the AR scoring system

2. Skin scratch testing according to the European Academy of Allergy and Clinical Immunology

3. Allergenic extracts used for cluster immunotherapy

The inclusion criteria were typical signs of AR in exposure with inhaled allergens, positive results in the standard questionnaire, and a positive skin prick test with at least 3 standard inhaled allergen extracts. Patients were excluded in case of consuming beta blockers, accompanying uncontrolled asthma, and concomitant conditions including autoimmune disease, psychotic disorders, pregnancy and other forms of rhinitis. For lab studies 5-7 cc blood was taken from the brachial vein and lymphocyte markers regarding the phenotype of TH1, TH2 TH17 and Treg and specific markers of GITR, FOXP3, IFN-γ, IL17, IL10, IL4, TGFβ were evaluated with RNA extraction, cDNA production and eventually RT-PCR with the standard method (TaqMan for FOXP3 and cyber green for other cases).

For patients with allergic rhinitis who received a minimum of immunotherapy to treat pollen allergies using Allergovit (composition: 015 grass/cereals-100%; or composition: 108 Birch-35%, 115 Alder-30%, 129 Hazel-35%, Allergopharma, Reinbek, Germany).

The sample size was calculated as 25 cases concerning similar studies. Immunotherapy based on a planned schedule was conducted by the subcutaneous injection of mixed inhaled allergens extracts (Hollister, USA) with a concentration of

1:1000 of the original vial each week for 10 sessions, 1:100 every two weeks for 10 sessions and finally 1:10 monthly for one year. After a year after immunotherapy initiation, clinical and laboratory findings were once again evaluated. Wilcoxon test was used for IFN-γ, IL17 and IL10 measurements analysis whereas Paired T-test and the SPSS software version 11 were used for FOXP3, GITR, TGFβ, and IL4 measurements.

The study protocol was approved by the Research Council of Mashhad University of Medical Sciences and written informed consent was obtained from each participant before study entry.

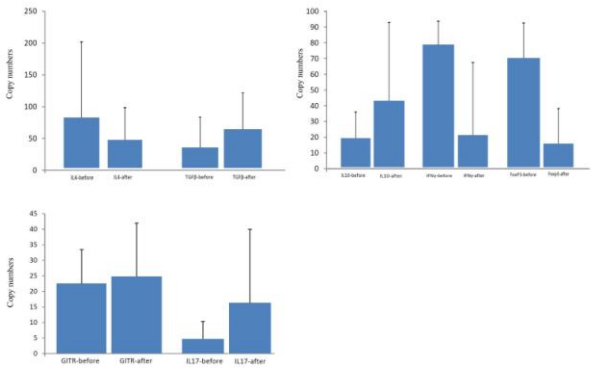

Figure 2: Expression of Cytokines before and after treatment

Results

Thirty patients with the diagnosis of allergic rhinitis underwent immunotherapy for one year. Twenty three (23) individuals, 9 males and 14 females, completed the study. Seven patients did not consent to continue treatment. The participants' age ranged from 15 to 45 years, and most patients experienced significant improvement in their clinical symptoms after the treatment period. Based on the clinical indices of TNS and TSS, disease severity was highest in the 30 to 39-year age group whereas the best therapeutic response was achieved in the 30 to 34-year age group (Figure 1).

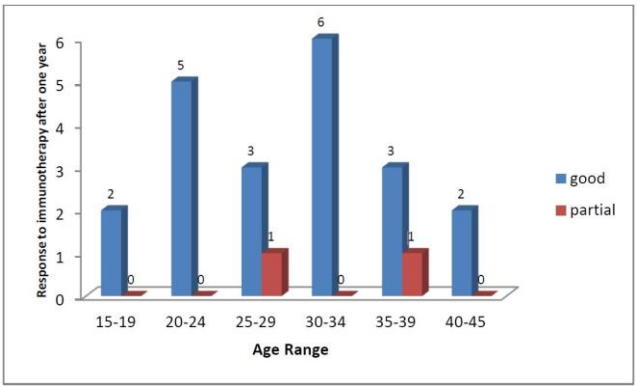

Figure 1: Response to immunotherapy after one year according to age

Regarding sex and response to immunotherapy; no meaningful response was obtained (P = 0.7). Considering the studied variables a year after treatment, an increase in the IL17 level was detected which was not statistically significant (P = 0.81). IFN-γ levels showed a reduction but with no meaningful significance (P = 0.21). However, the rise in TGB and IL10 levels was statistically significant, (P = 0.013, P = 0.05). Finally, a decrease in the level of IL4, GITR, FOXP3 was obtained, none showing a statistical significance (P > 0.05) (Figure 2).

Discussion

Immunotherapy if performed in an appropriate way in the right patient will be a safe and effective treatment that not only prevents the symptoms but will also result in reduced disease severity beside preventing disease progression [3] [6] [13]. Immunotherapy with allergens is a slow-process treatment which alters the immunologic mechanisms of disease. Meta-analysis of the performed studies comparing the therapeutic effects of subcutaneous immunotherapy with medical therapy has shown that immunotherapy has equal effects on medical therapy and can even be considered at treatment initiation [14]. The induction of tolerance in peripheral T lymphocytes resulting in a change in the immune system pathway from TH2 to Treg cells is the main goal in immunotherapy which is accompanied by elevated IL10 and TGF beta levels [4]. Eventually, the production of IL10 will lead to the inhibition of allergen-specific IgE production and on the other hand will result in IgG4 production [15]. Although at the start of immunotherapy a transient rise in the allergen-specific IgE level is observed, subsequently a gradual decrease takes place in the specific IgE level over years which prevents the rise of IgE in the mating season of plants in such patients [4].

In recent studies, it has been reported that the blocking effects of IgG in immunotherapy are FCR IIB receptor-mediated [16] [17] [18]. Attention should be paid to the fact that IgG4 immunoglobulin levels decrease the following immunotherapy, but their protective and biologic effects remain constant [15].

Nevertheless, IL10 can reduce the release of pro-inflammatory cytokines from mastocytes. Also, IL10 can reduce the function and activity of basophils and suppress IL5 production by TH0 and TH2 lymphocytes [9] [16] [18].

Cezmi et al. studied the therapeutic mechanisms of Treg cells in immunotherapy and published their results in 2009 in JACI. They stated that an increase in the number of Treg, CD25+ and CD4+ cells in allergen-specific immunotherapy (SIT) has a significant role and that immunotherapy with the grass group will result in increased IL10 and TGF-β expression in T cells and the mucosa.

One of the main characteristics of Treg cells is the expression of FOXP3 in them. In case of receiving signals from pro-inflammatory cytokines such as IL6, the FOXP3 function is inhibited, and the TH17 pathway is activated. For the induction of both the Treg and TH17 cells, TGF-β is required; but the function of these two cell groups are at odds with each other [19]. Although FOXP3 is expressed to a lesser degree by T-effector cells, the amount is very small, and it is expressed transiently; therefore FOXP3 is considered as a specific marker for Treg cells only [13] [15].

Bacchetta reported the principle role of Treg expressed FOXP3 as tolerance induction and prevention of effector T cell responses. In the mentioned study those Treg cells originating from the thymus were named as nT-reg cells, and those forming outside the thymus were called aT-reg (adaptive) cells [15] [17].

nT-reg cells react toward self-antigens expressed in the thymus whereas nT-reg or iT-reg cells including Trl (producing IL10) and TH3 (producing TGFB) result from the differentiation of naive T cells following antigenic stimulation in the environment. In several studies, it has been proved that the number of CD4+CD25+FOXP3+ cells increases in the nasal mucosa following immunotherapy, inconsistent with the improvement of the symptoms of allergic rhinitis [10].

The deviation of allergen-specific effector cells towards the Treg phenotype is the key to successful immunotherapy even in healthy individuals' immune responses. The inhibitory effect of IL10, known as an inhibitory cytokine for T cells, has been well proved in inducing tolerance towards allergens, autoantigens, bonding antigens and tumoral antigens. In 2009 Ciprandi et al., from Italy evaluated the TGF-β and IL17 levels in 23 allergic rhinitis patients before and after immunotherapy. TGF-β was 12.503 ± 23.354 ng/ml and 43.305 ± 31.861 ng/ml before and after immunotherapy, respectively, (P = 0.0016). This study showed a remarkable rise in TGF-β levels one year after immunotherapy. It also confirmed a rise in IL10 following immunotherapy [5].

Recently SLIT (sublingual immunotherapy) has found its place in immunotherapy. In a study by L. Cosmi et al., on the effects of SLIT in allergic rhinitis patients and mite-sensitive asthma, immunologic changes included reduced allergen-specific IgE and increased TGF-β, IL10 and IFN-γ levels [20]. In a study by Jutel et al., in 2003 in Switzerland, the mechanism of immunotherapy was studied in HDM-sensitive patients after 70 days of immunotherapy. In a part of this study, the IL10 and TGF-β receptors were blocked and re-evaluated. Eventually, it was observed that the inhibitory responses of T-cells by IL10 and TGF-β are the key mechanism of the mucosal immune response to allergens. Also, the inhibitory effects of IL10 on T-lymphocytes were due to changes in the CD28 mediated signalling pathway while the inhibitory effects of TGF-β were due to its inhibitory effect on TCR/CD3 and the inhibition of the CD28 pathway. Moreover, IL15 which is one of the main factors in the survival and growth of T-cells is inhibited by IL10 and TGF-β [7].

In our study, a significant decrease was observed in the clinical symptoms of allergic rhinitis patients after immunotherapy. In lab studies similar to Cezmi and Ciprandi studies, a rise in TGF-β and IL10 was detected in our patients. However, in contrast to previous studies, the FOXP3 was reduced although not to a significant level. Moreover, an increase in IL17 was detected following immunotherapy which could be suggestive of TH17 induction by TGF-β following the increase in this inhibitory factor.

Regarding the obtained results from this study, it seems that the immunologic mechanism leading to the alleviation and improvement of symptoms in AR patients mainly includes a rise in the number of inducible Treg cells accompanied by increased TGF-β and IL10 levels.

Limitation of the study: The limitation of the current study was small sample size because only 30 patients had inclusion criteria.

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Factors Associated with the Choice of Peritoneal Dialysis in Iran: Qualitative Study

Seyedeh Masoumeh Ahmadi[1], Amir Jalali[2*], Rostam Jalali[3]

[1]Department of Nursing, School of Nursing and Midwifery, Kermanshah University of Medical Sciences, Kermanshah, Iran; [2]Department of Psychiatric Nursing, School of Nursing and Midwifery, Substance Abuse Prevention Research Centre, Kermanshah University of Medical Sciences, Kermanshah, Iran; [3]Department of Nursing, School of Nursing and Midwifery, Kermanshah University of Medical Sciences, Kermanshah, Iran

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Keywords: Peritoneal Dialysis; Decision-making; Content Analysis; Qualitative Research

***Correspondence:** Amir Jalali. Department of Psychiatric Nursing, School of Nursing and Midwifery, Substance Abuse Prevention Research Centre, Kermanshah University of Medical Sciences, Kermanshah, Iran. E-mail: a_jalali@kums.ac.ir

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Abstract

BACKGROUND: Decision making about the type of dialysis in renal failure is a matter of great importance involving the patients, his/her family, and the health care team. Identifying the factors influencing decision making for undergoing peritoneal dialysis (PD) helps the development of this therapeutic method in patients**.**

AIM: The present study aims at explaining the factors influencing decision making about undergoing PD in End Stage Renal Failure patients

METHOD: The present study is a qualitative research, which used content analysis method. A semi-structured and in-depth interview was conducted with the 19 participants that selected by purposefully sampling. All interviews were recorded subsequent to receiving consent of the participants and were analyzed using content analysis method.

RESULTS: The first codification process resulted in 345 codes, which finally decreased to 278 codes by continuous reading and removal of duplicates. Overall, two main categories and eight categories namely facilitating factors (viz. family atmosphere, hemodialysis problems, PD advantages, and social environment) and inhibitory factors (viz. inefficient family, PD requirements, attitudes towards hemodialysis, and the country treatment system) were selected from the total 19 sub-categories and 278 codes.

CONCLUSIONS: Results indicated that various personal, family-related, psychological, social, and economic factors could affect the decision on the type of dialysis in patients. Therefore, basic infrastructures such as social support, education, and even the specialist and positive perspective of the Ministry of Health are required to choose this therapeutic method.

Introduction

End-stage Renal Diseases (ESRDs) are rapidly developing throughout the world [1]. Nowadays, appropriate alternative therapeutic methods for patients with ESRD or Chronic Kidney Disease (CKD) are reliant upon three main treatments namely Hemodialysis, Peritoneal Dialysis (PD), and Kidney Transplantation [2] [3]. In Iran, by the end of 2012, 32686 patients were identified with renal disease; of this number, 15957 patients were treated with hemodialysis, 15592 patients with transplantation, and 1137 patients were treated with the PD method [4].

Alternative treatments for these patients have to be predicted in advance and the willingness of the patients has to be preferentially intervened in choosing the therapeutic method. The selection of type of treatment may depend on the physician's judgment and diagnosis, access to medical care centers, the clinical state of the patient, pre-dialysis training, age, gender, personal beliefs and values, past experiences, and family support [1] [2]. Pre-dialysis training may help patients in better decision making on the type of dialysis. The aforementioned

training is of particularly crucial importance for CKD patients for whom dialysis treatment is essential to helping them face minimum disruption in their lifestyle or family situation [5]. Patients' intervention in decision making related to their health has been rarely common throughout history; traditionally, medical-related decisions have been frequently made by physicians. Such a style of Medical Decision Making (MDM) is frequently the dominant style [6].

Recognition of the perspectives of patients, caregivers, and significant agents affecting decision making is required for helping patients in making the best choice [6] [7]. The aforementioned trend may be highly helpful as well as effective in finding out the reasons behind the selection of PD by patients [5]. Therefore, the best method for understanding the reasons behind selecting PD by these patients is their own explanations, which could prove extremely helpful [7] [8] [9].

Regarding the investigations carried out in terms of the decision making process and related influencing factors, qualitative research was found to be the best study method [10]. In qualitative research, the words and explanations statements of participants are analyzed by the researcher, and their experiences in terms of the study under-scrutiny are reported as explained personally by the patients [11]. Qualitative research is done in a variety of ways; qualitative research using the content analysis method is one of these ways [12]. In content analysis, the researcher may reduce data, endow it with favorable structure and order, and subsequently facilitate theory development. Content analysis is a research methodology investigating the words and phrases in a given text [11].

The researcher determines the duplications, and the meaning and relevancies of the words and concepts of the text, and then concludes the messages embedded in the text, the messages of the author and audiences, and even the culture and the era to which the words and concepts are dedicated [11] [12]. According to statistics of Kermanshah province and its neighboring cities, patients have a strong desire toward hemodialysis. On PD, according to existing statistics of the educational center of Imam Reza (AS) in this province (the only PD center in the west of Iran), by January 2014, the number of PD patients under supervision in this center were reported as approximately 32. In the present study, by using the aforementioned method, the experiences of participants were dealt with to present strategies for policy makers to encourage patients to undergo PD; additionally, the facilitating factors and existing barriers to choosing PD for continued treatment were also determined. The present study aims at explaining the factors influencing decision making about undergoing PD in ESRD patients.

Methods

This study was approved by the Ethics Committee of Kermanshah University of Medical Sciences (KUMS.REC.1394.476). In the present study, according to the research objective, all the patients under treatment with PD or patients with kidney transplantation who had experienced PD as a treatment method before kidney transplantation - at the time the of the study - were selected as the study participants. Moreover, a sampling with maximum variation was done to obtain richer information. Additionally, the patients' acquaintances, their family and some nurses and specialist physicians who could participate in decision making as the therapists of the patients were also included as participants of the study. Using purposeful sampling patients with appropriate mental and physical conditions without the experience of mental disease were chosen as the participants. A deep and semi-structured profound interview including open-ended questions was used as the main method of data collection. The main interviews with participants were carried out separately and face-to-face at different times – whether on the morning or afternoon- in the PD ward of Imam Reza Hospital, Kermanshah at the appropriate time and place based on the participants' preferences.

Upon participant consent, their interviews were recorded. During interviews, some notes were taken, so that tone of the voice, word pronunciations, laughing, crying, and pauses of the contributors were also recorded. For the sake of facilitation in data gathering, guiding questions were also used. Before each new interview, the previous handwritten and codified interview including some notes as well as guiding questions were also reviewed by the researcher to help him/her to more profoundly and perfectly investigate the issues in the following interviews. Meanwhile, the contributor's address and telephone numbers were received by their own permission for verify their statements or for invitations for participating in later interviews, if needed. An interview guide was used for leading the interview in the proper direction and extracting the facts, mindsets, processes, and perspectives of contributors. At the very beginning of the interview, questions in terms of the onset of kidney failure and its symptoms were asked to pave the way for proceeding with the main questions and establishing proper connection with the contributors. Then they were asked to explain about whatever happened to them after the time from final diagnosis confirmation of the disease to the treatment onset.

Some questions used during the interview include:

1. What led you to choose PD as your therapeutic method of choice?

2. Who and what affected your decision?
3. What are the advantages and disadvantages of this method?
4. Why did you choose hemodialysis?

According to the responses delivered by participants, more questions were posed to clarify the details of their responses. When the participants started to speak freely, the researcher led them by asking deeper and more persistent questions at the right time to better clarify the phenomenon under-scrutiny. The interview frequently ended with: “Is there anything else you want to talk about? Any questions?"

The interview time varied depending on the participants’ power to respond. The average time of interviews in each session varied from 40-60 minutes. Meanwhile, as the researcher (the first author) was familiar with the native language of the studied region, richer information was collected.

After each interview ended, relevant content analysis was performed using the conventional method. To this end, after each interview, first, it was precisely listened to by the researcher; second, the interview was listened line by line; then the whole interview was transcribed in this way followed by codification. Following codification, the codes were classified according to the conceptual content, similarities and differences. Finally, the main categories were provided after reviewing the codes and their relevance.

Four criteria of authenticity (credibility, confirm ability, dependability, and transferability) by Lincoln and Guba (1985) were used to ensure the accuracy and reliability of data [12]. To that end, the researcher had a long-term connection with the place of the study, which led to appropriate understanding of the study environment and winning the trust of participants. Member check was applied to confirm the accuracy of data and codes, that is to say, after coding the interview text, it was returned to the participants to verify the accuracy of codes and interpretations. Accordingly, the codes that failed to represent the perspectives of the participants were modified. Moreover, the sampling method covering a wide range of clients, their family and the medical team from the perspective of age, gender, and work experience in that ward helped to increase validity of data. To increase the level of verification of the findings, the researcher attempted to completely explain the whole process of research (e.g. data collection, analysis, and content formation) to enable others to evaluate the research by reading it. To investigate the transferability of findings, attempt was made to thoroughly explain the field of the study to enable readers to comment on the transferability of findings.

The text of some interviews was also revised by the observers; that is to say, codes and extracted categories were investigated by several therapists and faculty members in addition to the researcher.

Results

In the present study, a total 22 interviews with 19 participants including 13 dialysis patient's that had demonstrated in table 1. (10 patients with PD and three patients with hemodialysis), three family members of patients (e.g. husband/wife, patient’s daughter/son), and three therapists including three male specialists having experience in the treatment of kidney disease were interviewed. Moreover, two main categories, eight categories, 19 sub-categories, and 345 codes that reduced to 278 after duplicate removal and continuous reviewing were developed .

Table 1: Sociodemographic characters of participants

NO	Age (Y)	Sex	Marital S.	Interval of PD (Y/M)	Interval of Hemodialysis (Y/M)	Job	Graduate S.
P1	46	Male	Married	1 Y	-	Deriver	Diploma
P2	50	Female	Married	4 Y	9 Mon	House Wife	Elementary School
P3	26	Female	Single	6 Y	4 Mon	Student	Diploma
P4	25	Female	Single	3 Y	1 Mon	Unemployment	Bachelor
P5	40	Female	Married	3 Y	-	House Wife	Diploma
P6	61	Male	Married	2 Y	2 Mon	Retired	Bachelor
P7	59	Male	Married	1 Y	-	Retired	Elementary School
P8	50	Female	Married	1 Y	1 Mon	House Wife	High school
P9	39	Female	Single	10 M	1 Y	Retired	Master of Science
P10	27	Female	Single	5 M	-	Unemployment	Diploma
P11	56	Female	Married	-	3.5 Y	House Wife	Elementary School
P12	32	Female	Married	-	2 Y	House Wife	Diploma
P13	51	Male	Married	-	17 Y	Retired	Diploma

Grounded on the research data, patients cited two main factors namely inhibitory and facilitating in choosing their therapeutic method. The aforementioned factors were obtained under the title of general categories resulted from the codes, sub-categories, and categories.

Facilitating factors:

Four categories and 10 sub-categories were resulted from the total number of codes and interview analyses. These factors will be introduced by the order of categories.

Table 2: Categories and subcategories

Categories	Sub-categories
Facilitating factors	Family atmosphere
	Hemodialysis disadvantages
	Peritoneal dialysis advantages
	Social atmosphere
Inhibitory factors	Inefficient family
	Requirements of PD
	Attitudes towards hemodialysis
	The treatment system of the country

Family atmosphere

Data analysis in the present study indicated that patients are overshadowed by their family atmosphere for choosing their main treatment method following confirmation of diagnosis. Family atmosphere could enact a binary impact on the acceptance or refusal of PD as the therapeutic method by patients. In the present study, family

atmosphere comprises four sub-categories namely interactions, family structure, cultural level, and life expectancy. Qualitative analysis of data indicated that if the given family has appropriate interactions and internal structure stability, it could be considered as a helpful as well as effective factor for choosing PD as the therapeutic method. Accordingly, knowledge and culture of the family is also assumed to be a significant as well as effective factor, so that it could act as an inhibitory factor when it fails to be insufficiently high. In this respect, one of the patients stated that "*my mother helps me a lot. In 90% of cases she does my affairs, although I can handle my daily work, my mother does not let me do them, she says*".... (P10).

Another patient also suggested that "*my wife did all my dialysis affairs, I could do them, I have no problem, but she extremely helped me. Indeed, she was the person who recommended me to choose this therapeutic method*".... (P1).

Disadvantages of Hemodialysis

The disadvantages of Hemodialysis are considered to be an important category, which enact a highly significant role as facilitator in choosing PD. This category constitutes three sub-categories namely physical problems, psychological problems, and lifestyle disorder. Analyses of the quotes of patients are representative of numerous physical, mental, social and even financial problems of hemodialysis. Most patients and their families are reluctant to choose hemodialysis due to its physical and mental problems; rather, they tend to choose PD as their therapeutic method. In this respect, one of the patients pointed out that "*when I did hemodialysis, I felt extremely lethargic; it was very difficult for me to cope with it. When I returned home, my mood totally changed, I really hate to*"*….* (P9).

Advantages of Peritoneal Dialysis

PD advantages are another significant category included in the general categories of facilitating factors. Analysis of the statements of patients revealed that the present category could play a significant role in choosing the therapeutic method by the patients. This category constitutes two sub-categories, namely welfare and family accompaniment. Patients choosing PD is more compatible with the stress of the respective disease, for the patient could individually handle his/her disease easily and without being hospitalized; the patient may even feel healthy and satisfied with life, and notably, continue with their career. In fact, the patient feels responsible for caring for him/herself. One of the patients observed that "*I feel at ease with PD. I even go out, visit my relatives, go for shopping, and go to parties. Frankly speaking, I have no problem at all, that is*".... .(P13).

Another patient said "*since I have done PD and have stayed at home with my wife and children, I have a good feeling; I never feel that I'm sick*".... (P6).

Social Atmosphere

Currently, there is a low level of recognition and awareness about PD, and hemodialysis is known as an alternative therapeutic method. The results obtained from content analysis indicated that the public has a compassionate look at hemodialysis patients. In fact, the majority of people consider these patients to be frail and weak individuals who excessively need the help of others for their daily life. Most patients well perceive such a feeling, which is why they conceal themselves from the eyes of others, particularly from relatives and acquaintances. The aforementioned issue may fail to be seen in PD patients, and this is one of the reasons it is selected by patients. This category includes two sub-categories namely the perception of the society and sense of ostracism. In this regard, one of the patients stated that "when my relatives, acquaintances and neighbors found that I undergo hemodialysis, their behavior toward me changed completely. When they confronted me on the street, their look was full of pity "....(P5).

Another patient said that "*my friends, relatives, and even the members of my family treated me in a way as if I was a hapless patient. They continuously said that throw 'him' away, we should merely give 'him' service; while 'he' can do nothing for us, irrespective of the costs of 'his' medications* "...(P7).

Inhibitory Factors

This main category constitutes four categories and nine sub-categories. These categories are presented as follows.

Inefficient Family

Inefficient family is one of the effective categories in decision making for treatment. Family conflicts and personal orientations may lead to numerous challenges for the selection of treatment that may lead to delay in patients' decision and lead them toward hemodialysis. This category includes three sub-categories namely inappropriate interactions of the family, limited financial ability, and limited knowledge and culture. One of the patients stated that "*my family had nothing to do with me, they always say that we can do nothing for 'you', as all physicians say 'you should undergo hemodialysis, so do it'. But I consulted with many; I stood against them and chose PD*"... (P10).

Requirements of PD

Analysis of the interview carried out with participants indicated that PD fails to be appropriate for all patients. The need for personal health care, self-care, and other factors related to care and treatment of PD are factors that may require more attention. However, the above-mentioned factors may lead patients to a sense of added responsibility. Correspondingly, in cases where there is a low level of self-confidence and weakness in patients, the sense of self-efficacy may become strengthened in them and subsequently lead the patients toward PD provided that they receive adequate education and family support; otherwise, the patients may tend to choose hemodialysis. This category includes two sub-categories namely self-care and mental challenges.

One of the patients stated that "*PD is a good method but you should highly care for yourself; you should check your bandage, and should highly observe hygiene to avoid infection. Generally, you should be your own nurse and it is better if your family helps you in this regard".... (P3).*

Attitudes toward Hemodialysis

In explaining this category, it must be mentioned that hemodialysis is a traditional method that is widely known and accepted by the majority of people. On the basis of the remarks outlined by patients and therapists, in hemodialysis, toxins and waste products are quickly excreted from the body. Moreover, due to the regular presence of hemodialysis patients in the hospital and their visits with other patients -unlike the condition for PD- they are in close contact with the problems of this group of patients. Furthermore, the unfavorable history of PD in the public mind, such as infections, may considerably affect the refusal of this method by patients. More to the point, according to the emergently clinical condition of some patients with ESRD at the time of arrival to the hospital and health-care centers, hemodialysis is the only possible therapeutic option for them. Additionally, a plethora of hemodialysis centers in cities and a limited number of PD centers (only in provincial centers) consider hemodialysis as the best solution for patients requiring dialysis.

This category includes two sub-categories namely accessibility and pervasiveness. One of the patients asserted that "*hemodialysis units are very large with numerous beds and patients. When I was hospitalized for the first time for dialysis in the upper units, with that excessive population, I never thought there may be another type of dialysis available, or, if any, where it is offered*".... (P6).

Treatment System of the Country

Analysis of the remarks mentioned by participants indicated that ESRD patients referred to different units of healthcare centers fail to receive sufficient information about their disease on behalf of the therapeutic team; even medical students of different levels fail to provide considerable information to these patients. Unfortunately, nurses of non-specialized units fail to considerably help these patients. Most physicians and nurses are somewhat well familiar with hemodialysis. Moreover, the huge number of dialysis units in most hospitals is representative of the preference of the treatment system of the country for hemodialysis. This category comprises two sub-categories namely focus on hemodialysis and financial support to the therapeutic team in hemodialysis.

Discussion

The present study aimed at explaining the factors influencing decision making on choosing PD in ESRD patients in Kermanshah. Attempts were made to identify the factors influencing decision making of patients under PD as well as the factors enacting the role of barriers to their decision.

The findings indicated that the family atmosphere and its different areas appear to be significant factors in decision making on the type of therapeutic method in ESRD patients, particularly in Iran and even the west part of the country where patients receive high support from their families [13]. Different areas of family atmosphere could enact either a facilitating role or inhibitory role in the decision-making process on choosing PD as the therapeutic method. In the present research, different family-based areas including high family interactions, good structure, high knowledge level, up to date culture, and high life expectancy may play a facilitating role. On the contrary, inefficiency of the family, limited financial ability, and limited knowledge and culture may play an inhibitory role in this regard. In different studies, the role of the family in supporting and rehabilitating patients with kidney disease in the selection of their therapeutic method [10] [14] Hope (2013), in his study, investigated the profound impact of the decision-making process on the selection of an appropriate therapeutic method in chronic diseases and the impact of the family on this matter. He also believed that such decision making may provide the best quality of life for the patient [15].

Hemodialysis disadvantages, physical and psychological problems and lifestyle changes other significant factors affecting the decision making of patients. The aforementioned issue has a more considerable role in hemodialysis compared to PD. The analysis results of the remarks mentioned by participants who firstly undertook hemodialysis and subsequently shifted to PD are representative of the aforementioned claim. Hope (2013) also introduced

physical symptoms and the incompatibility of hemodialysis with school attendance or social activities as the prominent problems of this therapeutic method. In their study, Berger et al. (2016) pointed out the amount of life-threatening infections among hemodialysis patients due to application of permanent vascular catheters and manipulations applied on arteries and veins. They also emphasized on selecting PD at the onset of kidney alternative therapies [16]. The present studies showed that physical and psychological symptoms resulting from hemodialysis are assumed to be significant influencing factors in the decision making of patients .

Results indicated that the advantages of PD are another significant factor that could affect the decision making of patients and their families on the type of treatment. Sense of self-confidence induced on the patient, absence of limitations the patient would face in hemodialysis, the patient's ability in doing daily life activities, and granting permission to patients for carrying out social activities are assumed to be highly important and effective factors that emphasized on the high satisfaction of patients and their quality of life. The results presented by some studies also emphasized on the life quality of patients during the PD process [2] [17]. In their study, Rubinsky et al. (2015) also concluded that providing patients with good consultation about self-care in physical, mental and emotional areas could improve their quality of life [18]. Other studies also concluded that PD may lead to satisfaction of patients and their families due to the fact that PD is done daily and more significantly in the home environment [10] [19] [20]. Similarly, in the present study, the authors also found that the home-driven nature of PD and no need to be present at the hospital three times a week is two of the reasons encouraging patients to choose this therapeutic method.

Self-management is another considerable factor in PD selection. The fact of the matter is that, PD, per se, may lead to a sort of self-management in patients. The results showed that self-care ability in patients, education, the hope induced on them, and also the mental support received on behalf of their families, may lead patients to choosing PD and promoting their ability in performing self-care activities in this therapeutic method. Another study concluded that enabling patients in choosing PD and accompanying them during the treatment process are considered to be significantly effective factors [9]. Wang (2013) also emphasized the observation of hygiene and self-care in dialysis patients as an important factor in promoting quality of life [20]. He also concluded that appropriate education and guidance on behalf of nurses and regular planning in terms of self-care activities, and the promotion of patients' knowledge and skills in addition to self-management may promote the quality of life in patients [9] [16] [20].

Results indicated that PD is a caring method in which the patient and his/her family are required to play active roles. Therefore, related requirements could face most patients with fear as well as a challenge. Due to lack of knowledge about the given disease and the expectations of the medical team of self-care activities, a number of patients may suffer anxiety and may face the dilemma whether to choose PD or hemodialysis. In a systematic review study, researchers found that a plethora of studies may focus on the prioritization of problems occurring during hemodialysis and dialysis, self-management ability and prioritizing its levels may be considerable factors in decision making about the type of dialysis by the patient [9]. Another study investigated and compared PD and hemodialysis problems faced also by Japanese patients and demonstrated that they too may experience similar challenges. Considering the Japanese culture where people are accustomed to eliminating problems and difficulties with the help of each other, the members of the family, wife/husband, and relatives supportively work in line with the patient and intervene with the decision of the of the patient regarding the type of treatment [8]. In the Iranian society, the family also plays an extensively considerable role and most patients choosing the PD method have the backing of their family members.

Results of the study indicated that the state of treatment system in our country, widespread hemodialysis units throughout the country and pervasiveness of hemodialysis among people are considered inhibitory factors for the selection of PD by patients. The results presented by Ghaffari et al. (2010) may also reveal the popularity of hemodialysis among people. Moreover, the results presented by studies are representative of the pervasiveness and popularity of this treatment among patients and even the medical team [21].

The existing problems in treatment tariffs for PD and also the treatment system approach of our country toward hemodialysis are considered to be influencing factors in the unwillingness of physicians toward this therapeutic method, finally leading to less encouragement of patients to choose this method.

In conclusion, results indicated that personal, family-related, psychological, social, and economic factors could highly affect the treatment state of patients and the type of dialysis chosen by them. Selection of the type of dialysis, particularly PD in this study was closely related to the family atmosphere, personal perceptions, and financial and social support for patients. Moreover, knowledge and awareness of patients and their families about PD and hemodialysis was considered to be a significant as well as influencing factor. Unfortunately, the enhancement of hemodialysis units and their development in the Medical Education Units (MEU) has led to limited attention and education about PD, and has also led most people and particularly patients to inadequate knowledge of PD. Furthermore, the attitudes of physicians and their advices about the type of dialysis

were highly influencing. In the present study, it was revealed that most patients and their families follow the advices and decisions of their physicians for a variety of reasons. Therefore, appropriate planning in the country and development of the PD unit in treatment, education, and research units, it could be expected that public awareness promotes this therapeutic method so that more patients will choose the method based on their power and family status.

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Case Report

Brucellosis as the Cause of Non-Viral Bacterial Hepatitis: A Case Report

Bilger Çavuş[1*], Özge Çaydaşı[2], Ahmet Aktan[3], Ercan Ayaz[3]

[1]Bingöl State Hospital, Gastroenterology Department, Turkey; [2]Bingöl State Hospital, Infectious Diseases, Turkey; [3]Bingöl State Hospital, Radiology Department, Turkey

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***Correspondence:** Bilger Çavuş. Bingöl State Hospital, Gastroenterology Department, Turkey. E-mail: dr_bilgercavus@yahoo.com

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Abstract

BACKGROUND: Brucellosis can lead to different clinical pictures such as hepatomegaly, granulomatous liver disease, hepatic abscess, and it can cause mild hepatic transaminase elevation in the laboratory investigations.

CASE REPORT: We found out that the etiologic agent was Brucella in our two cases admitted with acute hepatitis presentation in the investigations conducted. Liver transaminases were as follows for case 1 and case 2; AST: 306/187U/L, ALT: 368/312U/L, ALP: 355/264U/L, GGT: 116/197U/L, LDH: 887/549U/L, respectively. Sacroiliitis also accompanied the clinical picture in our first case. Our patients showed clinical and laboratory improvement with rifampicin, doxycycline, (additional streptomycin for 21 days in the patient with sacroiliitis) treatment.

CONCLUSION: Brucellosis which may manifest as a clinical picture regarding numerous medical branches should be considered in case of acute hepatitis, especially in endemic regions, along with viral hepatitis.

Introduction

Although hepatotropic viruses are more commonly responsible for the aetiology of acute hepatitis, the clinical picture of acute hepatitis may occur during the courses of many bacterial infections. Brucellosis is a zoonotic infection caused by Brucella, a gram-negative coccobacillus, and is estimated to affect about 500,000 humans annually in the world [1]. Brucellosis can manifest itself with very different clinical presentations such as high fever, fatigue, sweating, generalised body pain, arthralgia, depression, and therefore some difficulties may be experienced during its diagnosis [2]. Brucellosis can lead to different clinical pictures such as hepatomegaly, granulomatous liver disease, hepatic abscess, and it can cause mild hepatic transaminase elevation in the laboratory investigations [3] [4]. Brucellosis, especially in endemic areas, should be kept in mind along with viral hepatitis regarding the aetiology of acute hepatitis.

Case presentation

A 55-year-old female patient admitted to the outpatient clinic of internal medicine with the complaints of increasing fatigue, nausea and high fever recently. Her past medical history revealed that she did not have any known chronic disease and that she underwent the hysterectomy due to myoma uteri six months ago. The patient who was dealing with ovine breeding and living in a village was not on any continuous medication. On her physical examination, she was alert, her general status was good. There was an increased tenderness in the epigastric region and right upper quadrant during palpation. The liver was palpated for about 1 cm below the rib. Traube's space was open. Breath sounds were normal bilaterally, arterial blood pressure (BP) was 100/60 mmHg, fever was 37.8^{o}C, S1 and S2 were normal on the auscultation of cardiac sounds. No additional sound and murmur were heard. Tenderness was detected in the left sacroiliac region. The patient had increased fever episodes reaching up to 38^{o}C during clinical follow-up. Her blood and urine cultures were

collected during these febrile periods. In the laboratory investigations, the hemogram showed WBC: 5000 mm^3, HGB: 12.7 g/dL and PLT: 184000/mm^3. The biochemical parameters were as follows; CRP: 1.89 mg/dL (upper limit: 0.08 mg/dL), AST: 306 U/L, ALT: 368 U/L, ALP: 355 U/L, GGT: 116 U/L, LDH: 887 U/L, Total Bilirubin: 0.4 mg/dl, creatinine: 0.78 mg/dl, albumin: 3.8 g/dl. Of the coagulation tests, INR was 1.21, prothrombin time was 13.7 s. The viral serology of the patient whose complete urine analysis revealed no pathological finding was as follows; HBsAg: Negative, anti HbcIgM: negative, anti HAV IgM: negative, anti HCV: negative, antiHIV: negative. The craniocaudal length of the liver was found to be increased in the abdominal ultrasonography. The Rose Bengal test requested from the patient who was dealing with breeding resulted in positive. It was found to be positive at the titer of 1/1280 in the Brucella tube agglutination test. Brucella spp grew in the blood culture. Apart from the tricuspid insufficiency in a trace amount, transthoracic echocardiography revealed no pathological finding. The sacroiliac MRI requested due to the tenderness of the patient in the left sacroiliac region revealed the finding consistent with sacroiliitis.

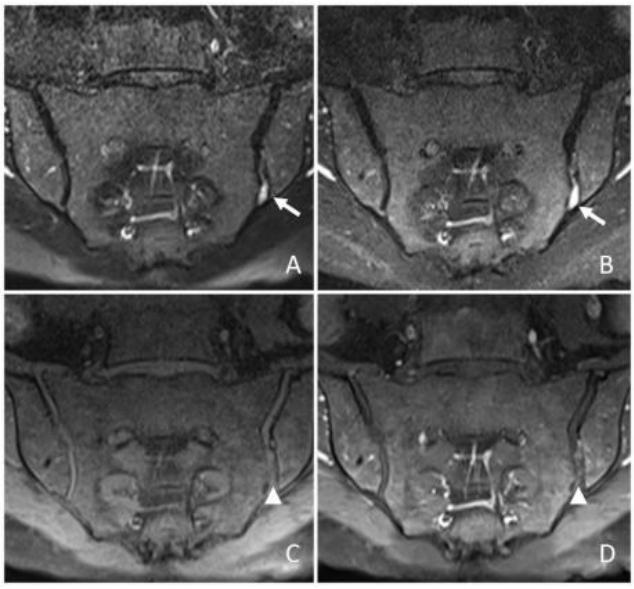

Figure 1: Coronal oblique fat-suppressed T2-weighted (A) and coronal oblique STIR; (B) MR images showed the left sacroiliac joint effusion (arrows) at the lower level; Coronal oblique fat-suppressed T1-weighted MR images obtained before (C), and after (D) the administration of paramagnetic contrast medium showed enhancement (arrowheads) at the left sacroiliac joint consistent with synovitis. There was no bone marrow oedema and joint irregularity

Doxycycline 100 mg 2 x 1, streptomycin 1 g 1 x 1 IM, rifampicin 300 mg 1 x 2 were initiated for the patient. Doxycycline, rifampicin treatment of the patient was given for six weeks while streptomycin treatment was given for three weeks. Improvements were observed in the clinical and laboratory findings of the patient's follow-up.

A 53 -year-old male patient was admitted to the outpatient clinic of internal medicine with the complaints of pain in the right upper quadrant for ten days. The patient who did not have a history of chronic illness and did not have any history of drug use did not have complaints such as fever, weight loss, nausea and vomiting. On the physical examination of the patient, he was alert, cooperated and his general condition was good. Bilateral breath sounds were normal; BP was 130/70 mmHg, S1, S2 were normal during the auscultation of heart sounds, no additional sound and murmur were heard. Tenderness was detected in the right upper quadrant of the abdomen. The liver could be palpated up to 2 cm below the rib. Traube's space was closed laterally. In the laboratory investigations of the patient, hemogram showed that WBC was 4600 mm^3, HGB was 14.5 g/dL, PLT was 220000/mm^3. Sedimentation was observed to be 16 mm/h. The biochemical values were as follows; AST: 187 U/L, ALT: 312 U/L, ALP: 264 U/L, GGT: 197 U/L, LDH: 549 U/L, Total Bilirubin: 0.5 mg/dL, creatinine: 1.16 mg/dL, albumin: 4.4 g/dL. INR was found to be 1.19. The viral serology of the patient whose complete urine analysis revealed no pathological finding was as follows; HBsAg: Negative, anti HbcIgM: negative, anti HAV IgM: negative, anti HCV: negative, antiHIV: negative. Although the patient had back pain, the sacroiliac MRI of the patient in whose physical examination no pathology was detected was observed to be normal. The Rose Bengal test of the patient who was found out to deal with breeding in the village he lived and that he had relatives being treated due to Brucellosis was positive. The Brucella tube agglutination test was positive at the titer of 1/320. No growth occurred in the blood culture. Doxycycline 100 mg 2 x 1, rifampicin 300 mg 1 x 2 were initiated for the treatment of the patient. An improvement was observed in the tenderness of the right upper quadrant after two weeks, and the liver transaminases were observed to be normal at the end of the patient's treatment which was completed at six weeks.

Table 1: Laboratory findings in the cases with brucellosis

	AST/ALT U/L	ALP/GGT U/L	LDH U/L	T.Bil/D.Bil mg/dL	INR/PTT(s)	WBC/NEU	HGB g/dL	PLT	CRP (mg/dL)
CASE 1 Admission	306/368	355/116	887	0.6/0.25	1.21/14	5000/2800	12.7	184000	1.89
CASE 1 Treatment Second week	79/119	267/104	633	0.3/0.1	1.17/13.5	6400/3500	12.2	410000	1.16
CASE 1 End of Treatment	22/34	180/98	330	0.4/0.1	1.14/13.1	6600/3300	13.1	300000	0.27
CASE 1 Treatment protocol	Doxycycline 100 mg 2 x 1, Streptomycin 1 g 1x1 IM, Rifampicin 300 mg 1 x 2 were initiated. Doxycycline, Rifampicin treatment for six weeks, Streptomycin treatment for three weeks								
	AST/ALT U/L	ALP/GGT U/L	LDH U/L	T.Bil/D.Bil mg/dL	INR/PTT(s)	WBC/NEU	HGB g/dL	PLT	CRP (mg/dL)
CASE 2 Admission	187/312	264/197	549	0.5/0.2	1.19/13.4	6000/4300	15.3	221000	1.45
CASE 2 Treatment Second week	106/298	162/129	253	0.4/0.2	1.16/13.4	4500/2.42	14.2	213000	1.12
CASE 2 End of Treatment	27/45	109/85	300	0.4/0.2	1.09/12.5	7000/4600	16.3	204000	0.6
CASE 2 Treatment protocol	Doxycycline 100 mg 2 x 1, Rifampicin 300 mg 1 x 2 was completed in six weeks.								

Discussion

The incidence of brucellosis, a zoonotic infection considered to affect 500,000 humans annually in the world, is reported to be about 13 in a hundred thousand in our country [5] [6]. Brucellosis, which can manifest with many different clinical presentations, can relate to different scientific branches such as infectious diseases as well as internal diseases, orthopaedics, physical therapy, psychiatry, urology and gastroenterology. Mild transaminase elevation is reported during Brucellosis in 25% of the cases [3]. We observed that liver transaminases were 5-8 times higher than the normal upper limit in two cases that we presented. In a study conducted by Cervantes F. et al. in which liver involvement of brucellosis was assessed in 40 patients, the mean serum AST and ALT levels were 1.5 times higher than the normal upper limit (NUL), and the mean alkaline phosphatase (ALP) was found to be 158U /L [4]. In a study conducted by Gursoy et al. in which they examined 140 patients, mild transaminase elevation was detected in 27.9% of the patients whereas transaminase elevation was detected in 25% of 60 patients with brucellosis in a recent study by Isılak Demir M. et al., [7] [8]. Although mild transaminase elevation is frequently encountered during brucellosis, we obtain our data on moderate-high levels of transaminase elevations rather from the articles published as case reports. Denk A. et al. found that AST was 771 U/L, ALT was 471 U/L, and total bilirubin level was 2.61 mg/dL in a brucellosis case with acute hepatitis and bicytopenia [9]. The bilirubin levels in both of our cases were normal while hepatic transaminases were elevated at acute hepatitis levels and also, lactate dehydrogenase (LDH) levels were observed to be elevated 7-8 times higher than NUL in both of our cases, indicating that this may be primarily associated with increased cell destruction by Brucellosis, which is an intracellular bacteria. Although ALP and LDH levels were 4 to 8 times higher than NUL in both cases, both enzyme levels were higher in our case with sacroiliitis. The treatment of our patient with sacroiliitis and growth in blood culture was arranged as doxycycline 100 mg 2 x 1, rifampicin 300 mg 1 x 2 and streptomycin 1g 1 x 1. The treatment of our second case was completed as doxycycline 100 mg 2 x 1, rifampicin 300 mg 1 x 2 for six weeks. No drug-related hepatotoxicity occurred during the treatment of both cases, clinical and laboratory cure was achieved in both patients.

In conclusion, brucellosis which may manifest as a clinical picture regarding numerous medical branches should be considered in case of acute hepatitis, especially in endemic regions, along with viral hepatitis.

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Case Report

The Novel Surgical Margin for One Step Melanoma Surgery (OSMS) (Without Using Ultrasonography Preoperatively): The End of Conformity! "Vivere militare est!"

Georgi Tchernev[1,2*], Ivanka Temelkova[1]

[1]Medical Institute of Ministry of Interior (MVR), Department of Dermatology, Venereology and Dermatologic Surgery, General Skobelev Nr 79, Sofia, Bulgaria; [2]Onkoderma, Policlinic for Dermatology and Dermatologic Surgery, General Skobelev 26, Sofia, Bulgaria

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***Correspondence:** Georgi Tchernev. Medical Institute of Ministry of Interior (MVR), Department of Dermatology, Venereology and Dermatologic Surgery, General Skobelev Nr 79, Sofia, Bulgaria; Onkoderma-Policlinic for Dermatology and Dermatologic Surgery, General Skobelev 26, Sofia, Bulgaria. E-mail: georgi_tchernev@yahoo.de

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Abstract

BACKGROUND: Innovations in medicine are often due to the simplicity of a certain activity, interaction, even counteraction, or a mistake leading to a subsequent final optimal outcome. Innovations could also be due to conclusions based on targeted clinical or sporadic, as well as completely random observations. The genius of an approach or statement is often based on the “iron logic”, which in turn is based on irrefutable data or facts. These are often observations or results from actions that happen right before our eyes and provide advantages or prerequisites for the better future development of things (in this case, disease) concerning certain groups of people (in these case-patients). When the clinical results achieved following an inevitable introduction of certain methods or innovations speak eloquently of a number of advantages in terms of 1) spearing effect on the patients, 2) better control or prevention of possible local and/or distant metastatic spread 3) better financial balance for the health institutions and patients, ..., then even the "Gods of certain latitudes" should be silenced. We at this moment present a completely new method or approach for surgical treatment of cutaneous melanoma that once again proves the effectiveness of one-step melanoma surgery, which was successfully first officialised in the world literature again by the Bulgarian Society of Dermatologic Surgery, (BULSDS). In some cases, this method does not even require the preoperative use of a high-frequency ultrasound for determining the tumour thickness.

CASE REPORT: In patients with advanced stage of cutaneous melanoma, removal of a primary draining lymph node and/or locoregional lymph nodes is often performed simultaneously. However, it remains unclear why in patients with early-stage (or intermediate, with moderately thick melanomas) disease high-frequency ultrasound is not applied as a routine method of determination of tumour thickness? Meanwhile, re-excision is required following histopathological verification? Is it necessary to have 2 surgical interventions? The two surgical interventions are a burden for the patients and create prerequisites for contradicting opinions, statements, and subsequent results, which ultimately slows down the patient’s staging and the introducing more precise treatments. Based on the logic (and further aided by the clinical picture and dermatoscopy), we decided to operate selected cases of patients with cutaneous melanomas with a field of surgical security of 1cm in all directions when clinical, and dermatoscopic data are indicative of melanoma in situ or thin melanomas (less than 1 cm). Optimal results were achieved, with one surgical intervention and subsequent rehospitalisation spared for the patient.

CONCLUSIONS: An answer to the question whether it is better not to follow the guidelines strictly (since, as a rule, they are generally recommended and somewhat misleading in certain circles of specialists, and as we have already found, also lead to unjustified logical secondary excisions), or update them at least annually when data for better tumor control is available (using a new method such as the one we mentioned above), should be searched for. This is a method not derived from AJCC/USA or other similar/equal or equivalent organisation’s “recesses”! Acceptability of innovations depends to a large extent on the latitude or territory where they originated?! Something that should be changed! Or in other words, something that has already been changed! The End of Conformity, and the beginning of a New Era!

Introduction

The melanoma surgery is based on certain guidelines according to which the main therapeutic steps are: 1) primary excision of melanoma with margins of surgical safety of 0.5 in all directions, 2) histological examination of the removed lesion and determination of the tumor thickness according to the Breslow method, on the basis of which follows 3) determination of the necessity of reexcision with extended margins of surgical safety with or without removal of the draining lymph nodes [1]. The current recommendations of the American Academy of Dermatology are: for melanoma in situ excision with margins of safety from 0.5 -1.0 cm in all directions; for

tumor thickness <1 mm, 1.0 cm in all directions, by thickness 1.01 and 2.0 mm: 1.0-2.0 cm and by tumor thickness >2 mm: 2.0 cm (1). This variability in the centimeters of surgical safety (0.5-1.0 cm for melanoma in situ and 1.0-2.0 cm for tumor thickness between 1.01 and 2 cm) can be often confusing for the clinician and often this comprehensiveness or to some extent definitiveness (without specification or personalization depending on the clinical and dermatoscopic characteristics of the lesion) leads to the loss of the individual approach towards the patient [2]. One step of melanoma surgery is an approach which corrects this drawback of the guidelines and considers each patient as an individual set of characteristics [3].

Case report

We present a sixty-three-year-old patient in good general condition. He is admitted to the clinic for the first time due to surgical removal of a melanocytic lesion located in regio abdominalis sinistra and many year duration. From the dermatological examination was established the presence of oval hyperpigmented lesion (Figure 1a). This discovery met the requirements for a malignant melanocytic lesion clinically and dermatoscopically. Additional tests were performed and from the paraclinic was noticed the light form of dyslipidemia and PSA- 13.900 (< 4.0). The apparat diagnostic was without indication for process dissemination.

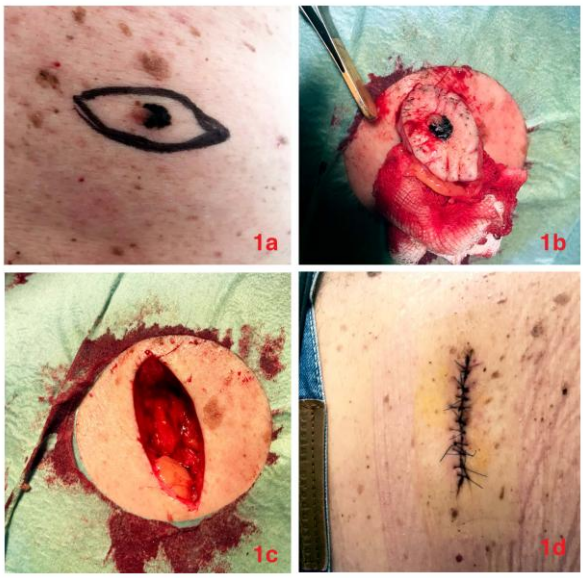

Figure 1: a) Clinical manifestation of melanoma and preoperative surgical skin marking with 1.0 cm filed for safety in all directions; b, c) Elliptical surgical excision of the lesion under local anaesthesia; d) Wound closure with simple interrupted sutures

A conversation with the patient about the advantages and disadvantages of the one step melanoma surgery was conducted, and accordingly, preoperative informed consent of its execution in outpatient conditions was signed.

The suspected for malignant melanoma lesion was radically removed under local anaesthesia (Figure 1 b, d). By clinical and dermatoscopic data (indicative for melanoma under 1mm), the operation was performed with a field of surgical safety of 1cm in all directions (Figure 1 b, c). The closure of the surgical defect followed via an expandable plastic (Figure 1d). The histological test confirmed the originally clinically and dermatoscopically set diagnosis: melanoma in situ, clear resection lines.

Discussion

In that case, if the therapeutic approach was based on the current guidelines, the treatment should start with a primary excision of the melanocytic lesion with a field of safety of 0.5 cm in all directions followed by postoperative measurement of the tumour thickness [4]. Depending on the histologically established thickness, during the so-called second stage, an assessment of the reexcision necessity with othe r without removal of the draining lymph nodes has to be performed [4]. In the case of our patient, the decision for only one initial excision of 1cm in all directions was taken by clinical experience and dermatoscopic data. Both indicated malignant melanoma < 1 mm. The described case is a vivid consecutive example of how, with only one surgical intervention, diagnosis and surgical treatment are achieved simultaneously without the patient is subject to a second surgical session [3].

In given cases, the determination of the surgical margins can be supported by preoperative ultrasonographic examination of the tumour thickness [5]. This should not be a prerequisite by patients with thin melanomas (determined by definitive clinical and dermatoscopic criteria), and in that case, the many year's clinical experiences are enough. Although one step melanoma surgery does not follow the guidelines, it provides a radical removal of the melanoma via adequately respecting the surgical safety margins for the respective tumour thickness by considering the recommended by the guidelines resection fields [6].

The main advantages of this one-stage model are 1) melanocytic lesion removal with one operative (surgical) session, instead of two, in cases of thin melanomas which inevitably 2) decreases the risk of complications which every additional surgical intervention contains and 3) leads to significant financial relief.

It should be noted though that this method is

applicable only to certain groups of patients. This fact should be considered critically and individually assessed for each patient.

Figure 2: a), b) The conformism leads to depersonalization and loss of individualisation. When the respective person does not understand the group's ideas but notices that the latter has an influence, then that person seeks to join it. The reason for this affiliation is the most often the fear of isolation and the lack of competence on certain issues or topics. Uniting with the group solves this problem and the individuals in question enter their area of comfort as they do not want to be disturbed. They make themselves equal (I would say them descent too) with the group's ideas and problems only within the pseudo-identification. They attend congresses and have 20 years of work experience, but they are not aware of what the fields of surgical security in melanoma patients are or should be!? They nod assent approvingly on themes that are totally unclear to them!? They make presentations on topics they do not understand. They strive for the group to solve all the problems. They implicitly obey. This is the so-called or the "flock" in question. This situation is analogous and identical in medicine, politics and the social sphere. That is why we use it as a model for determining a given individual in a given situation and under certain conditions. The conformism is a 100% model for problem generation but also a model within which we find solutions too! And we present them in "all their glory"!; c) Fluctuations and differences are not tolerated if they are noticed. The reason is that they can break the status quo and cause turmoil, doubt, and so on. And the turmoil creates anxiety, revolution, and dissent. The turmoil leads to the provocation of questions, and hence inevitably explanations should also be given. The turmoil leads to a loss of capital in certain lobbies. And this eventually ends up remodelling the "artificially created landscape!" "To become sober from the heavy hangover!". To the public shame of the revealed unconventional approaches that have been applied by the recent references of imitation. Therefore, it must be suppressed and destroyed. The turmoil is harmful; d), e) At the basis of conformism is the manipulation, brainwashing, and the intrusion of the intrusive ideas, that you feel good, that you are most appreciated that it is also so for all your friends and relatives and that you should be pleased. But actually, you live in a pseudo-realism, you have pseudo-desires, have pseudo-ideas (knowing nothing about them), do pseudo-actions (not knowing what they will bring), pseudo-family, pseudo-sensations, even pseudo-therapy? And all the desires and messages directed to you have one, though a "slightly or softly transmitted" form of order, which is meant to "pacify and tame" you! To put you into the "right order" of the law or the guideline! Given from the top" finely and elegantly, or a little bit "roughly and insensitively". But instructive and threatening! Or in the form of party assemblies, secret societies, international guidelines, symposia, congresses, dinners, romantic walks in Venice or the Greek islands, etc.? However, the stimulation of independent thinking is again strictly prohibited. It is defined as risky. That is why it should be turned in the right direction

It is not clear why, for the time being, these innovative opportunities are overlooked? It is not the good perception of innovations (but namely the lack of any arguments available against them) that would determine to a certain extent the current surgical approach conducted in melanoma patients (albeit it is too extreme as a definition) to a certain extent as „populistic, unconsidered and deliberate".

The conformism is (sickness and weakness) spread not only in politics, fashion and the social sphere but also in medicine. The human desire to perceive him/herself, or to be falsely accepted or perceived as a part of a large, a whole, „complete society", not to be isolated, misunderstood, ignored-this is the perpetual generator of energy for the conformism (Figure 2) [7] [8] [9]! This gives rise to and "feeds" the conformism, or otherwise said: our fears lead us to the loss of our individuality and identity:" To be afraid to be wrong! Not to break the "basic, accepted norms" that are created by someone and for a certain purpose? In order not to be isolated and understood in a wrong way?! (Figure 2c)".

It could be summarised as follows: "When you have nothing to offer when you are afraid to be different then you are allowed to be cowardly, to have no face and go against yourself (Figure 2 a, b). You're even allowed to put a bell on your neck and to shout. You are allowed to be conformist and to be a part of the society which probably does not care for you, as long as you are quieter than the grass. The advantage is that you are allowed to graze from time to time (Figure 2 a)".

It is a paradox that even the sheep beware themselves of the wolf for all their life but are slaughtered and eaten by the shepherd? The one who supposedly takes care of them? And preserves them from the monsters' evil (which 98% of them will never even meet)? That's the idea-not to be eaten by the wolf. Finally, however, they are eaten by the shepherd, their guardian!

Isn't it analogically in medicine-the question is only rhetorical.

If we optimise the baseline positions for diagnosis and treatment of melanoma, ...if we smooth out the possibilities for a wrong or inadequate primary approach? Then an enormous number of patients would not reach a terminal stage where the means for innovative treatments are sometimes out of control? Out of limits? Out of reason?

The individualisation of the therapy in the early stages of melanoma should be our duty and priority as clinicians. The rough arithmetic indicates that a change of the baseline conditions or the treatment rules for melanoma would result in an inimitable optimisation of amounts (grossly or grosso modo) of no less than 300 to 500 million dollars (worldwide). For some units of the "food chain", these would not be advantages (saved fates), but simply-losses! We are letting readers guess who they are! These are the ones, who would be interested in the pharmaceutical industry to flourish and to take care of carefully prepared innovations!

Is it also interesting what feeds the conformism? The manipulations of the people around us (mainly), the inferiority complex within us (a necessary condition), the weakness of our characters (often an available condition), the fear of being ourselves (often an available condition)? And in fact, it turns out that our differences, ... this is our biggest advantage? Differences, this is the progress! "Vivere militare est"-"To live means to fight!".

Since ancient times, the progress has been made with fire and sword (Figure 3 a, b, c)! The lack of acceptability and discutability of the proposed innovation necessitates their application (as if you are certain in your actions-you do not wait for approval), which is successful as already shown by us. And when the results are indicative, then also the “gods” are silent! That is why the one step of melanoma surgery is successful. It is because of this-it takes place without the need for the consent of certain circles and lobbies. That is why we meet the silence of these circles, but also the smiles of the satisfied patients!

Figure 3: a), b) and c) Duplicity and manipulation are characteristic of human traits. They have accompanied humanity since prehistoric times and have been the cause of countless wars and losses of human lives. Unfortunately, even today, we are again in a continuous state of war as well as a complete information blackout. The reason is again in the fact that the changes and the different thinking give rise to the revolutions. They should not be allowed, although they provide or could provide benefits to certain groups. Manipulation and disinformation are the main cause of the decay of the society and human civilisation as a whole. A civilization that does not learn from its mistakes! A civilization that does not allow the innovation unless it originates from certain circles or is refracted through certain prisms.Regardless of the fact that this would cost human fates. That is why innovation in all spheres should not be subject to control. But to debates, proof and quick perception. However, the important thing is: Innovations can be carried out without the consent of certain circles, whether it is “the Pacific influences” or “Some Western European trends!” But then the first step should be made by ourselves. And then - we should not expect approval from the “strongest of the day” or from the “pseudo-reality”! We just have to be ourselves: “Vivere militare est!”

We must not forget that the conformism, aided by the manipulations of the people around us or those surrounding us, and the “flock mental make-up” -these are the factors that are mutually potentiated, are stuck in a diabolical circle and are inevitably linked with severe consequences to us and to others (especially in medicine), namely: 1) the loss of individuality, 2) the inability to progress in general, and sooner or later, though it sounds cruel, selfish and extreme ... and 3) the loss of a human life (Figure 2d).

The ability to simplify means to eliminate the unnecessary so that the necessary may speak (Figure 4a). This is the reason why we have opened the way for one step melanoma surgery (OSMS) for our patients. Because simple ideas as the OSMS could solve (in a high per cent of the cases) complex problems (Figure 4b).

Figure 4: Simplicity and geniality? Probably connected?

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Case Report

Forced Diuresis and Expedient Blood Pressure Control in the Management of Quetiapine Induced Neuroleptic Malignant Syndrome: A Case Report

Neville Aquilina*, Vincent Bugeja

Karen Grech Rehabilition Hospital - Geriatrics, Pieta, Malta

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***Correspondence:** Neville Aquilina. Karen Grech Rehabilitation Hospital - Geriatrics, Pieta, Malta. E-mail: nevaqui@gmail.com

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Abstract

BACKGROUND: This case report intends to highlight the importance of safeguarding renal function from rhabdomyolysis in neuroleptic malignant syndrome (NMS) by concomitant administration of parenteral fluids at a high rate together with high doses of parenteral loop diuretics (we utilised 6 mg bumetanide daily) and tailed over a few days, in order to preserve glomerular/renal medullary perfusion and nephron function.

CASE REPORT: This case describes an elderly lady previously diagnosed with Lewy body dementia who had been started on low dose quetiapine a few days previously and presented with an acute 24 – 48 hour onset of fever, generalised stiffness, rapidly becoming uncommunicable and with high blood pressure. Haemoglobinuria was present prompting intravenous treatment with labetalol to address the BP, whereas rapid isotonic saline fluid infusions together with intravenous high dose bumetanide were instituted to safeguard the kidneys against damage due to nephron deposition, both from haemoglobinuria as well as possibly myoglobin from rhabdomyolysis. A working diagnosis of the neuroleptic malignant syndrome with secondary malignant hypertension was made, and the quetiapine withdrawn. Blood pressure was after that subsequently controlled on amlodipine, and the haemoglobinuria quickly settled within 24 hours, with large amounts of dilute urine being passed on account of the forced diuresis. The fact that renal function and creatinine kinase remained normal is testimony to how these expedient measures averted progression to both rhabdomyolysis and renal failure in this case, thereby ameliorating prognosis.

CONCLUSION: The patient was kept on infused fluids with maintenance bumetanide alone, achieving a full clinical recovery within the following 3 days.

Introduction

This case report intends to highlight the importance of safeguarding renal function from rhabdomyolysis in neuroleptic malignant syndrome (NMS) by concomitant administration of parenteral fluids at a high rate together with high doses of parenteral loop diuretics (we utilised 6 mg bumetanide daily) to preserve glomerular/renal medullary perfusion and nephron function, thereby encouraging a good urine output. It also reminds us of how blood pressure (BP) may rise inordinately in NMS and that in this situation rapid control is imperative, since high BP not only acts synergistically with rhabdomyolysis to cause renal damage but may lead to other complications, as in our case by setting up a microangiopathic intravascular haemolysis, the latter leading to haemoglobinuria which adds further insult to the nephron.

We also need to have a very low threshold of suspicion for NMS in the elderly, particularly those with degenerative neurological disease processes, who, because of their low central nervous system reserve, remain eminently susceptible to its' development.

Case Report

An 81-year-old lady with Lewy body dementia (LBD) and only on bendroflumethiazide 2.5 mg for well-controlled mild hypertension was admitted under our care, where she had been started on low dose quetiapine at 25 mg daily for behavioural symptoms one week before. Unable to volunteer any coherent history, she was noted to be rather apathetic, albeit responsive when prompted, with mild hypertonicity in

the left arm, in keeping with her LBD diagnosis. Four days later she was found febrile at 41°C, severely obtunded, completely and symmetrically stiff all over from muscular rigidity, tachycardic at 120 beats per minute and tachypnoeic at 33 breaths per minute. Other vital signs included 89% oxygen saturation on room air, while her blood pressure (BP) was around 300/160 mmHg. Upon catheterisation, the urine was clear but had a crimson hue, resembling cranberry juice. Bedside dipstick urine testing with a reagent strip immediately registered full blood, with high bilirubin, moderate protein and ketones, but negative nitrites, no leucocytes and no glucose, while the pH was 6.

Formulating a working diagnosis of the neuroleptic malignant syndrome (NMS) the quetiapine was withdrawn immediately and we set up a running intravenous (IV) saline infusion, after about one hour we administered a 6 mg bolus of bumetanide IV. The idea behind this forced diuresis being to allow the nephron to flush out any toxic products from erythrocyte and skeletal muscle breakdown. Such an intervention was intended to avert progression to renal failure, considering the very real possibility of rhabdomyolysis, compounded by the present danger constituted by intravascular haemolysis from microangiopathic haemolysis, which may be attributed to the acutely and inordinately elevated BP. High flow oxygen, was administered whereas BP was addressed via labetalol 50 mg by slow IV infusion. She was kept on IV bumetanide at 6mg daily in three divided doses while 0.9% isotonic saline infusion at a high rate (150 mLs/hour via infusion pump) was administered. Her urine output was monitored and noted to remain above 50 mLs/hour at all times.

Table 1: Laboratory findings

Blood index	*Baseline*	*Day 1*	*Day 3*	*Reference range*
White cell count (x 10^9/L)	6.4	6.7	7.5	3.5 – 11
Haemoglobin (g/dL)	9.9	10.1	10.2	11.5 – 16.5
Platelets (x 10^9/L)	227	212	217	140 – 400
Urea (mmol/L)	9.7	8.6	8.1	1.7 – 8.3
Creatinine (µmol/L)	91	82	79	44 – 80
Sodium (mmol/L)	141	139	138	135 – 145
Potassium (mmol/L)	4.7	4.3	4.1	3.5 – 5.1
eGFR (mLs/min/ 1.73 m^2)	55	61	62	
Creatinine kinase (IU/L)	104	97	99	26 – 192
Lactate dehydrogenase (IU/L)	476	337	269	140 – 280
Bilirubin (µmol/L)	35	30	27	1.72 – 17.1
Alkaline phosphatase (IU/L)	66	60	61	40 – 104
Gamma-glutamyl transpeptidase (IU/L)	23	21	24	5 – 36
Aspartate transaminase (IU/L)	17	19	17	5 – 40
Alanine transaminase (IU/L)	19	22	21	10 – 35
Haptoglobulin (mg/dL)	38	41	43	45 – 165

Following the parenteral labetalol infusion, her BP stabilised at 160/90 mmHg, and we instituted the dihydropyridine calcium channel blocker amlodipine at 5mg daily for maintenance control of blood pressure. The following day all parameters remained stable, her BP fell to 145/85 mmHg, and the IV fluids were decreased to a slower 12 hourly rate (84 mLs/hour); she continued to improve clinically, registering a full recovery of mental status to the premorbid baseline within 4 days. Subsequently, we kept her on IV 0.9% saline alternating with 5% dextrose for 5 more days, following which they were withdrawn, meanwhile, the bumetanide was tapered to 2 mg daily for maintenance treatment.

Discussion

The autonomic lability associated with NMS accrued an extremely high blood pressure (BP). In this state, damage from elevated hydrostatic pressure to the intima of arterioles causes fibrinoid necrosis within the arteriolar wall, itself triggering off the intrinsic coagulation cascade, which, in turn, results in the formation of fibrin strands, straddling the lumen like a cobweb. These slash and traumatise bypassing red blood cells (microangiopathic haemolysis) resulting in free haemoglobin which, following filtration through the glomerular sieve, impart this characteristic colour to the urine observed. Moreover, the serum haptoglobin level came back low, together with elevated lactate dehydrogenase (LDH), consistent with an intravascular haemolytic process. However, this resolved fairly quickly with the LDH returning to normal levels within 3 days.

A pre-emptive measure to prevent acute nephrotoxic tubular necrosis by induction of forced diuresis was successful, the aim of this being to wash away any haemoglobin and any myoglobin crystals that accumulate in the nephron tubule lumen. This not only discourages their precipitation out of solution, thereby safeguarding luminal patency, but also reduces contact time of these toxic products with the basolateral tubule membrane, insofar that damage is minimised and a rapid re-establishment of physiological exchange mechanisms is promoted. Prognosis in NMS worsens significantly with the onset of renal failure. In our case, the pigmented urine cleared within 24 hours of starting the rapid saline infusion and blood pressure responded well to the nonspecific α/ß blocker labetalol, also readily returning to within normal range following its administration. All this, together with the fact that the serum creatinine kinase (CK) failed to rise, are positive prognostic indicators that rhabdomyolysis has been effectively averted and bode well for a quick and full recovery.

Autonomic instability is inherent to NMS insofar that both low and high blood pressure (BP) may occur; indeed there may also be rapid fluctuations in it throughout this state. However, in our case, the BP was sufficiently high to present an additional clinical adverse factor (which, if left unaddressed, may have led to malignant hypertension) being severe enough to have already initiated microangiopathic haemolysis (as attested by the low haptoglobin and isolated hyperbilirubinaemia), with subsequent haemoglobinuria (highly positive blood marking on the urine reagent strip) presenting the real possibility of compounding any nephron damage in the event of

rhabdomyolysis. Such a high BP risked causing other macrovascular complications such as acute stroke, myocardial infarction, retinal disease and acute renal failure. It followed that aggressive control of this added complication must be taken as seriously as the management of the NMS itself and was indeed crucial to ensure a positive outcome. On the other hand one must also be weary of low BP in NMS (in view of the autonomic lability) such that close BP monitoring is mandatory, so that drops in the BP may be countered with increased IV hydration to safeguard end organ perfusion (whilst withdrawing any BP lowering medication).

Literature review

Second generation antipsychotics (SGAs) or atypical antipsychotics have been known to result in correspondingly 'atypical' NMS where the classical features are altered. Traditionally NMS has been postulated to originate from central dopaminergic D1 and D2 receptor blockade, leading to muscle rigidity and thermogenesis. In fact frequency of NMS incidence was found to be proportional to the potency of dopamine blockade in the mesocortical, mesolimbic, nigrostriatal and hypothalamic tracts [1]. However atypical antipsychotics function more via serotonergic 5HT and adrenergic receptor blockade, bearing little or no central antidopaminergic activity. Quetiapine has been demonstrated to produce an NMS variant where autonomic manifestations (tachycardia, tachypnea, diaphoresis, BP fluctuations) seem to predominate over extrapyramidal symptoms (EPS); this has been attributed to the noradrenaline reuptake inhibition, histaminergic antagonism and serotonin toxicity that feature in quetiapine pharmacology [2] [3]. SGA induced NMS (SGA-NMS) has been shown to be somewhat more benign than the first generation antipsychotic (FGA) subtype, carrying a mortality of 3% for SGA, as opposed to 16.3% for FGA induced NMS [4]. However, when outcomes of SGA - NMS were compared amongst the different individual, causative atypical antipsychotics, quetiapine was associated with the highest fatality rate at 7.7%, together with the lowest complete recovery rates (61.5%), despite carrying the least tendency towards EPS [5]. In this latter finding it was comparable only to clozapine where NMS cases occurred in the total absence of hypertonicity or rigidity, however, the other main features of NMS (fever, CK rise, autonomic dysfunction) remain ubiquitous amongst both FGA and SGA induced forms [6] [7] [8].

Review of case reports has demonstrated that SGA - NMS may occur equally with atypical antipsychotic monotherapy, as well as in combination with other psychoactive drugs [1]. Specifically, quetiapine has been implicated in NMS when combined with the likes of venlafaxine [9] [10], fluvoxamine [11], other antidepressants [1] and sulpiride [12]; paradoxically, however, quetiapine has also incurred NMS when associated with muscle relaxant drugs such as benzodiazepines and valproate [1]. Additionally, it has also been reported with lithium [1] and with antiparkinsonian drugs in a case report which led to a fatal outcome [13]. In this respect, the fact that antidepressants, lithium and other mood stabilisers may precipitate NMS, in combination with antipsychotics, led to the hypothesis that the serotonin excess they produce could determine a 'relative hypodopaminergic state' such that this serotonin - dopamine disequilibrium favours NMS [14] [15]. One study even reports onset of NMS purely on the administration of an SSRI (selective serotonin reuptake inhibitor) combination, in the absence of dopamine inhibition [15].

The fact that quetiapine-induced NMS is completely idiosyncratic has been demonstrated in many studies, whereby it has been shown to occur equally with patients on steady-state doses of the drug without fluctuations, as well as when another psychoactive drug is introduced, or with dose titration of quetiapine [8]. Cases had occurred after patients had been many months stable on a fixed dose regimen of quetiapine, who before the onset of NMS had their primary psychiatric condition well controlled and who did not display any previous history of drug-induced EPS [8]. Around 50% of NMS cases occurred with patients on stable doses of SGAs in another study considering all atypical antipsychotics across the board, whilst 60% of those cases of SGA - NMS (including participants on both steady state as well as recently altered doses of atypical antipsychotics) were on some other concomitant psychotropic medication [16]. However, certain epidemiological factors did emerge that effectively rendered one more susceptible to quetiapine-induced NMS [9] [17] [18] namely: males are twice more likely than females, age range of between 20 – 50 years and mental impairment or learning disability. Other pharmacological and pathophysiological factors were also implicated in increasing the likelihood of NMS with patients on antipsychotics in general [19] [20] [21]: history of previous NMS, rapid dose escalations, intramuscular depot antipsychotic injections (particularly upon dose increase), high potency neuroleptics, states of decreased absolute or effective circulating blood volumes, such as dehydration, third spacing, sepsis or other states of shock, and catabolic states such as malnutrition.

Our case description presents underlying cognitive impairment as the sole risk factor; however, we argue that such elderly patients, particularly those with cognitive impairment, remain relatively more susceptible to the development of NMS compared with the general population. This challenges the commonly held belief that NMS occurs mostly in younger schizophrenic patients [22] and that presence of dementia seems to confer a degree of 'immunity' from the syndrome. One must bear in mind that old age brings about with it an increased permeability of the

blood-brain barrier and a lower threshold for neurotransmitter decompensation (lower neural mass, together with a decreased enzymatic degradation of psychoactive drugs and neurotransmitters at the synaptic level); additionally a lower hepatic metabolism rate and renal clearance rate, a lower lean muscle mass and increased fat to water ratio are all pharmacokinetic factors that potentiate the predicted effect of psychoactive drugs [23]. Moreover, Lewy body dementia is notorious for its associated neuroleptic sensitivity, which is potentially fatal and afflicts 50% of LBD patients [24]. As such we believe that this case is a reminder to the clinician that geriatric cases, particularly those with heightened sensitivity to neuroleptics as with LBD, remain eminently susceptible to not only the 'extrapyramidal' or striatonigral manifestations, but also to the other central manifestations of dopamine blockade, as resultant from neuroleptics, with the former giving rise to parkinsonism, whilst the latter to NMS.

In conclusion, we wish to highlight how the strategy adopted above of forced diuresis with expedient BP control seems to be successful in promoting a positive outcome with NMS patients. We also suggest that any elderly who already have some form of extrapyramidal dopamine insufficiency state (idiopathic or drug-induced parkinsonism, LBD, Parkinson plus syndromes) ought to be earmarked for the other central dopamine deficiency complications, namely NMS, thereby lowering the clinicians' threshold of suspicion further in such cases.

Learning points: NMS is often subacute and presents insidiously a low suspicion threshold is needed for NMS in an elderly on antipsychotics who presents with psychomotor retardation or deterioration; cognitive impairment, particularly LBD, presents an additional risk factor for NMS. The usual features of NMS may be lacking in cases induced by atypical or second-generation antipsychotics (SGAs); early treatment is crucial in safeguarding against renal damage where BP must be closely monitored, and both excessively high or low values treated accordingly, since prognosis improves in cases where rhabdomyolysis has been averted or minimized.

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Case Report

A Case of the Co-Existence of Subcorneal Pustular Dermatosis and Pyoderma Gangrenosum and a Review of the Literature

Maria Ferrillo[1], Alessia Villani[1*], Gabriella Fabbrocini[1], Massimo Mascolo[2], Matteo Megna[1], Claudia Costa[1], Maddalena Napolitano[3]

[1]Section of Dermatology, Department of Clinical Medicine and Surgery, Dermatology Unit, University of Naples Federico II, Napoli, Italy; [2]Department of Advanced Biomedical Sciences, Pathology Section University of Naples Federico II, Napoli, Italy; [3]Department of Medicine and Health Science "Vincenzo Tiberio", University of Molise, Campobasso, Italy

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Keywords: Subcorneal pustular dermatosis; Pyoderma gangrenosum; Sneddon-Wilkinson disease; neutrophilic dermatoses

***Correspondence:** Alessia Villani. Section of Dermatology, Department of Clinical Medicine and Surgery, Dermatology Unit, University of Naples Federico II, Napoli, Italy. E-mail: ali.vil@hotmail.it

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Abstract

BACKGROUND: Subcorneal pustular dermatosis, also known as Sneddon-Wilkinson disease, can be classified as one of the neutrophilic dermatoses together with pyoderma gangrenosum. The development of both SPD and PG in the same patient has rarely been reported and may be a strong indicator of IgA dysglobulinemia

CASE REPORT: We report the case of a 34-year-old woman with a 2-year history of relapsing pustular eruptions mainly affecting the abdomen, gluteus region, elbows, and the extremities. Four years after the onset of subcorneal pustular dermatosis (SPD), she developed pyoderma gangrenosum (PG) on her right hand. In literature, the coexistence of SPD and PG in the same patient has already been described. This co-occurrence might indicate a certain predisposition for immune dysregulation.

CONCLUSION: Although the two NDs are often associated with systemic diseases, these patients should be followed up for any malignancy because of the strong association between these disorders.

Introduction

Subcorneal pustular dermatosis (SPD), also known as Sneddon-Wilkinson disease, can be classified as one of the neutrophilic dermatoses (NDs) together with pyoderma gangrenosum (PG). The development of both SPD and PG in the same patient has rarely been reported and may be a strong indicator of IgA dysglobulinemia [1].

We describe the case of a patient exhibiting SPD lesions associated after two years' duration with typical PG.

Case report

A 34-year-old woman was admitted to our hospital in 2015 with 2 years-history of relapsing pustular eruptions mainly affecting the abdomen, gluteus region, elbows, and the extremities (Figure 1). Clinical examination showed multiple small painful flaccid pustules varying in size from 0.5-2 cm that tended to coalesce to form an annular or circinate pattern and superficial crusts on the normal or mildly erythematous skin. A skin biopsy specimen showed subcorneal neutrophilic infiltrate with occasional eosinophils and absence of spongiosis or acantholysis. In the dermis, there was a dense perivascular infiltrate of neutrophils and occasional eosinophils (Figure 2). Direct immunofluorescence was negative.

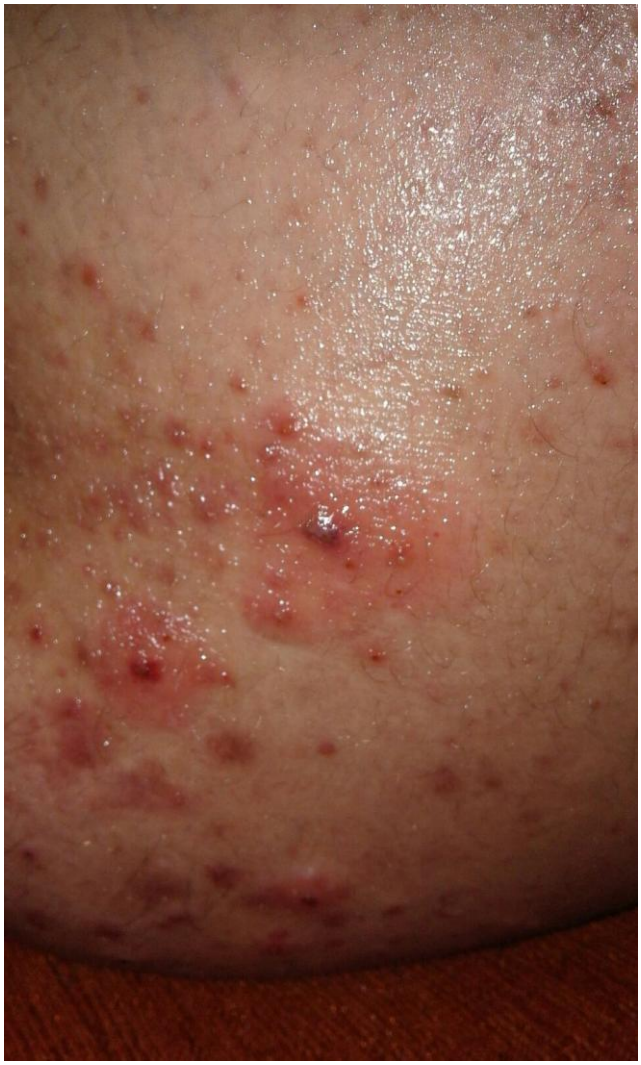

Figure 1: Subcorneal pustular dermatosis. Small flaccid pustules varying in size from 0.5-2 cm that tended to coalesce to form annular or circinate pattern

These findings were consistent with SPD diagnosis. Dapsone was introduced at a dose of 100 mg/daily resulting in gradual improvement of SPD with few residual scarring. She remained on dapsone for 20 months with almost complete remission of the neutrophilic disease.

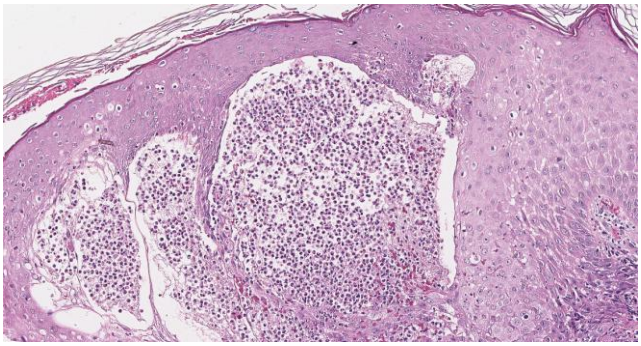

Figure 2: SPD histological examination: subcorneal pustule below the stratum corneum containing mainly neutrophils

In 2017, the patient developed on the right hand an area of painful, rapidly enlarging, ulceration (Figure 3). Clinical examination showed a 2.5 cm diameter-ulceration with violaceous borders and surrounding erythema. Similar lesions were also present on the lower extremities.

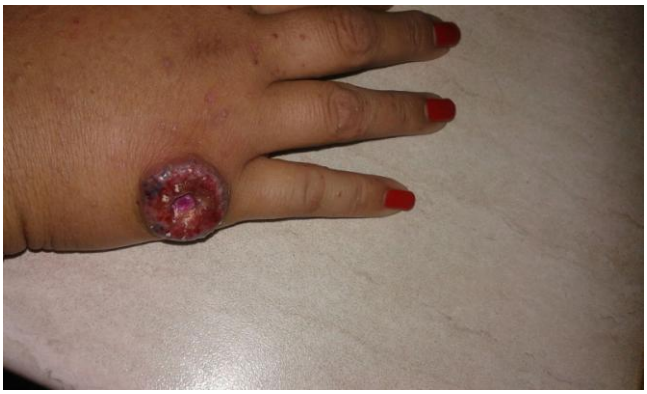

Figure 3: Pyoderma gangrenosum. An area of ulceration with violaceous borders and surrounding erythema

A skin biopsy found a mixed inflammatory infiltrate of neutrophils and lymphocytes, extending into the panniculus (Figure 4).

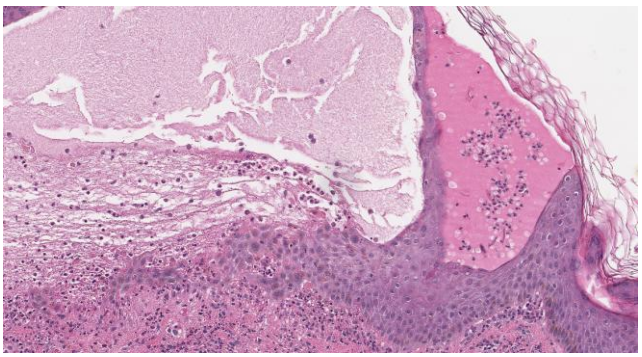

Figure 4: PG histological examination: an intensive mixed inflammatory infiltrate including mainly neutrophils associated with vessel destruction and necrosis of the epithelium

Direct immunofluorescence was negative. Clinical and histopathologic features ware compatible with PG diagnosis, according to Delphi Consensus criterion [2]. Laboratory investigations showed an increased white cell count (11,000/mm^3) with a normal differential. Erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) were elevated (ESR: 20 mm; CRP: 0.72 mg/dL). The blood chemistry, liver function and renal function tests were within the normal limit. Abdominal and pelvic ultrasound and computed tomography were normal. A gastroenterological visit excluded the presence of inflammatory bowel disease. The patient did not respond to topical and oral corticosteroids (prednisone 50 mg/daily for a month); cyclosporine was introduced at a dosage of 150 mg/daily (2 mg/kg) in association with tacrolimus 0.1% ointment, thus resulting in gradual improvement of PG, while SPD remained in remission [3].

Discussion

NDs are a group of heterogeneous skin diseases, with common features and a common histopathologic characterised by polymorphonuclear leukocyte infiltrates at various levels within the epidermis, dermis, or panniculus [4] (Table 1).

Table 1: NDs classification

NEUTROPHILIC DERMATOSIS [4]		
Superficial (epidermal)	En plaque (dermal)	Deep (dermal and hypoderma)
Subcorneal pustular dermatosis	Sweet's syndrome	Pyoderma gangrenosum
IgA pemphigus	Neutrophilic eccrine hidratenitis	Neutrophilic panniculitis
Bullous neutrophilic dermatoses	Erythema elavatum diutinum	Aseptic abscesses
Other pustuloses	Neutrophilic rheumatoid dermatitis	-
-	Granulomatous pyoderma gangrenosum	-

SPD is a rare chronic and relapsing skin disease, characterised by symmetrical sterile pustular eruption typically involving the flexural sites of the trunk and proximal limbs [1]. Although it is typically seen in women over the age of 40 years, there have been a few cases found in children [4] [5]. The etiopathogenesis of SPD remains unknown [6]. A trigger for aberrant neutrophil chemotaxis in SPD has not been identified. Faulty immune mechanisms and genetic susceptibility have been proposed, but no widely accepted hypothesis has been substantiated [3]. PG is a neutrophilic, non-infective skin disorder characterised by pustules or nodules, in which sterile destructive inflammatory process leads to necrotic cutaneous ulceration with violaceous borders and central necrosis. Lesions are mainly localised on the lower legs, especially on pretibial area [7] [8].

In our patient, the SPD preceded the PG onset by 2 years. In literature, the coexistence of SPD and PG in the same patient has been described previously in 12 patients (Table 2) [1]. This co-occurrence might indicate a certain predisposition for immune dysregulation. Although the neutrophilic accumulation appears to be the hallmark of neutrophilic disorders, the exact pathophysiology is still unknown. Probably, it is implicated the production from T cells activated by polymorphonuclear cells of several chemotactic factors, including the pro-inflammatory cytokines: tumour necrosis factor (TNF)-a, interleukin (IL)-8 and complement fragment C5a [4] [9]. Indeed, a complete remission of clinical manifestations, with the off-label use of TNF blocking agents in 29-years old man with SPD, refractory to first-line therapy, has previously been described [10].

The two NDs are often associated with systemic diseases, including inflammatory bowel disease, rheumatoid arthritis, paraproteinemia or haematological malignancy, such as monoclonal gammopathies, lymphomas and multiple myeloma [11].

Table 2: Articles reporting the coexistence of the two neutrophilic diseases published in the literature

Authors	Sex	Age	Associated disease	Therapy	Outcome
Wolff K. *1971 [12]*	-	-	IgA gammopathy	-	-
Marsden JR, et al. *1986 [15]*	-	-	IgA gammopathy	-	-
Venning VA, et al. *1986 [16]*	F	59	IgA gammopathy	dapsone	remission
Freire Murgueytio P, et al. *1989 [17]*	-	-	-	-	-
Kohl PK, et al. *1991 [18]*	F	60	IgA gammopathy	-	-
Scerri L. *1994 [14]*	F	89	-	CCS, dapsone, minocycline	remission
Cartier H, et al. *1995 [19]*	F	54	IgA gammopathy	CCS, dapsone	remission
Stone MS, et al. *1996 [20]*	F	72	Multiple myelomas	CCS, dapsone	Remission
T.A.Chave, et al. *2001 [21]*	M	82	IgA paraproteinaemia and IgG antiepithelial antibodies.	CCS	-
Puechguiral-Renaud I, et al. 2006 [22]	M	67	IgA and IgG gammopathy	CCS	-
Ahmad K, et al. *2009 [1]*	F	57	IgA and IgG gammopathy, multiple myeloma	CCS, dapsone, tacrolimus, acitretin, colchicine, clofazimine, nbUVB cyclosporin, cyclophosphamide	Remission
Audemard A, et al. *2012 [23]*	M	82	Spleen abscess	CCS	Remission

It is of interest that this diseases combination has been reported in the presence of an IgA monoclonal gammopathy [12]. However, in 2006 a case of a 67-year-old man, affected by both PG and SPD simultaneously, who developed a biclonal benign IgA and IgG kappa gammopathy have been reported [13]. Furthermore, *Ahmad et al.* described the case of a 57-year-old woman, with a 5-year history of PG before she developed SPD, associated with biclonal IgA and IgG gammopathy, that 12 years later, developed myeloma [1]. In our patient, no evidence of myeloma or myeloproliferative disorders has been found. Although PG and SPD may occur without the association of an underlying malignancy as in our case¸ these patients should be followed up for any malignancy because of the strong association between these disorders [14].

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Case Report

Primary Cutaneous CD30+/ALK- ALCL with Transition into sALCL: Favourable Response after Systemic Administration with Brentuximab Vedotin! Unique Presentation in a Bulgarian Patient!

Tanya Naskova Popova[1], Atanass Radinov[1], Konstantin Stavrov[2], Ivanka Temelkova[2], Ivan Terziev[3], Ilia Lozev[4], Detelina Lukanova[5], Hristo Mangarov[2], Uwe Wollina[6], Georgi Tchernev[2,7*]

[1]Department of Clinical Hematology, University Multiprofile Hospital for Active Treatment "Sveti Ivan Rilski", Sofia 15, Acad. Ivan Geshov Blvd., Sofia 1431, Bulgaria; [2]Department of Dermatology, Venereology and Dermatologic Surgery, Medical Institute of Ministry of Interior (MVR-Sofia), General Skobelev 79, 1606 Sofia, Bulgaria; [3]Universitetska Mnogoprofilno Bolnitsa za Aktivno Lechenie "Tsaritsa Yoanna", Common and Clinical Pathology, Sofia, Bulgaria; [4]Medical Institute of the Ministry of Interior, Surgery, Sofia, Bulgaria; [5]National Hospital of Cardiology, Clinic of Vascular surgery and Angiology, Sofia, Bulgaria; [6]Städtisches Klinikum Dresden, Department of Dermatology and Allergology, Friedrichstrasse 41, Dresden, Germany; [7]Onkoderma, Policlinic for Dermatology and Dermatologic Surgery, General Skobelev 26, Sofia, Bulgaria

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Keywords: Anaplastic large; T cell lymphoma; Brentuximab; Remission; CD30

***Correspondence:** Georgi Tchernev. Department of Dermatology, Venereology and Dermatologic Surgery, Medical Institute of Ministry of Interior (MVR-Sofia), General Skobelev 79, 1606 Sofia, Bulgaria; Onkoderma - Policlinic for Dermatology, Venereology and Dermatologic Surgery, General Skobelev 26, 1606, Sofia, Bulgaria. E-mail: georgi_tchernev@yahoo.de

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Abstract

BACKGROUND: Modern drugs could sometimes be a good solution even to problematic patients. The cutaneous and systemic forms of the CD30 positive anaplastic large T-cell lymphoma could often be described as a suitable target for therapy with Brentuximab vedotin.

CASE REPORT: We present the first case of a Bulgarian patient with a histologically confirmed primary cutaneous T-cell CD30+/ALK- large anaplastic cell lymphoma-cALCL (therapeutically resistant to therapy with Methotrexate, radiation therapy and systemic corticosteroid therapy) who was successfully treated with Brentuximab vedotin. In several years, the patient has developed a comparatively fast skin progression as well as an initial systemic one which impacts inguinal and mediastinal nodes. After the implementation of 4 therapy cycles with Brentuximab vedotin, complete regression of the described by previous hospitalisations lymph nodes as well as 80% reduction of the cutaneous and subcutaneous located tumour formations were observed.

CONCLUSION: The therapy of CD30+/ALK- anaplastic large T-cell lymphoma is a significant challenge for oncologists and dermatologists because it requires maximally efficient and minimally traumatic treatment in parallel. Therapy with Brentuximab is a new direction which shows extremely good clinical results and can be applied to the cutaneous as well as to the systemic form of anaplastic large-cell CD30 positive lymphoma. The key element by treatment with Brentuximab is suppression of the CD30- expression which, in turn, could be the cause of relapses. On that ground, patients with these lymphomas should be strictly monitored.

Introduction

Cutaneous lymphoma treatment is a challenge for the clinician and requires an accurate assessment of the therapy to achieve optimal therapeutic results and a minimum rate of side effects [1]. CD-30 and ALK positivity in lymphomas is important for the prognosis and choice of treatment regimen [1].

Case Report

We at this moment report the case of a patient with histologically confirmed primary cutaneous T-cell CD30+/ALK- anaplastic large cell lymphoma with an available histological verification from 2 years ago. From the immunohistochemical tests, CD45+, CD3+, CD2+, CD30+, CD20+, EMA+, ALK- and Ki67+ reactions were detected.

Complaints initially began with wide-surface erythema in the right lower leg area. Subsequently, tumorous papules and a pigmentary solitary tumour formation appeared which were treated surgically. Concomitant computer tomographic scans did not reveal any initial data on the transition to systemic lymphoma. Over the last 2 years, 1) 2 corticosteroid treatment regimens, 2) 3 radiotherapy courses with a total focal dose of 30Gy, and 3) attempted Methotrexate 20 mg/week therapy over a total period of approximately 2.5 months, were performed, all of the currently listed options not leading to any symptomatic improvement. Clinical evidence from the latest outpatient examination indicated a progression of skin symptoms and a slight worsening of the general status expressed in subfertility, weakness, and severe pain in the muscles and joints.

The patient was hospitalised for a second reevaluation of the diagnosis, ruling out a transition to systemic lymphoma, and recommendations for introducing a more effective treatment regimen.

During the clinical examination, multiple, partially grouped, as well as single standing nodular tumor-like formations with a diameter of between 0.5 and 3 cm were observed on the skin of the right lower leg, which were elastic and dense at palpation, most of them with a centrally ulcerated surface, located on an erythematous base (Figure 1a). The primary skin large anaplastic cell lymphoma (pALCL) diagnosis was confirmed again in histologically and immunohistochemically. Bone marrow puncture/bone marrow flow cytometry analysis showed evidence of normocellular and hypercellular bone marrow with no immunomorphologic evidence of the involvement of the latter from lymphoma. Paraclinical examinations showed the following results that made an impression: ESR - 22 mm/h; uric acid-plasma - 471.0 µmol/l; glycated hemoglobin% - HbA1C - 8.4%; cholesterol-6.6 mmol/l; LDL - 4.6 mmol/l.

Computer Tomography scan showed the presence of a single 14/11mm paraesophageal lymph node, while in the previous scan the latter had a size of 18 mm. Additionally, bilaterally enlarged inguinal lymph nodes were observed. Primary skin anaplastic CD30+ T cell lymphoma was diagnosed with possible involvement of the regional lymph nodes. The patient received a systemic Brentuximab vedotin therapy, and within 3 cycles (each at a 21-day interval), he showed a significant improvement of the clinical symptoms and involution of the nodular formations in the lower limb, as well as normalisation of the lymph node dimensions (Figure 1a, 1c). A total of 12 Brentuximab cycles were planned.

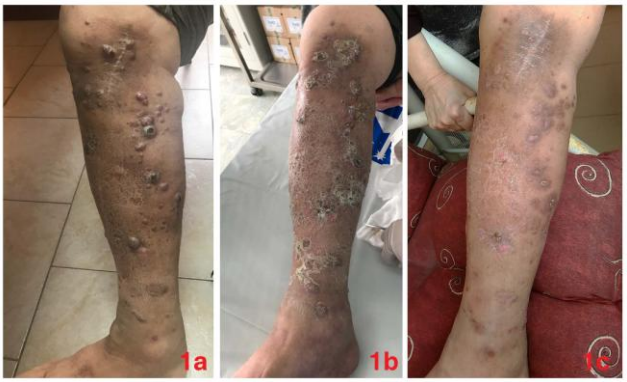

Figure 1: a) Clinical picture before starting therapy in a patient with Methotrexate resistant anaplastic large CD 30 positive ALK-negative primary cutaneous T cell lymphoma; b) Clinical picture after the second Brentuximab infusion; c) Clinical picture after the 3 Infusion with Brentuximab showing significant clinical improvement of the tumourous formations

Discussion

Anaplastic large cell lymphomas comprise a group of CD30-positive non-Hodgkin lymphomas that generally are of T-cell origin and share common morphologic and phenotypic characteristics [2].

The World Health Organization recognises 3 entities: 1) primary cutaneous ALCL (pcALCL), 2) anaplastic lymphoma kinase (ALK)-positive ALCL, and, provisionally, 3) ALK-negative ALCL [2]. pcALCL presents in the skin and, while it may involve locoregional lymph nodes (as in our case suspected), rarely disseminates [2]. Outcomes typically are excellent [2].

Anaplastic large-cell lymphoma (ALCL), especially anaplastic lymphoma kinase-negative (ALK^-) ALCL (as in the case described by us), is a rare CD30-expressing aggressive subtype of peripheral T-cell lymphoma [3]. The CD30 expression on the cell surface of ALCL is creating the unique possibility for anti-CD30 therapy with Brentuximab (3). Brentuximab vedotin (BV; Adcetris® Takeda Pharmaceuticals) is a CD30-directed antibody-drug conjugate that received FDA approval in August 2011 for the treatment of systemic ALCL (sALCL) [3].

The advantage of Brentuximab therapy is that it is available to patients with both cutaneous and systemic form of anaplastic large-cell CD-30 positive lymphoma [4] [5]. Both indications (systemic and cutaneous) for introducing systemic treatment are of significant importance about the clinical management

in “problematic patients” [4] [5]. The patient we have described did not agree to a biopsy of the paraesophageal-localized lymph node and bilaterally localised inguinal enlarged lymph nodes.

During the comparisons of the CT images, it was found that the case was most likely related to a transition from the cutaneous to the systemic form of ALCL. Meanwhile, the two indications for treatment of ALCL ensured some freedom of action relating to the initiation of innovative Brentuximab therapy, as well as optimal clinical outcomes (with complete regression of the paraesophageal and bilateral inguinal located lymphatic nodes) after 4 cycles with Brentuximab vedontin.

The lack of effectiveness due to the loss of CD-30 expression in the course of the targeted treatment is a possible reason for relapses in ALCL patients, as well as a serious obstacle to continued Brentuximab therapy [6].

Therefore, the progression of the disease during the therapy or discontinuation of treatment in patients treated with Brentuximab should be accompanied by re-testing for CD30 expression in the lesional tissue and redefine the therapeutic strategy [6]. The patients should be closely monitored.

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Case Report

A Case of Orbital Myositis Presenting With Dizziness

Neville Aquilina*, Vincent Bugeja, Charmaine Zahra

St Vincent de Paul, Ingiered Road, Luqa, Malta

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Keywords: Pain; Complex ophthalmoplegia; Unilateral; Steroids; Dizziness

***Correspondence:** Neville Aquilina. St Vincent de Paul, Ingiered Road, Luqa, Malta. E-mail: nevaqui@gmail.com

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Abstract

BACKGROUND: Orbital myositis is an idiopathic, inflammatory, non-infectious condition, typically confined to more than one extraocular muscle and usually targeting young females in their third decade.

CASE REPORT: We describe a case of orbital myositis uncommonly afflicting an adult male. He initially presented with a sensation of dizziness when turning his head and mobilising, together with right-sided orbital pain that failed to respond to the vestibular sedative cinnarizine. Failure to resolve and development of diplopia initially prompted a working diagnosis of ocular myasthenia gravis. On further investigation using HESS charting, paresis in the inferior and medial rectus and superior oblique ocular muscles of the right eye were elicited correlating with clinical examination. However, the lack of response to low dose steroids and acetylcholinesterase inhibitors, together with a negative screen for myasthenia antibodies, precluded a diagnosis of ocular myaesthenia gravis from being made. Other investigations included a high creatinine kinase and lactate dehydrogenase which indicated pathology in the extraocular muscles themselves. An MRI scan showed normal extraocular muscle thickness while excluding other orbital pathology. Exclusion of a variety of other conditions subsequently led to a diagnosis of orbital myositis.

CONCLUSION: Response to high dose steroids consolidated this diagnosis with a rapid response ascertained clinically by resolution of pseudo-vertigo and pain, as well as the ophthalmoplegia with follow-up on HESS charting. We aim to raise awareness of this rare condition that carries a good response to steroids.

Introduction

Orbital myositis (OM) is an idiopathic inflammatory, non-infectious condition confined to more than one extraocular muscle (EOM), usually targeting young females in their third decade. It occurs suddenly and affects commonly one eye with pain that is worse on eye movements, in more severe forms eyelid swelling (blepharitis), proptosis and conjunctival injection become prominent. Visual acuity is preserved. Focal or diffuse homogeneous enlargement of EOMs and surrounding structures is often observed on neuroimaging [1] [2].

Some conditions can mimic or have been linked to OM [2] [3] [4]. In our discussion, we will highlight the importance of excluding other diseases before making a diagnosis of OM.

Case presentation

We report a case of a 38-year-old male patient whose initial presentation was a mild sensation of dizziness when turning his head and when mobilising, which he described as a feeling that the room moved about as he shifted his gaze, but this was absent at rest and if he kept his gaze fixed. He also started complaining of pain in the right orbit, which increased with eye movements. These symptoms were attributed to vertigo secondary to acute inner ear pathology by the general practitioner who prescribed the vestibular sedative cinnarizine to no effect. Initially, eyesight was intact however he soon developed diplopia on looking down and medially. This diplopia was intermittent while the motion-related dizziness remained the most frustrating symptom. It was noted that this 'vertiginous-like' dizziness was not

of vestibular origin because if he stopped turning his head, it subsided immediately and was never associated with nausea. Instead, it was associated with a dull right-sided orbital pain and also failed to respond to cinnarizine.

Upon review, the neurologist suspected ocular myasthenia gravis (OMG) and started him on pyridostigmine 60 mg, 4 hourly, together with a small dose of atropine 2 mg twice daily. He was also prescribed 7.5 mg daily of prednisolone (in 3 divided doses) set to titrate up slowly over the following weeks to 20 mg. There was no improvement in the patient's symptoms. Nicotinic acetylcholine receptor antibodies were negative. Other antibodies typical of myasthenia gravis such as anti-Musk (muscle-specific kinase) and anti-striational antibodies were also negative.

Table 1: Investigations

Test	Result	Normal range
S-immunoglobulin G	13.73	7.01-13.05
LCR-immunogobulin G	46.00	< 40
S-Albumin	45.20	40.84-51.72
LCR-Albumin	327.00	< 350
Link index (IgG index)	0.46	< 0.65
ACE Liquor	0.34	0.06-0.25
Muscle specific tyrosine kinase (MuSK) antibodies	< 0.01	< 0.05 - negative
Calcium channel antibodies (P/Q)	negative	
Acetylcholine receptor antibodies	< 0.1	< 0.25
Thyroid stimulating hormone receptor antibodies	< 0.4	< 0.4 - negative
Angiotensin-converting enzyme	5.3	65-114
Creatinine kinase	286	38-174
Alpha-hydroxybutyric acid	259	72-182
Lactate dehydrogenase	274	135-220
Ferritin	423	28-365

A battery of blood tests was done including renal, liver and thyroid function, inflammatory markers, creatinine kinase (CK), full blood count together with a fasting blood glucose and glycosylated haemoglobin (HbA1c). The CK was somewhat elevated; however, this was initially attributed to his muscular build. Moreover, when compared to previous baseline levels this was the first instance an elevated CK was recorded. Otherwise, all other blood tests, including the diabetes screen, the thyroid function and an autoimmune screen (anti-nuclear and anti-neutrophil cytoplasmic antibodies plus rheumatoid factor) were also normal. Clinically, he had no features of Graves' disease ophthalmopathy. Chest X-ray showed no abnormality in the anterior mediastinum, together with a normal CT (computed tomography) of the head and thorax thereby excluding thymoma. A lumbar puncture was performed whereby his cerebrospinal fluid failed to show any oligoclonal bands and the IgG index was normal thus excluding acute inflammatory demyelinating conditions such as multiple sclerosis. Additionally, a normal MRI (magnetic resonance imaging) of the brain further evidenced such preclusion of these possibilities.

An ophthalmic consultation was made, and HESS charting showed deficits in the medial rectus, inferior rectus and the superior oblique of the right eye. At this point, OMG was considered as most plausible. However, the movement deficits in the right eye remained fixed, though at times clinically he would show variable features appearing like a 4th (trochlear) nerve palsy.

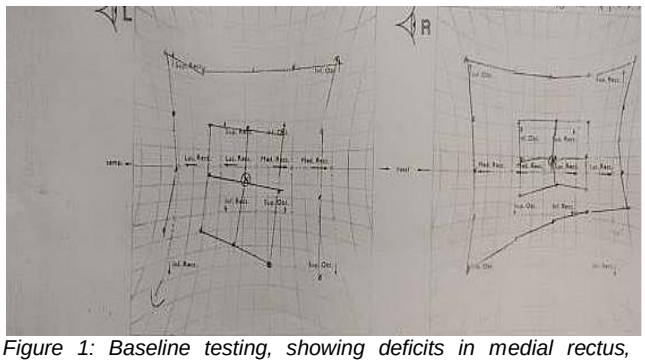

Figure 1: Baseline testing, showing deficits in medial rectus, inferior rectus and the superior oblique of the right eye

Presence of unremitting orbital pain then started to entertain the possibility of other diagnoses. Due to the high CK, chronic progressive external ophthalmoplegia (CPEO) was considered, but lack of other features and a unilateral predominance vouched against mitochondrial cytopathy.

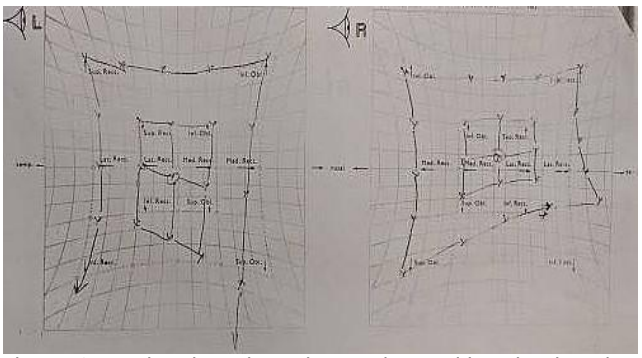

Figure 2: During low dose (7.5 mg) steroids, showing the persistence of the right-sided deficit

Further diagnoses such as Miller Fisher syndrome, Tolosa-Hunt syndrome, oculopharyngeal muscular dystrophy and vasculitides where all excluded [the latter in view of his negative ANCA (anti-neutrophil cytoplasmic antibodies)]. Lambert-Eaton myasthenic syndrome was excluded since a negative voltage-gated calcium channel antibody screen also came back negative, while there were no signs of any associated bronchial malignancy. Neurosarcoidosis was excluded despite a marginally elevated cerebrospinal fluid ACE (angiotensin- converting enzyme) level following lumbar puncture testing. Normal serum ACE levels were also consistent with this.

The only diagnosis left to consider was orbital myositis, which possibility was strengthened given his serum creatinine kinase (CK) and lactate dehydrogenase (LDH) rise. Crucially, since

being administered 50 mg prednisolone daily, the symptoms had resolved completely within 3 days. Then the prednisolone was slowly tapered down over a period of 3 months and stopped. The condition did not recur, and CK returned to normal, further corroborating a working diagnosis of orbital myositis (OM). In this case, the OM would be classified as the milder LOOM (limited oligosymptomatic ocular myositis) variety, considering the absence of scleral injection, ptosis, proptosis and signs of inflammation in this case [9].

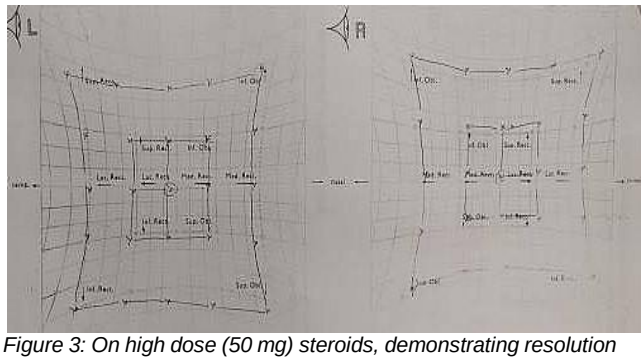

Figure 3: On high dose (50 mg) steroids, demonstrating resolution

We report a case with orbital myositis (OM) presenting initially with a movement related vague dizziness or 'vertiginous-like' sensation due to extraocular muscle weakness, which is a way how diplopia may manifest, from failure of the binocular tracking mechanism when shifting gaze. Diplopia sometimes produces a sensation of movement of the environment which may mimic genuine vertigo. True vertigo could not be present in this case because on sitting still there was no hallucination of movement of the patient himself or his surroundings (which is the *bona fide* description of vertigo), moreover the symptom failed to respond to cinnarizine (a vestibular sedative), whilst there was the added complication of right-sided orbital pain.

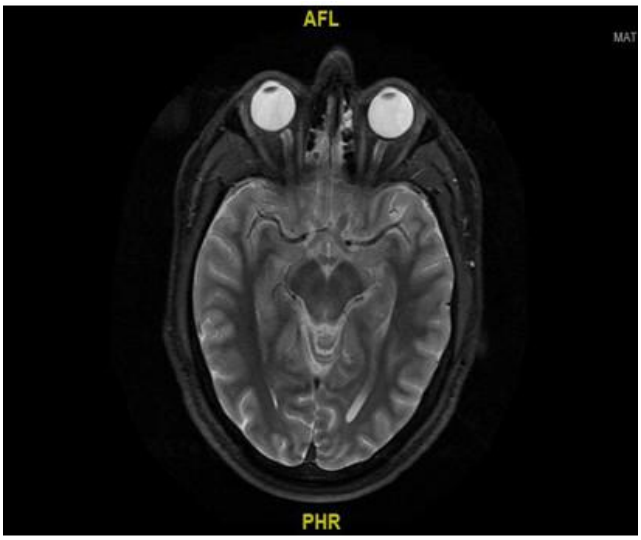

Figure 4: Plain MRI performed while the patient was not on steroid therapy

After a comprehensive exclusion, he was diagnosed with OM. We emphasise that OM should be included in the differential when considering a diagnosis of OMG (especially when atypical features are present). Imaging although helpful is not always useful in diagnosing OM, as in this case, in which enlargement of the EOM or surrounding structures was not visible (Figure 4). However, there was an immediate response to high dose systemic steroids, whereas conversely, in OMG this would have precipitated a myasthenic crisis.

Discussion

Features suggestive of orbital myositis, as opposed to ocular myasthenia gravis include:

-The features were unilateral;

-There was pain especially with eye movements;

-Muscle enzymes were all high (CK, LDH);

-No response was elicited to low dose steroids and pyridostigmine;

-Immediate resolution with high dose steroids;

These cases mandate a full workup including ophthalmic examination, routine blood, inflammatory markers and thyroid function.

Further workup may consist of MRI orbits, CT or ultrasound. Typical radiological features are thickening and contrast enhancement of the EOM and possible surrounding structures such as myotendinous insertion and fat. In a significant number of cases, the orbital muscles appear normal.

Other diagnoses need to be excluded. Bilateral inflammation of EOMs, with sparing of the myotendinous junction is seen in dysthyroid eye disease. Features of Graves' ophthalmopathy comprise proptosis, chemosis and complex diplopia and would be confirmed by positive thyroid-stimulating hormone (TSH) receptor antibodies.

In IgG4-related disease, thyroid function tests are normal, but an enlargement of inferior rectus and both lacrimal glands are observed [3].

In cases of orbital cellulitis or tuberculosis, imaging would show pus collections or caseous necrosis respectively, in addition to the fever and leucocytosis [2] [5]. In our patient, TB was immediately excluded as the patient did not have a history of foreign travel or any TB contacts. Contrast-enhanced MRI is the most sensitive modality for showing individual EOM signal hyperintensity and swelling [7].

Neoplasm/metastasis is a differential in the absence of pain; imaging would show a focal mass or increase in signal intensity of EOM in lymphoma [2]. However, a definite diagnosis would need to be confirmed via extraocular muscle biopsy.

Inflamed EOM due to venous congestion may be diagnosed in patients with low-flow carotid cavernous fistula. Another feature of this condition is enlarged superior ophthalmic veins [4], and there may also be pulsatile exophthalmos.

In lesser severity, orbital myositis of the LOOM (limited oligosymptomatic OM) variety, treatment in non-diplopic patients, or in those having only slight diplopia with mild to moderate pain, utilising high dose non-steroidal anti-inflammatory drugs (NSAIDs) may be considered. Systemic corticosteroids are nevertheless necessary, except perhaps for the mildest non-diplopic forms, because they accelerate recovery and prevent recurrence, albeit relapse rates are still relatively high, being up to 81% in one study [6] despite steroids. In refractory, chronic, or recurrent cases, steroid-sparing agents such as immunosuppressants or radiation therapy may also be considered.

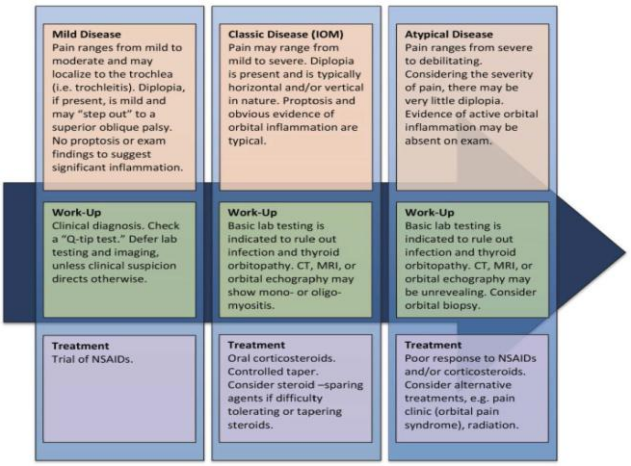

Figure 5: A clinical algorithm for orbital myositis [8]

"Q-tip test": A cotton-tipped applicator applied to the area over the trochlea, and other extraocular muscles may reveal point tenderness that the patient was otherwise unable to describe. This suggests trochleitis, superior oblique myositis/tendonitis, or focal myositis over the tender muscle or tendon.

In conclusion, to our knowledge this is the first published case report of OM presenting with dizziness as the primary symptom. Moreover, HESS charting is sensitive in mapping out any ophthalmoplegia that may not be prominent clinically. We have emphasised the importance of considering this rare inflammatory disease confined to the extraocular muscles and orbital structures, once ocular myasthenia gravis, dysthyroid eye disease and organic causes (cellulitis, tumours, carotid-cavernous fistula) have been excluded, especially as prognosis with high dose steroid therapy is favourable.

Learning points: (i) the isolated finding of a complex ophthalmoplegia requires ocular myasthenia gravis to be excluded *a prima face*; (ii) atypical features such as unilateral involvement, EOM or orbital pain, failure to respond to low dose steroids and a negative response to acetylcholinesterase inhibitors preclude ocular myasthenia gravis from the differential; (iii) MRI is essential to exclude other infiltrative conditions of the EOMs, albeit muscle biopsy would provide a definitive histological diagnosis; (iv) elevated muscle enzymes (CK, LDH) indicate a myositis; (v) once ocular myasthenia and organic causes have been excluded a trial of high dose steroids would be appropriate and thence a positive response effectively renders the diagnosis of orbital myositis highly plausible.

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Case Report

Key Issues in the Management of Multi-Drug Resistant Tuberculosis: A Case Report

Zorica Nanovic[1]*, Biserka Kaeva-Jovkovska[2], Gorica Breskovska[2], Milena Petrovska[3]

[1]Institute of Lung Diseases and Tuberculosis, Skopje, Republic of Macedonia; [2]University Clinic of Pulmonology and Allergology, Faculty of Medicine, Ss Cyril and Methodius University of Skopje, Skopje, Republic of Macedonia; [3]Institute of Microbiology and Parasitology, Faculty of Medicine, Ss Cyril and Methodius University of Skopje, Skopje, Republic of Macedonia

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***Correspondence:** Zorica Nanovic. Institute of lung diseases and tuberculosis, Skopje, Republic of Macedonia. E-mail: znanovic@hotmail.com

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Abstract

BACKGROUND: Global tuberculosis (TB) epidemic is being driven to an increasing extent by the emergence and spread of drug-resistant strains of Mycobacterium tuberculosis complex (MTBC). We present a case of primary multidrug-resistant tuberculosis (MDR-TB), highlighting Macedonian MDR-TB management issues.

CASE REPORT: A 39-year old previously healthy Caucasian male, with no previous history of TB or close contact to TB, was admitted in referral TB-hospital due to respiratory bleeding. Chest X-ray revealed opacity with cavernous lesions in the right upper lobe. Sputum samples showed no presence of acid-fast bacilli (AFB) on fluorescence microscopy, but molecular tests (real-time PCR-based assay and multiplex PCR-based reverse hybridisation Line Probe Assay) confirmed the presence of MTBC, also revealing rifampicin and isoniazid resistance and absence of resistance to second-line anti-tubercular drugs. The strain was considered multidrug-resistant, lately confirmed by conventional methods in liquid and solid culture. Following the protocol of the World Health Organization, we started the longer treatment of MDR-TB comprised of at least five effective anti-tubercular drugs. Due to patient's extreme non-adherence, we had to delay and modify the regimen (i.e. omitting parenteral aminoglycoside) and to discharge him from the hospital a month after directly observed therapy (DOT) in negative pressure room. As there is no legal remedy in our country regarding involuntary isolation, our patient continued the regimen under ambulatory control of referral TB-hospital. Ignoring the risk of additional acquisition of drug resistance and prolonged exposure of the community to MDR-TB strain - for which he was repeatedly advised - he decided to cease the therapy six months after beginning.

CONCLUSION: The benefit of molecular tests in the early diagnosis of TB and drug resistance is unequivocal for adequate treatment of resistant forms of TB. Whole genome sequencing ensures additional knowledge of circulating strains and their resistance patterns. These are essentials of effective TB control programs and can provide evidence to medical and legal authorities for more active policies of screening, involuntary confinement and compliance with therapy, and alternative modalities for successful treatment, as a part of infection control.

Introduction

Global tuberculosis (TB) epidemic is being driven to an increasing extent by the emergence and spread of drug-resistant strains of *Mycobacterium tuberculosis* [1] [2]. Multidrug-resistant TB (i.e. TB resistant to rifampicin and isoniazid, MDR-TB) presents major risk to global tuberculosis control, due to the great discrepancy between real and estimated burden, long-term (18-24 months) and expensive treatment, with less effective and more toxic drugs then first-line treatment, and low treatment success rate (54% globally) [3] [4] [5] [6].

According to World Health Organization (WHO), of the estimated 600,000 people newly eligible for MDR-TB treatment in 2016 (490,000 cases of MDR-TB and an additional 110,000 with rifampicin-resistant TB, RR-TB), only 129,689 (22%) received appropriate treatment. Of these MDR-TB cases, an estimated 6.2% will develop extensively resistant tuberculosis (i.e. MDR-TB plus resistance to at list one quinolone and list one parenteral anti-tubercular drug, XDR-TB) [6]. Hence, timely and accurate diagnosis of

MDR-TB, particularly among new TB-cases, is essential to initiate appropriate treatment, which can prevent further amplification of drug resistance and break the chain of infection [7].

Exciting developments in the field of molecular diagnosis of TB and drug resistance have led to an era in which rapid testing for TB and rifampicin (RIF) resistance, almost always associated with MDR-TB, have been rolled out on a global scale [8] [9]. The WHO has recommended molecular testing as the standard of care for diagnosis of TB and MDR-TB around the world [8] [10] [11]. Furthermore, recently a new shortened treatment has been introduced for selected MDR-TB patients in terms to the redintegrate optimal balance of adherence and cure of MDR-TB patients, with adverse drug events and overall cost of the treatment [12].

The Republic of Macedonia is a country with a low total number of notified cases of MDR-TB over a ten years period, low incidence of MDR- TB (0.28/100,000 in 2016) and continuously declining TB incidence [6] [13]. However, this enviable epidemiological setting interpreted regarding intensive migration processes in the recent years should raise the vigilance for future.

Here we present a case of primary resistant MDR-TB and most likely imported from abroad, that is particularly useful in highlighting the Macedonian MDR-TB management issues.

Case Report

A 39-year old previously healthy Caucasian male, a smoker, employed as a professional driver on international truck transport, sought medical care at the local hospital due to respiratory bleeding. After initial assessment and supportive treatment, he was referred as an outpatient at referral hospital for TB in Macedonia-the Institute of lung diseases and tuberculosis. Initial examination of two sputum samples showed no presence of acid-fast bacilli (AFB) on fluorescence microscopy, but real-time PCR based molecular method (Xpert MTB/RIF; Cepheid, Sunnyvale CA, USA) confirmed the presence of *Mycobacterium tuberculosis* complex (MTBC) along with detection of RIF resistance (i.e. *rpoB* gene mutation). On patient's request, both analyses were repeated 20 days later in another two samples of sputum, with the same result. By national guidelines and protocol, molecular analyses were extended to testing of susceptibility to isoniazid (INH) and subsequently to second-line anti-tubercular drugs, by using multiplex PCR-based reverse hybridisation Line Probe Assays (LPA). Both assays confirmed the presence of MTBC, revealing mutations of the *rpoB, katG and InhA* genes, i.e. RIF and INH resistance, as well as an absence of resistance to second-line anti-tubercular drugs and ethambutol (GenoType MTBDR*plus* and GenoType MTDRs*l*, respectively; Hain Lifescience GmbH, Nehren, Germany). Patient's illness was notified as MDR-TB, and the strain was considered to be multidrug-resistant. In the month that followed, *Mycobacterium tuberculosis* growth was verified with conventional methods in liquid and solid culture (Becton-Dickinson BACTEC MGIT 960 TB system, and Löwenstein Jensen medium, respectively). Resistance to the most important first-line anti-tubercular drugs-RIF and INH- was confirmed by conventional phenotypic drug-susceptibility testing (DST), using the liquid-culture-isolate from the very first sputum sample (Table 1).

Table 1: Microbiological analyses of sputum from the MDR-TB patient during 2016 (before and over the course of treatment)

Date	Sputum specimen	Conventional methods				Molecular methods		
		AFB smear micro-scopy[a]	Solid medium[b]	Liquid medium[c]	Resistance pattern[d]	Xpert MTB/RIF *VG*4	HAIN MTBDR*plus* *V*2.0	HAIN MTBRs*l* *V*1.0
Apr 15	A	Negative	Negative (result released on May 31)	Positive (result released on May 04)	INH –R RIF - R EMB - S SM – S (result released on June 03)[e]	MTB detected, low; RIF resistant (*rpoB*)		
	B	Negative	Negative (result released on May 31)					
May 04	A	Negative	Positive (2+) (result released on May 31)	Positive (result released on May 16)		MTB detected, low; RIF resistant (*rpoB*)	INH –R (*katG mut2*) RIF – R (*rpoB mut1*)	FLQ – S AG/CP – S EMB – S
	B	Negative	Negative (result released on May 31)					
May 30	A	Negative	Positive (1+) (result released on Jul 18)	Positive (result released on June 15)				
	B	Negative	Positive (1+) (result released on Jul 18)					
Jun 30	A	Negative	Negative (result released on Aug 22)					
	B	Negative	Negative (result released on Aug 22)					
Aug 22	A	Negative	Negative (result released on Oct 10)					
	B	Negative	Negative (result released on Oct 10)					

Abbreviations: AFB = Acid Fast Bacilli; INH = isoniazid; RIF = rifampicin; EMB = ethambutol; SM = streptomycin; FLQ = fluorochinolones; AG = aminoglycozides; CP = cyclic peptides; MTB = *Mycobacterium tuberculosis*; A = early morning sputum; B = spot sputum specimen; R = resistant; S = sensitive; [a]Auramine-rhodamine staining for fluorescence microscopy (Merck, Darmstadt, Germany); [b]Löwenstein Jensen medium; [c]BACTEC MGIT 960 TB system (Becton-Dickinson); [d]Phenotypic drug-susceptibility test (DST, conventional proportion method on Löwenstein Jensen medium); [e]Resistance pattern from the isolate in liquid medium (Apr 15).

Although the patient was repeatedly informed and advised about the seriousness of his health condition and health hazard for his family and community, it took a month and a half before obtaining consent and starting an appropriate anti-tubercular regimen. The major obstacles in this way were the stigma (i.e. denying the presence of disease) and behaved aggressively toward health care providers. At

hospital admission, the patient presented in good health condition, complaining only to rare productive cough. He had no personal or familial history of TB/TB treatment or known close contact, i.e. exposure to TB. Laboratory analyses showed only slightly raised erythrocyte sedimentation rate (ESR) of 36 mm in the first hour, without deviation in complete blood counting and routine blood analyses, and with HIV negative status. Chest X-ray revealed opacity with cavernous lesions in the right upper lobe (Figure 1).

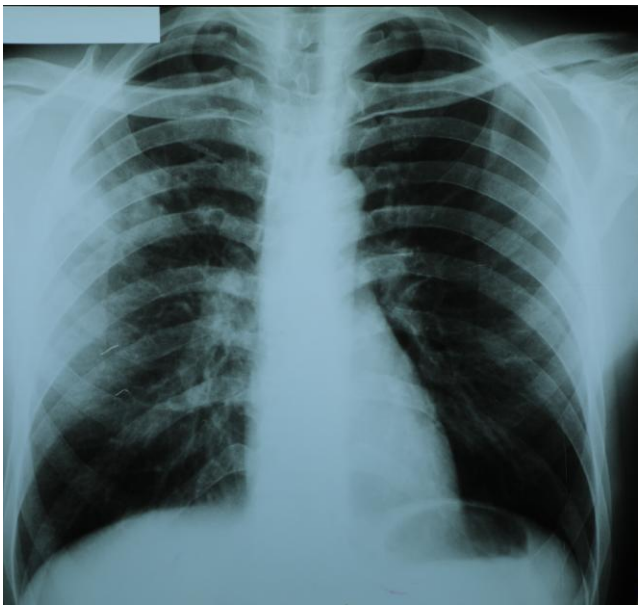

Figure 1: At hospital admission, opacity with cavernous lesions in the right upper lobe

Following the guidelines of the World Health Organization (WHO), we started the so-called longer treatment of MDR-TB composed of at least five effective anti-tubercular drugs. Due to the patient's strictly declining of parenteral drugs, we had to modify the regimen and conduct the intensive phase with three (instead of four) core second-line anti-tubercular drugs (levofloxacin, cycloserine, prothionamide) plus pyrazinamide and ethambutol (i.e. omitting the parenteral aminoglycoside) (Figure 2).

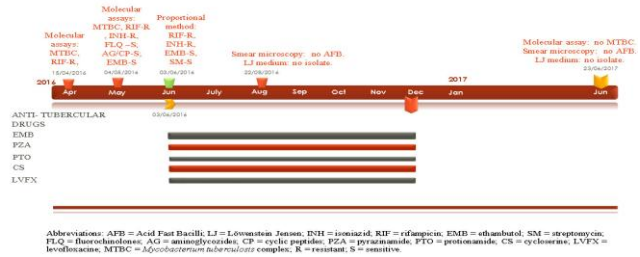

Figure 2: Timetable of therapy

After a month of directly observed therapy (DOT) and isolation in negative pressure room, two follow-up samples of sputum were again smear-negative for AFB, sedimentation rate dropped into the normal range (6 mm in the first hour) and control chest X-ray showed no regression of changes. The patient was discharged from the hospital against medical advice and advised to continue the intensive phase of the regimen under ambulatory supervision (i.e. monthly control and take up of medicines in the Institute).

Three months after the beginning of the treatment, he stopped making controls. The last available information provided by medical patronage system confirmed that he ceased the therapy early in December 2016. The only control after cessation took place in June 2017: routine laboratory analyses didn't show any deviations, and microbiological examination of two sputum samples (smear microscopy, real-time PCR, liquid and solid medium culture) yielded no positive results. But, the changes in the right upper lobe on chest X-ray were still present, indicating the activity of the process (Figure 3).

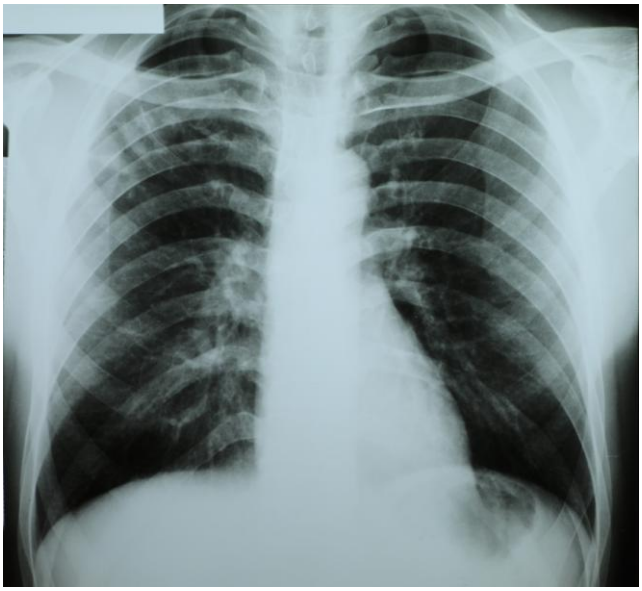

Figure 3: Six months after cessation of treatment: opacity with cavernous lesions and fibrotic changes in the right upper lobe

Discussion

This case report emerges several important issues pertinent to the overall management of MDR-TB.

Sputum smear microscopy is a commonly used test for diagnosis of pulmonary TB in low- and middle-income countries. In addition to the key disadvantage-the low sensitivity of 50-60% - it requires trained staff and at least two sputum samples to analyse while providing no information about drug resistance and distinction of non-tuberculous mycobacteria [14]. In the last decade, WHO set out

the efforts to establish infection control and overcome the problem with drug-resistant forms of TB, by implementing new molecular techniques for detection of *Mycobacterium tuberculosis* complex and drug resistance to anti-tubercular drugs. There are several molecular assays for rapid diagnosis of TB and determination of drug susceptibility profile, endorsed by WHO: Line Probe Assays (LPA) in 2008, and Xpert MTB/RIF assay in 2011 [10] [11]. Shortly after WHO approval, Ling, Zwerling and Pai conducted a meta-analysis to evaluate the performance of most frequently used LPA-GenoType MTBDR [15]. GenoType MTBDR assays demonstrate excellent accuracy for rifampicin resistance (pooled sensitivity and specificity estimates of 98.1% and 98.7%, respectively). While specificity was excellent for isoniazid (99.5%), sensitivity estimates were modest and variable (84.3%). Similarly, the Cochrane review of 27 studies of adults with pulmonary TB, indicated that Xpert MTB/RIF is accurate for detecting rifampicin resistance, sensitive (95%) and specific (98%) [16]. Also, it is more accurate than smear microscopy for diagnosing TB: it is highly sensitive (89%), detecting almost all cases, and specific (99%). Authors concluded that Xpert MTB/RIF provides accurate results which can allow rapid initiation of MDR-TB treatment, pending results from conventional culture and DST. Likewise, the latest release of WHO Manual for Xpert MTB/RIF implementation (2014) contains an extended recommendation for implementing the Xpert MTB/RIF assay, i.e. to be used rather than conventional microscopy and culture as an initial diagnostic test in all adults suspected of having TB [17].

Our case is a good confirmation of accuracy, precision and reproducibility of TB molecular diagnostic tests. Determined by two different molecular (genotyping) methods, drug resistance profile was identical in two separate sputum samples and subsequently confirmed by conventional DST. This gains importance when making therapy decision, especially if based only on conventional methods. Due to sputum low bacterial load (i.e. AFB negative on fluorescence microscopy), one should wait for the culture results (2-6 weeks) to start anti-tubercular therapy, and another 6 weeks to modify the regimen respecting the results of conventional DST. Conversely, molecular tests used in our case allow detection of MTB and RIF resistance in 2 hours, and detection of MTB and resistance to first-and second-line anti-tubercular drugs in 24 hours [15] [18]. This emphasises the importance of molecular tests for the timely and accurate diagnosis of TB and resistance pattern, particularly in smear-negative microscopy [6] [8] [19] [20].

Drug resistance in MTBC is caused by mutations in restricted regions of its genome [21] [22]. As technical possibilities in our laboratory don't allow sequencing of drug-resistance associated regions of MTBC genome and subsequent detailed description of mutations, detection is limited to mutations already included in molecular tests mentioned above. In terms of this, a clinician must count on occasional false-positive RIF-resistance result on molecular test, which has to be promptly resolved as it seriously implicate therapy decision-to start first line anti-tubercular regimen and take the risk of resistance amplification to pyrazinamide and ethambutol, to conduct the lengthy and much more toxic MDR-TB treatment, or to defer starting any treatment while awaiting results of DST? [23] [24] [25] [26]. This complex problem could be overcome by advanced genotyping methods (DNA fingerprinting, whole genome sequencing). Studying the isolates at molecular level allows not only detection of MTBC and mutations in genes associated with drug resistance, but also discrimination between MTBC strains, distinction of co-infection with multiple strains, endogenous reactivation and exogenous re-infection, existence of subpopulations with different resistance profiles, assessing the possible treatment failure, as well as clustering of isolates to particular genotype family databases (sequencing libraries from genomic DNA)-which may implicate pathogenesis, virulence, propensity to acquire resistance more easily under conditions of suboptimal treatment, and to spread in the community [27] [28] [29] [30] [31] [32] [33]. The importance of former molecular tools for epidemiological tracking in the community (i.e. detecting the sources of infection, pathways of spread of MTBC strains, distinct geographic distribution) is unequivocal and has made the worldwide designing of prevention and control strategies to block further transmission possible [34].

The low bacterial load in spot sputum samples shouldn't underestimate the possibility of variable bacillary as a function of time, and so the persistence of the chain of infection [35]. Occasional sputum positivity in our patient is very likely, considering subjective sample collection for smear microscopy from a non-cooperative person. Moreover, due to negligent patient behaviour (postponing of hospital admission, shortening the regimen much earlier), we have to anticipate not only deterioration of his health condition over time, but the potential prolonged exposure of the environment to MDR-strain of *Mycobacterium tuberculosis* [18]. As the number of MDR-TB cases continues to rise worldwide, so does the amplification of MDR-TB strains during treatment. This amplification is generally assumed as a result from in vivo evolution of drug resistance caused by poor therapy compliance and/or inadequate treatment, considering the prior anti-TB treatment is a major risk factor associated with MDR-TB [7] [22] [36] [39]. Given the fact that our patient had no history of TB or TB treatment, nor he has other apparent risk factors or contacts for TB, it was reasonable to consider the case as TB with primary multidrug resistance [27] [28] [34] [40] [41]. To make the situation even more serious, our patient requested modification of intensive phase of the anti-tubercular regimen, and interrupted it early in the course, before initiation of

continuation phase. It has to be stressed that extreme non-adherence like this, could further compromise the infection control in an international context (i.e. continuous transmission of infection, additional acquisition of drug resistance via “amplification of resistance” and subsequent developing an XDR) [18] [34] [41].

This emphasises the critical importance of clinical monitoring during DOT, introduced and recommended by WHO [30] [42]. However, there are some articles, mainly from the African continent, reporting on the successful alternative modality of treatment of smear-positive pulmonary TB and MDR-TB instead of conventional DOT strategy. The essence of this home-based “family-member variant of DOT” approach, is to involve a reliably family-member with responsibility of providing appropriate conditions (i.e. isolation, natural ventilation), supplying and directly supervising the taking of medicines on regular basis, and providing feedback about patient’s condition to his attending physician [43] [44]. Although this kind of practice is primarily related to resource-limited settings and is not anticipated in current Program for TB control in Macedonia, it certainly deserves attention in future for extreme cases as we presented here.

The previous issue raises the crucial ethical dilemma: how to deal with dual standards and protect the human rights of the minority (i.e. non-adherent TB patient)-at the expense of majority (i.e. community)? Patients generally have the right to refuse to follow professional medical advice. Those with infectious TB disease, however, “may lose the right to refuse such advice if health officials believe these persons are putting the public at risk for infection” [45]. There are rare situations where, despite all reasonable efforts, patients do not adhere to the prescribed course of treatment, or are unwilling or unable to comply with infection prevention and control measures. For such cases, the interests of other members of the community may justify efforts to isolate the patient involuntarily, by a pre-existing law or policy. WHO states that involuntary isolation should never be a routine component of TB-program. Involuntary isolation should be limited to exceptional circumstances, concerning infectiousness and non-adherence of a patient to effective treatment, and after all reasonable measures to ensure adherence have been attempted and proven unsuccessful [46]. Governments have the legal authority to enact laws regarding infectious persons, and TB programs should develop policies in line with the guidance that clearly explains when and how involuntary isolation of TB patients is allowable. Some countries have already implemented the option for legal intervention, i.e. involuntary confinement in such cases [47] [48]. In our country, there is still no solution within the framework of existing legislation regarding involuntary isolation and compliance with the recommended combination of drugs, as well as interruption of therapy.

In the transmission of resistant strains, the greatest individual risk factor is sharing living quarters with individuals who have defaulted on their treatment of TB or relapse cases [49]. Fortunately, close contact screening among household members (spouse, and two children aged 3 and 7) showed no presence of latent tuberculosis infection (LTBI) or active TB. Nevertheless, it raises the question of preventive treatment for contacts with MDR-TB, especially when children involved in this problem, as well as the duration of the follow-up period. The current modest recommendation reflects the limited quality of evidence and comes down to strict clinical observation and close monitoring for the development of active TB for at least two years for contacts with MDR-TB cases [50].

In conclusion, the benefit of molecular tests in the early diagnosis of TB and drug resistance is unequivocal for adequate and in time treatment of resistant forms of TB. Whole genome sequencing of pathogens, also, is becoming part of routine practice for establishing resistance patterns and transmission tracking. To sum up, the current comprehensive technology has the power to clarify our understanding of the links in MDR-TB transmission between low- and high-incidence areas. Given these points, our report implies that MDR-TB represents not just a regional epidemic in some parts of the world, but also a challenge to international health, including countries with low prevalence of MDR-TB. Knowledge of circulating strains and their resistance patterns is essential to developing effective TB control programs within occupational health, to curtail the transmission of TB in the country and the region. The greater certainty of transmission data can provide evidence to justify more active policies of screening and isolation as part of infection control. Increased vigilance for TB among workers whose job description includes frequent border crossing should be surely taken into consideration as a part of these screening policies. Finally, we believe an urgent need is existing for in-depth discussion between medical and legal authorities about the problem of involuntary confinement and enforced treatment of non-adherent patients. While waiting for the legal resolution of this ethical and social problem, clinicians should consider alternative modalities for the successful treatment of patients with MDR-TB and protect the patients themselves, their families and the community. Regarding this, foreign experience in home-based DOT could be adapted to local conditions and would be a good starting point in moving forward.

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Dental Science

Assessment of Photodynamic Therapy and Nanoparticles Effects on Caries Models

Ali Saafan[1], Mohamed H. Zaazou[2], Marwa K. Sallam[3], Osama Mosallam[2], Heba A. El Danaf[2*]

[1]Dental Laser Applications Department, National Institute of Laser Enhanced Sciences (NILES), Cairo University, Cairo, Egypt; [2]Restorative and Dental Materials Department, Oral and Dental Research Division, National Research Centre, Cairo, Egypt; [3]Medical Microbiology and Immunology, Kasr El Aini Faculty of Medicine, Cairo University, Cairo, Egypt

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***Correspondence:** Heba A EL Danaf. Restorative and Dental Materials Department, Oral and Dental Research Division, National Research Centre, Cairo, Egypt. E-mail: happyeldanaf@hotmail.com

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Abstract

AIM: To assess the antibacterial competence of 650 nm diode laser, Methylene Blue (MB) and Silver Nano-Particles (Ag NPs) on *Streptococcus mutans* in biofilm-induced caries models.

MATERIAL AND METHODS: One hundred eighty specimens were prepared and equally divided into 6 groups. One group was untreated (control), and the others were subjected to either MB, laser, Ag NPs, the combination of MB and Laser or MB, laser and Ag NPs.

RESULTS: Comparison of the log10 mean Colony Forming Units per millilitre (CFU/ml) values of each of the treated 5 groups and the control group was found statistically significant (P-value < 0.05).The combination of MB, laser and Ag NPs recorded the greatest reduction (95.28%). MB alone represented the least capable (74.09%). The efficiency differences among the Ag NPs treated group; the Laser treated group and the combined MB/Laser treated group were found statistically insignificant.

CONCLUSION: The combination of MB, 650 nm diode laser and Ag NPs may be among the highly effective modern antimicrobial therapeutics in dentistry.

Introduction

Dental caries in humans is still among the most prevalent diseases [1]. Caries treatment traditional procedures do not eliminate all the microorganisms in the residual dental tissues [2] [3]. Residual organisms interfere with amalgam or veneer durability in restorative dentistry [4]. Systemic and topical antibiotics and conventional disinfectants together with mechanical cavity preparation of caries do not completely disinfect dental biofilms and caries-related lesions [4].

Experimental chemical induction of caries through enamel demineralisation and direct acid exposure has been criticised as it lacks the bacterial biofilm interactions that characterize real caries formation *in vivo* [5] [6]. The caries-like enamel lesions formed in *Streptococcus mutans (S. mutans)* biofilm models have been found to fulfil all of the principal histological features of natural caries and has been used as a pre-clinical model for evaluation of caries-preventive agents [7] [8].

Modern antimicrobial therapeutics including photodynamic therapy (PDT) and metal nanoparticles (NPs) during caries treatment reflects the need to find out protocols giving the least residual microorganisms after mechanical caries removal. Unlike conventional antimicrobial agents, PDT based on photosensitising agents' activation by a light source is non-invasive, repeatable without developing drug resistance and easily reaches deep situated areas. It may kill microorganisms in a few minutes when the proper energy density is delivered. Its precise lesion selectivity depending on photosensitizers' careful topical application and irradiation site can be further enhanced with optical fibres assistance [9] [10]. The interaction of laser light and dental hard tissues is determined by its wavelength, pulse energy, duration of exposure, and repetition rate [11]. Diode laser

irradiation can penetrate up to 1000 µm into the dentinal tubules; the penetration power of chemical disinfectants is limited to 100 µm [12].

NPs have a greater surface-to-volume ratio than non-nanoscale particles of the same material, and therefore are more reactive [13]. The antimicrobial energy of NPs has been attributed to their multi-cationic and multi-anionic large surface and its positive charge density [14]. NPs combined with polymers or coated onto surfaces show antimicrobial applications within the oral cavity [15] [16]. Silver compounds and NPs have been studied for dental applications including dental restorative material, and caries inhibitory solution [17]. Silver nanoparticles (Ag NPs) have been applied in many health care fields because of their broad-spectrum bactericidal properties [18].

This study aims to evaluate the bactericidal efficiency of the 650 nm diode laser, methylene blue photosensitizer (MB) and Ag NPs on *S. mutans* in induced caries model.

Material and Methods

One hundred and eighty (n = 180) dentin discs from crowns of sound extracted human molars and premolars were obtained using the high-speed diamond disc. Each disc measured in 4 x 5 x 6 mm. Its surfaces were ground flat and polished. All but one of each fragment surfaces was sealed with acid-resistant nail-varnish [19]. Fragments were sterilised in an autoclave for 20 minutes at 121^{o}C. Dentin specimens were pre-conditioned with sterile artificial saliva at 37^{o}C for 2 hours [20], and saliva was then gently aspirated.

The bacterial suspension was prepared from the reference strain of *S. mutans* 1815^{T} bacteria Cultured in Brain Heart Infusion (BHI) Broth, and then sub-cultured onto Mitis Salivaris Agar. The culture was grown under capnophilic conditions for 48 hours at 37^{o}C.

The bacterial suspensions were prepared in BHI broth containing sucrose by transferring colonies from Mitis Salivaris Agar to reach an optical density adjusted to the standard turbidity of 0.5 McFarland units containing 1.5 x 10^{8} CFU/mL.

S. mutans biofilm generation on dentin specimens was formed by mixing the incubated *S. mutans* suspension with the pre-conditioned dentin fragments in the test tube. The culture was daily replaced with fresh new BHI Broth media solution (BHIS) for seven days under appropriate condition to allow for *S. mutans* biofilm formation [19].

After the 7-day incubation, fragments with biofilm were transferred to sterile plain lavender-top tubes and were randomly and equally distributed according to the planned anti-bacterial therapy into the following six groups:

Group I: Two ml MB 0.02 mg/ml was added to each sample and shaken.

Group II: Two ml sterile physiological saline solution was mixed with each sample, shaken and for 3 minutes at 0.5 cm exposed to 650 nm diode laser with 200 mW power.

Group III: Two ml yellow coloured liquid spherical shaped Ag NPs with 200 µg/ml concentration and 19 ± 5 nm particle-size was added to each sample and shaken.

Group IV: After 2 ml MB addition to each sample and shaking, 650 nm diode laser with power 200 mW was swept for 3 minutes at 0.5 cm distance.

Group V: 2 ml MB and 2 ml Ag NPs were added to the samples, and 650 nm diode laser with power 200 mW applied for 3 minutes at 0.5 cm distance.

Group VI: Specimens with biofilm were assigned as a negative control. The sterile physiological saline solution was added to the samples and was shaken.

Groups containing MB were kept covered with aluminium foil in a dark environment at 37°C for 5 minutes.

Serial dilution up to 10^{4} of the inoculums was carried out in sterile Eppendorf tubes. The microbial biofilms were detached from the fragment in a sterile physiological solution (0.9% NaCl), and 25 µl of the dilution was plated onto the surface of BHI agar plates. Plates were then incubated anaerobically at 37ºC for 24 hours in a dark field candle jar for protection against light and air.

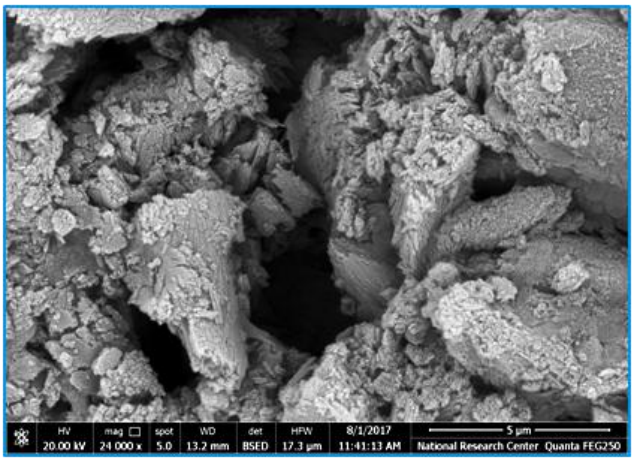

Figure 1: SEM- induced caries lesions on dentin discs

The antibacterial power of the tested therapeutic agents was assessed by:

- *Colony counting:* After incubation, the newly formed colonies were counted after 24 hours of incubation. Then the number of Colony Forming Units per millilitre (CFU/ml) was determined. Suspected colonies were confirmed to be *S. mutans* by Gram staining (crystal violet) under a light microscope.

- *Scanning electron microscope:* For evaluation of the induced caries lesions, dentin discs were rinsed with distilled water for 2 minutes to dislodge the attached biofilm. Dentin specimens were coated with a layer of gold in "S150A sputter coater" machine under vacuum. Then the outer surface was examined with a scanning electron microscope "QUANTA FEG 250" [19], (Figure 1)**.** Bacterial colonies were as well assessed under the same microscope to prove and recognise *S. mutans* bacterial shape Figure 2.

The CFU/ml results were log-transformed (log10) and analysed by the analysis of variance (ANOVA) followed by the Tukey test. A p-value < 0.05 was considered to indicate a statistically significant difference.

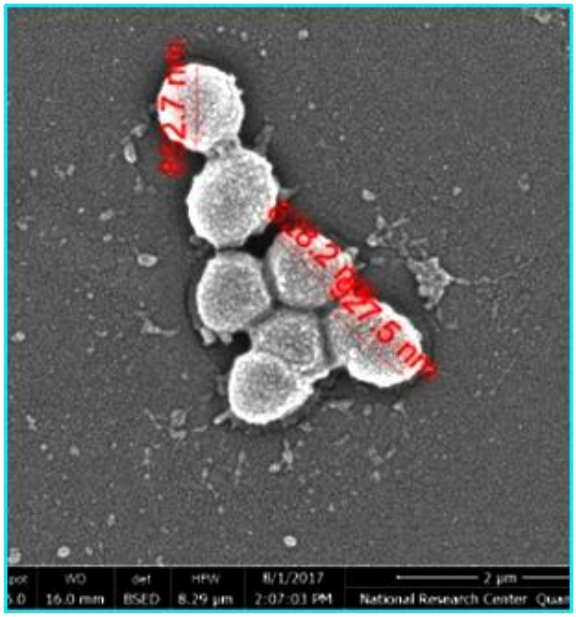

Figure 2: SEM picture of S. mutans

Results

Statistical analysis proved the high efficacy of the treatment with the combined three bactericidal agents namely Ag NPs, MB and Laser 650 nm in comparison with each of them alone. Specimens treated with Ag NPs, MB and exposed to 650 nm Laser recorded the greatest reduction percentage of CFU/ml (95.28%) compared to the control specimens. Group treated merely by 650 nm Diode Laser showed 94.27% reduction and the group treated with both 650 nm Diode Laser and MB gave 89.12% reduction. Specimens treated with MB solely recorded the lowest reduction percentage of CFU/ml (74.09%), Figure 3.

Table 1: Descriptive values of CFU/ml of the specimen's biofilms formed by exposure to S. mutans for different studied experimental conditions

	N	Minimum	Maximum	Mean	Reduction percentage of CFU/ml related to the control group
Control	30	20×10^6	140×10^6	53.23×10^6	-
Ag NPs	30	0.80×10^6	11.60×10^6	4.40×10^6	91.73%
MB	30	ND	52×10^6	13.79×10^6	74.09%
L650	30	ND	6×10^6	3.05×10^6	94.27%
MB+L650	30	0.80×10^6	20×10^6	5.79×10^6	89.12%
Ag NPs+MB+L650	30	ND	10×10^6	2.51×10^6	95.28%

ND means not detected (There were not a single CFU/ml).

There was a statistically significant reduction in the $\log_{10}$mean numbers of CFU/ml after using the combination of Ag NPs, MB and laser 650 nm irradiation in comparison to each of them separately. A significant difference was recorded by comparing the $\log_{10}$ mean CFU/ml values of the control group and the $\log_{10}$ mean CFU/ml values of each of the other groups.

Table 2: Mean values of CFU/ml ($\log_{10}$) and P-value

			P-value related to:					
Log^{10}	N	Mean ± SD	Control	Ag NPs	MB	L650	MB + L650	Ag NPs + MB+ L650
Control	30	7.65 ± 0.25	-	0.0001	0.0001	0.0001	0.0001	0.0001
Ag NPs	30	6.56 ± 0.29	0.0001	-	0.0001	0.785	0.654	0.0001
MB	27	7.07 ± 0.31	0.0001	0.0001	-	0.0001	0.0001	0.0001
L650	29	6.45 ± 0.21	0.0001	0.785	0.0001	-	0.062	0.036
MB+L650	30	6.68 ± 0.28	0.0001	0.654	0.0001	0.062	-	0.0001
Ag NPs+MB+L650	29	6.21 ± 0.44	0.0001	0.0001	0.0001	0.036	0.0001	-

All comparisons among different groups were statistically significant except the two comparisons between the Silver treated group and each of the laser treated group and the combined MB/ laser-treated group Table 1 and 2.

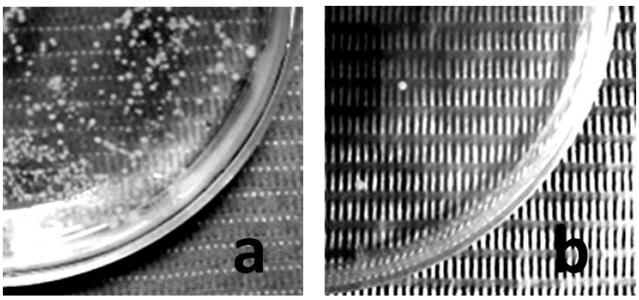

Figure 3: Bacterial colonies counted in different groups; a: control; b: Ag NPs+MB+L650

Discussion

Traditional mechanical cavity preparation of caries lesions does not guarantee complete eradication of bacteria. The principal objective of caries removal is to eliminate infected, necrotic hard tissues and microorganisms that may cause persistent

inflammation and treatment failure. Removal of the infected dentin has a direct influence on the clinical success of the restoration [4]. The caries treatment procedures widely practised presently does not eliminate all the microorganisms in the residual dental tissues [2] [3]. Cavity disinfection is an adjunctive approach to reduce the bacteria in the residual dental tissues left after cavity preparation. Considering the importance of efficient disinfection and elimination of microorganisms from the cavity, this study aimed to assess the efficacy of five different disinfection methods, which could be used as an adjunct to mechanical debridement.

S. mutans was used in this study because it is generally believed that this bacteria is known as the principal cariogenic pathogen [21]. Dental caries involves the adherence of bacteria and development of biofilms on a tooth surface [22]. Biofilm is an aggregate of microorganisms in which cells adhere to each other and to a surface [22]. Formed biofilm confers resistance against antibodies and antimicrobial agents to this microorganism [23]. Different in vitro biofilm models have been described [24] because of their easy handling and simplicity; monospecies batch cultures are preferred in in-vitro secondary caries models. For investigating antimicrobial agents, the cariogenic challenge is induced by the addition of sucrose. Steiner-Oliveira et al., [25] used a *S. mutans* monospecies biofilm model with initial specimen immersion in artificial saliva, growth medium change every 24 h and periodical exposures to sucrose, the same was used in our experiment. Hetrodt et al., 2018 [26] used dentin-enamel discs subjected to a *S. mutans* monospecies biofilm model with 0.5% sucrose to a McFarland turbidity standard of 0.5.

The mechanism of disinfection by PDT is via the irradiation of a photosensitizer. PDT mechanism of bactericidal action may include bacterial cell wall damage; cytoplasm membrane augmented permeability and nucleic acid strand breakage [27]. It is a host-friendly technique for the elimination of microorganisms whereas other methods such as the direct application of antibiotic therapy can be potentially harmful to the host [28]. MB as a photosensitizer combined with the laser irradiation kills the bacterium [29]. Azizi et al., 2016 [30] found that combination of MB and laser irradiation decreases the final number of *S. mutans* colonies more than MB without laser irradiation, which is in agreement with our results. PDT can effectively decrease the number of bacteria present in the biofilm. PDT and Ag NPs have become a trustworthy alternative antibacterial therapy for plaque-related diseases such as dental caries [9] [10]. In the current study, the addition of Ag NPs to the PDT (650 nm Diode Laser and MB) significantly reinforced the bactericidal potential of the applied therapy. In their study, Afkhami et al., [31] used conventional PDT with indocyanine green ICG (1 mg/ml)/810 nm diode laser (200 mW, 30 seconds), modified PDT Ag NPs/ICG/810 nm diode laser (200 mW, 30 seconds) against *Enterococcus faecalis*, diode laser alone 810 nm (1 W, 4 times for 10 seconds), Ag NPs alone (100 ppm). Matching with our results, they found the greatest reduction in colony-forming units with the modified PDT and the lowest with the conventional PDT. Pagonis et al., [32] also used poly (lactic-co-glycolic acid) nanoparticles with MB and red light at 665 nm supportive of combining NPs with PDT.

In 2016, Gomez et al., [33] claimed that *S. mutans* contain endogenous photosensitizer porphyrins and that just 5-minute exposure to 380-440nm wavelength violet-blue light killed *S. mutans* biofilm without any photosensitizer. This may explain the laser 650nm bactericidal effect when used alone in our current work. In the current experiment, the red diode laser was used with sucrose-biofilm, according to the advice given by Gomez et al., [33]. Our results showed laser bactericidal effect (6.45 *log10* and 94.27% CFU/ml) more than laser/MB (6.68 *log10* and 89.12%) with an insignificant difference. This may be due to the presence of endogenous photosensitizer in *S. mutans*. The used MB (0.02 mg/ml) with incubation period (5 minutes) might be insufficient. It has been used with higher concentrations [34] and longer incubation periods [32] for allowing adequate penetration in S. mutans. Afkahami et al., in 2016 [31] reported results showing 97% bacterial (Enterococcus faecalis) reduction with laser (810 nm, 1 W, 4 times for 10 seconds) alone versus 68% with laser/photosensitizer (Indocyanine green (1 mg/mL)/810 nm DL (200 mW, 30 seconds).

Pereia et al., [35] used 0.1 mg/ml MB and 660nm laser, alone and conjugated. They reported that photodynamic inactivation with MB and laser showed a great reduction in CFU/ ml of the *S. mutans* biofilm while laser alone and MB alone did not yield a noticeable bactericidal effect. MB, when used alone in our experiment, showed antibacterial activity. Using broth dilution assay, Soria-Lozano et al., [36] needed 0.025 mg/ml MB and metal halide lamp emitting 420-700 nm to reach 99.9% *S. mutans* inhibition. Araujo et al., [37] showed *S. mutans* bacterial reduction of 73% for MB when these photosensitizers were used at 25 mg/L, and a reduction of 48% was observed for MB at 5 mg/L using a red laser for one minute. Our results were comparable and showed 89.12% bacterial reduction using MB 0.02 mg/ml and 650 nm laser for 3 minutes. Neves et al., [38] assessed the clinical effect of PDT against *Lactobacillus spp.* And *S. mutans in* deciduous molars. They used 0.01% MB dye followed by irradiation with an In GaAlP diode laser (λ-660 nm; 40 mW; 120 J/cm2; 120 seconds). They did not find any significant difference in the number of colony-forming units (CFU) for any of the microorganisms. In our study, MB 0.02 mg/ml and 650 nm laser for 3 minutes 200 mW showed 89.12% bacterial reduction. In our study, 3 minutes, 200 mW were made. In their study, the specimens were only irradiated with a laser

for 120 seconds, 40 mW. The highest bactericidal power depends definitely on the energy density. With the used parameters in our experiment, the score 95.29% was achieved when Ag NPs, MB and 650 nm diode laser co-worked.

Ag NPs can lyse the cells and prevent their proliferation via several mechanisms [39] [40]. Ag NPs show multiple antibacterial mechanisms such as adherence and accumulation on the bacterial surface. Ag NPs damage cell membranes are leading to structural changes, which render bacteria more permeable [41]. Ag NPs prevent DNA duplication and the expression of ribosomes and other cellular proteins. It also interferes with energy transfer cycles of the bacteria [42]. We used Ag NPs with 200µg/ml concentration and 19 ± 5 nm particle-size, and results showed 91.73% decrease in the bacterial count. Cristóbal et al., 2009 [43] detected the minimum inhibitory concentration of AgNPs against *S. mutans* in with sucrose addition as 101.98 ± 72.03µg/ml for 8.4 nm, 145.64 ± 104.88 µg/ml for 16.1nm and 320.63 ± 172.83 µg/ml for 98 nm. Sucrose addition enhances the cariogenic power of *S. mutans* and gives more realistic results because it's almost always present in our diet [43]. The smaller the nanoparticle, the more it releases Ag+ ions, and their antibacterial effect can be better [43]. Holla et al., in 2012 [20] set 40 µg/ml as the minimum inhibitory concentration and minimum bactericidal concentration needed for nano-silver base inorganic anti-microbial agent against *S. mutans in vitro* using broth dilution assay.

In vitro biofilm caries models have been widely used to study the carious process under laboratory controlled conditions, in an attempt to simulate the clinical development of carious lesions [7] [44]. Dental caries results from interactions among different cariogenic microorganisms. In our study *S. mutans,* monospecies biofilm model was used. Differences in experimental conditions may explain discrepancies between in vitro under controlled conditions and in vivo studies. Whereas monospecies biofilm models based only on the cultivation of *S. mutans* [45] [46] do not mimic the metabolic interactions that occur among the diverse microbiota of a clinical dental biofilm, the outcomes obtained by high-complexity microbial models based on microcosm cultivation are directly dependent on inoculum source [47]. Even though a model cannot capture all of the details involved with caries formation, it can give us a means of performing reproducible experiments under controlled conditions [48].

In conclusion, this in vitro study recognises that the addition of Ag NPs to diode laser and MB enhance their antibacterial efficiency against *S. mutans* in caries models. This modern therapeutic combination has a high potential for use in operative dentistry for *S. mutans* eradication.

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Prevalence of Malocclusion among Male School Children in Riyadh City

Fahad M. Albakri*, Navin Ingle, Mansour K. Assery

Advance Education General Dentistry and Dental Public Health, Riyadh, Saudi Arabia

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***Correspondence:** Fahad M Albakri. Advanced Education General Dentistry and Dental Public Health, Riyadh, Saudi Arabia. E-mail: f.albakry_407@hotmail.com

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Abstract

BACKGROUND: Malocclusion is defined as irregularity of the teeth or a molar relationship between the dental arches beyond the range of what is accepted as normal.

AIM: To determine the prevalence of malocclusion among male school children aged 12-15 years old in Riyadh, Saudi Arabia.

MATERIALS AND METHODS: Five hundred (500) school children in Riyadh city, Saudi Arabia with an age of 12-15 years participated in this study. The prevalence of malocclusion among the students was determined using a clinical examination form specially prepared for this study. The required information was collected from each subject, and descriptive statistics were performed.

RESULTS: The Molar Class I relation involved the highest percentage of the sample (71.2%) while Class II relation involved only 23% which was four times of Class III (5.8%). The maxillary arch crowding was present in 23.2% of the sample which was double than that of spacing. Whereas, the mandibular arch crowding was present in 28% of the sample which was three times more than spacing (8.8%). The open bite was present in 4% of the sample while deep bite was present in 9.6%.

CONCLUSION: The prevalence of malocclusion involved the highest percentage in Class I in comparison with other malocclusions.

Introduction

The high prevalence of malocclusion has made it a public health problem in the world; it is now considered the third highest oral health priority [1][2]. “A malocclusion is defined as irregularity of the teeth or a molar relationship between the dental arches beyond the range of what is accepted as normal [3]. Malocclusion is one of the most common dental problems as well as dental caries, periodontal disease, and dental fluorosis [1]. Also, maloccluded dentition can cause disturbances in oral function and psychosocial problems due to impaired dentofacial [4][5]. The prevalence of malocclusion during the mixed dentition period among different races and populations had been published by many authors [6] [7] [8] [9] [10] [11]. Despite the amount of literature on the subject, no study, to the best of our knowledge, was done across a huge geographically and ethnically identical Saudi male children in Central Region (Riyadh city) other than the study of the Prevalence of malocclusion and need for orthodontic treatment conducted by Al-Emran et al., (1990) [6]. Therefore, the purpose of this study was to determine the prevalence of malocclusion among male school children aged 12-15 years old in Riyadh city.

Materials and Methods

This cross-sectional study was conducted among 12-15 years old male school children in Riyadh city, Saudi Arabia. Sampling included sample size calculation, determination of age sample, selection of schools and subject. A multi-stage stratified random sampling technique was used in selecting the schools. A group of 500 Saudi male schoolchildren representing those age ranged between 12 and 15 were randomly selected from governmental and

private school for the study. These schools were categorised into five sections according to the geographic location of different parts in Riyadh City. These were Central (2 schools), Eastern (2 schools), Western (2 schools), Northern (2 schools) and Southern areas (2 schools).

The inclusion criteria were as follows; parent`s and child were originally from Saudi, child`s age between 12 and 15 years old, the child born and lived in the included area and of good health. The exclusion criteria were as follows: children or parents refused to participate in the study, children that have not completed the examination, craniofacial anomalies (clefts and syndromes), who were undergoing or had a history of orthodontic treatment and extracted of permanent teeth, impaction and delayed eruption of permanent teeth.

A full clinical examination was carried out in the school premises using a dental examination kit and special clinical form prepared for this study. During the examination, the following information was collected from each subject: molar relationship, canine relationship, incisors relationship, crowding, spacing, anterior and posterior crossbite, anterior overbite, open bite and overjet.

Ethical approval was officially obtained from the Scientific and Ethical Committee, Riyadh Elm University. After approval, the Riyadh Elm University has sent official letters to the Ministry of Education to justify the purpose and importance of conducting the study; explain the safety of the procedures; confirm the confidentiality of collected data and confirm that the participation is voluntary. Parents of the targeted children were contacted officially through letters which clearly and simply explained the study purpose, procedures, data confidentiality and voluntary participation in the study. All letters were received and signed by the parents of the participated children. Similarly, targeted children, whose offered parents' agreement, were verbally informed about the study purpose and procedures. Any child who has needed dental treatment was referred to Faculty of Dentistry, Riyadh Elm University for treatment.

All Information collected in the clinical forms were transferred into a spreadsheet and subsequently entered into the SPSS (Statistical Package for Social Sciences) software version 21. Descriptive statistics were generated to check for discrepancies and consistencies in the overall data.

Results

What the total number of the students participated in this study was 500 students aged 12-15 years. Table 1 showed the molar relationship of the study sample and the distribution of occlusion. Molar Class I relation involved the highest percentage of the sample (71.2%) while Class II relation involved only 23% which was four times more than Class III.

Table 1: Molar relationship

Type	Class I	Class II		Class III		Total
		Uni	Bi	Unl	Bl	
N=	356	34	81	10	19	500
%	71.2	6.8	16.2	2	3.8	100%
%	71.2	23		5.8		100%

The canine relationship showed the highest value among Class I relationships (68%). Class II bilateral is more than two times of that of unilateral (18.8% and 8% respectively), while bilateral Class III is more than three times of that of Unilateral (4% and 1.2% respectively). In general Class II canine relationship is about 5 times of that of Class III (Table 2).

Table 2: Canine relationship

Type	Class I	Class II		Class III		Total
		Uni	Bi	Uni	Bl	
N=	340	40	94	6	20	500
%	68	8	18.8	1.2	4	100%
%	68	26.8		5.2		100%

Regarding crowding and spacing of both arches (Table 3 & 4), the maxillary arch crowding was present in 23.2% of the sample which is double than that of spacing. Whereas, the mandibular crowding was present in 28% of the sample which is three times more than spacing (8.8%).

Table 3: Maxillary crowding and spacing

Type	normal	Crowding		Spacing		Total
		≤ 2mm	> 2mm	≤ 2mm	> 2mm	
N=	326	76	40	42	16	500
%	65.2	15.2	8	8.4	3.2	100%
Total %	65.2	23.2		11.6		100%

The anterior crossbite was found in 14 individuals who represent 2.8% of the total sample. The posterior crossbite was present in 30 individuals. Additionally, the posterior crossbite was present bilaterally in 23 students (4.6%) and unilaterally in 7 students (1.4%).

Table 4: Mandibular crowding and spacing

Type	Normal	Crowding		Spacing		Total
		≤ 2mm	> 2mm	≤ 2mm	> 2mm	
N=	316	120	20	34	10	500
%	63.2	24	4	6.8	2	100%
Total %	63.2	28		8.8		100%

Table 5 shows the anterior overbite relation. The prevalence of anterior open bite was observed in 4% (n = 20) from the overall subject. The overbite of 0:1/3 of the clinical crown was present in 45.2%, while that of 1/3:2/3 was seen in 41.2% of the sample. Cases of 2/3:3/3 of the deep bite was found in 8.8% of students, whereas, deeper bite (< 3/3 of clinical crown length) were present in 0.8% only. Vertical

relationship of the occlusion on the buccal segment (Posterior open bite) was found 0% (n:0) from the overall subjects.

Table 5: Anterior Overbite and open bite relation

Type	> 0	0: 1/3	1/3: 2/3	2/3: 3/3	< 3/3	Total
N=	20	226	206	44	4	500
%	4	45.2	41.2	8.8	0.8	100%

The sagittal relationship of the jaws (overjet) was summarised in table 6. A reverse overjet was observed in 14 students (2.8%). Normal overjet (0:4 mm) was seen in 377 students (75.4%) while a slight increase in overjet (4:6 mm) was found in 76 students (15.2%). Severe increase in overjet (6:9 mm) was seen in 33 students (6.6%).

Table 6: The overjet relationship

Type	> 0 mm (reverse overjet)	0: 4 mm	4:6 mm	6:9 mm	Total
N=	14	377	76	33	500
%	2.8	75.4	15.2	6.6	100

Discussion

In the present study, Class I molar relation showed 71.2% of the whole sample. This was in agreement with that of other studies conducted in Sweden [7] [8]. Higher proportions were found among Brazilian [9], Tanzanian [1] and Libyan [10] populations, whereas, lesser values were reported in Jordan [11], Kuwait [12], Turkey [13], Iran [14], Italy [15], Croatia [16], Hangiri [17] and Nigeria [18].

Class II division 1 in this study was seen in 17.4% of the sample. This was in line with the results obtained in Jordan [11], while a slightly lower value was seen in Libya [10]. Higher values were recorded among Kuwaitis [12], Turkish [13], Iran [14], Italy [15], Croatian [16], and Hungarian [17], whereas, fewer values were reported among Nigerian [18], Tanzanian [1] and Swedish population [7] [8].

Class II division 2 was 3.4% in this study which is in line with that of Iranian [14]. Fewer values were reported among Libyan [10] and Nigerian populations [18], whereas, higher values were found in Sweden [7] [8], Hungarian [17], and Turkey [13].

Class III cases (5.8%) in this study showed a similar value to that of Libyan [10]. However, it was less than that reported among Kuwaitis [12], Turkish [13], Iranian [14], and Hungarian [17] and higher than that found among Brazilian [9], Swedish [7] [8], Croatian [16], Italy [15], Tanzanian [1] and Jordanian [11].

The Canine relationship in the current study was 68% in Class I. lesser values were reported among Kuwaitis and Nigerian [12] [18]. Class II canine was found to be 26.8 in this study. This was in close relation to that of Nigerians [18] but lesser than that of Kuwaitis [12]. Class III canine was 5.2% in this study which is less than that of Kuwait [12] but higher than that Nigeria [18].

In the current study, crowding of the maxilla showed a lower value than that of the mandible (23.3% and 28% respectively). This was in agreement with the results observed among British [19], Libyan populations [10] and the Maxillary crowding of Sweden [7] [8]. Higher values were seen in Brazil [9], Croatia [16], Italy [15] and Jordan [11], whereas, lower values were seen in Tanzania [1], Hangiri [17], Nigeria [18], and Iceland [20].

In the present study, the spacing of the maxillary and mandibular arches are 11.6% and 8.8% respectively. These are higher than that found among British [19], Icelandic [20], Swedish [7][8] and Croatian populations [16], and lesser then that found among Hungarian [1], Tanzanian [1], Colombian [21], Iranian [14] and Libyan populations [10].

About anterior open bite, the Saudi sample show 4%. This was approximately similar to that found in Kuwait [12], Sweden [7] [8] and Croatia [16]. Very higher values were found among Tanzania [1], Brazil [9], Turkey [13], Colombia [16], British [19], French [22], and German [23], whereas, lesser values were found among Icelandic [20] and Jordanian populations [11]. In the current study, the deep bite (equal to or more than two-thirds of the clinical crown) was found to be 9.6%. Higher values were seen among Caucasian [24], Colombian [21], Nigerian [18], Kuwait [12], Icelandic [20], Turkish [13] and Iranian population [14] whereas, lesser values were seen Tanzanian [1], French [22], German [23], and Chinese populations [25].

Regarding overjet, the normal overjet was seen in 75.4% of the sample. An increase in overjet (4:6mm) was seen in 15.2% while more increase in overjet was seen in 6.6% of the sample. Higher overjet was seen among Caucasian [24], Chinese [25], Colombian [21], Kuwaitis [12], Jordan [11] Turkish [13], Icelandic [20] and German [23]. Lesser overjet were recorded among Tanzanian [1] and the Nigerian population [18]. The reverse overjet in this study was seen in 2.8%. higher values were seen in China [25], Colombia [21], Kuwait [12], Turkey [13], Tanzania [1] and Iran [14], whereas, lesser values were recorded among Jordanian [11], Nigerian [18], German [23] and Caucasian populations [24].

Anterior crossbite was found to be 2.8% in this study. Lesser values were found among Icelandic [20] and Croatian populations [16]. Much higher values were recorded in Jordan [11], Iran [14], Colombia [21] and Germany [23]. The unilateral and bilateral posterior crossbite in this study showed a prevalence of 1.4% and 4.6% respectively. Unilateral posterior crossbite showed higher values among

Kuwaitis [12], Turkish [13], Iranian [14], Croatian [16], Hungarian [17], Colombian [21] and German [23] populations. Bilateral posterior crossbite showed similar value to that of Turkish [13]. Lesser values were seen among Iranian [14], Hungarian [17], Colombian [21] and Caucasians [24], whereas, the higher value was recorded in Kuwait [12]. From the above, the differences in results could be attributed to the different ethnic groups and also to differences in age distribution as well as the sample size.

In conclusion, (i) the prevalence of malocclusion of Saudi male school children aged 12-15 years showed the highest percentage in Class I in comparison with other malocclusions and (ii) the baseline information outlined in the present study can be appropriately used for the future planning to meet the orthodontic treatment need among the Saudi population.

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Tuberculosis in the Prisons in the Republic of Macedonia, 2008-2017

Biljana Ilievska-Poposka*, Maja Zakoska, Karolina Pilovska-Spasovska, Ljiljana Simonovska, Vladimir Mitreski

Institute for Lung Diseases and Tuberculosis, Skopje, Republic of Macedonia

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***Correspondence:** Biljana Ilievska-Poposka. Institute for Lung Diseases and Tuberculosis, Skopje, Republic of Macedonia. E-mail: biljana.ilievska@yahoo.com

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Abstract

BACKGROUND: Tuberculosis (TB) is a major health problem in penitentiary institutions (prisons), and its prevalence was reported to be multiple times higher compared to that of the general population. Conditions such as overcrowding, malnutrition and limited access to medical care which often exist in prisons increase the risk of reactivation, transmission and poor prognosis of tuberculosis disease among inmates.

AIM: The main objective of this study was to present the epidemiological situation of TB in the prisons from 2008 till 2017 in the Republic of Macedonia (RM).

PATIENTS AND METHODS: There are 13 different penitentiary institutions in the RM with a total capacity to house 2600 prisons inmates. Management of TB in the prisons is part of the National TB program in RM, and the Institute for Lung Diseases and Tuberculosis in Skopje is in charge of it. All prisoners with TB in the RM are registered in the Central Register for TB within this Institute. We use the data from the Central Register, and with the method of description, we present the epidemiological data and clinical characteristics of the prisoners about TB in prisons in RM for 10 years' period.

RESULTS: From 2008 till 2017 there were 58 TB cases registered in prisons in total. The absolute number of TB cases in the prisons is not big, but the incidence rate is higher than 100/100,000 population, or several times bigger than in the general population (except in 2012 and 2016). In 2017 there were 10 TB cases registered in the prisons with an incidence rate of 323.9/100,000 population which is many times higher than in the general population in RM. The majority of inmates with TB were young men with risk factors for TB infection or TB disease before incarceration such as drug abuse, alcohol, smoking, but there was no association with HIV infection. The most of the patients diagnosed in prison were new cases (54), secondary TB due to reactivation from the latent TB infection or secondary TB due to the environment. From 2008-2017 there were 82.75% successfully treated TB cases in the prisons, and there were no cases of multi-drug resistant tuberculosis (MDR-TB).

CONCLUSION: The results from our study showed that the TB control in the prisons in RM is good with satisfactory treatment outcome. On the other hand, the high incidence rate showed that the prisons in RM provide conditions for TB transmission and with other additional risk factors present place for high TB prevalence. The study findings can be used for planning more effective TB control interventions for the prison population in RM.

Introduction

According to the World Health Organization (WHO), the prevalence of tuberculosis (TB) in prisons is very high, accounting for up to 25% of the TB burden in high-incidence countries, and is reported to be 10- to 100-fold higher than in the general population, in both low- and high-incidence countries [1]. In prisons located within developing countries, TB has been reported as the most common cause of death [2]. Conditions such as overcrowding, malnutrition and limited access to medical care which often exist in prisons increase the risk of reactivation, transmission and poor prognosis of tuberculosis disease among inmates [3] [4]. These factors could contribute to prisons to act as reservoirs of infection transmission [5]. Prisoners are overwhelmingly male, are typically aged 15–45 years, and come predominantly from poorly educated and socioeconomically deprived sectors of the population where TB infection and transmission are higher. Offenders often belong to minority or migrant groups and live on the margins of society. Prisoners are also more likely to suffer from other debilitating diseases and have additional health problems such as drug addiction, alcoholism and liver disease [6]. Prison health services are often minimal or nonexistent due to insufficient funding, and in many cases lack human rights. Prisoners are often admitted to cells without being given a health check and are mixed in confined settings ideal for the spread of disease [6]. Moreover, prisons represent a reservoir for disease transmission to the community at large; the TB infection may

spread into the general population through prison staff, visitors, and close contacts of released prisoners [7]. Overlooking TB prevention and control in prisons settings can carry serious consequences for both prisoners and the general community, in particular in those countries where poor TB control, lack of TB infection control measures, and incarceration rates are high [2].

In 2017, there were 220 new TB cases in the Republic of Macedonia (RM) with an incidence rate of 10.6/100,000 population [8]. We have reported continual decreasing of the TB incidence rate from 2002 till now [8]. But, prisoners are still high-risk groups with incidence rate several times higher than those in the general population [8]. An absolute level of TB prevalence or incidence may be used as a cut-off to define a risk group in a given epidemiological situation. For example, in Europe, where TB notification often is a good estimate of TB incidence, risk groups have been defined as those in which TB notification is more than 100/100,000 population, which is considerably higher than the incidence in the general population in the region [9].

The main objective of this study was to present the epidemiological situation of TB in the prisons from 2008 to 2017 in the RM.

Patients and Methods

All prisoners with TB in the RM are registered in the Central Register for TB within the Institute for lung diseases, and TB and all data regarding their diseases are kept there. For the aims of this study we use the data from the Central Register, and with the method of description, we present the epidemiological data and clinical characteristics of the prisoners regarding TB within the prisons in RM for 10 years" period. The analyses of the clinical characteristics are regard to the gender and age of the prisoners, present risk factors for TB, HIV status, and data of the previous TB treatment, localisation of the TB, bacteriological findings and treatment outcome.

Results

In Figure 1 we present the epidemiological data regarding the absolute number of TB cases in the prisons, the incidence rate of TB in the prisons and the incidence rate among the general population. During this 10 years' period, there were 58 TB cases within prisons in total. The absolute number of TB cases in the prisons is not big, but the incidence rate in the most of years is higher than 100/100,000 population, or several times bigger than in the general population (except in the 2012 and 2016). In 2017, there were 10 TB cases registered in prisons with an incidence rate of 323.9/100,000 population. In comparison, the incidence rate in the general population in RM was 10.6/100,000. There are a lot of variations during this period, but the prisoners belong to the risk group of TB in RM.

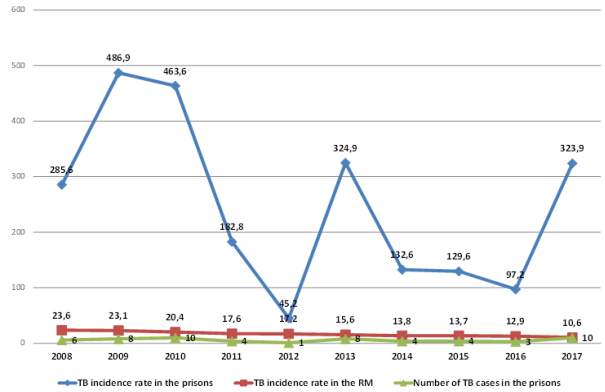

Figure 1: The absolute number of TB cases, TB incidence rate within prisons and the incidence rate among the general population

There was great variability among the prisons: the highest TB prevalence was observed in prison "Idrizovo" in Skopje which is the biggest prison in the country: 40 TB cases were from the prison "Idrizovo" in Skopje (68.95%), followed by prison "Skopje" in Skopje with 7 TB cases (12.06%) and 11 TB cases were from the other prisons (18.96%).

Among the total number of 58 prisoners with TB, 57 were male (98.2%), and only 1 case (1.72%) was a female.

Considering the most of TB cases were new-54, and only 4 were previously treated cases: 2 failures, 1 relapse and 1 after interruption of the treatment.

HIV testing had only 24 TB cases in the prisons, and all results were negative.

The youngest prisoner with TB was 20, and the oldest was 59 years old. In table 1, the distribution of the TB cases according to the age groups was presented. The most frequent were young cases between 25-34 years old (47.3%). 88.6% of prisoners are in their productive age group, from 25 to 54.

Table 1: Distribution of TB cases in prisons according to the age groups

Age groups	15-24	25-34	35-44	45-54	55-64	Total
Number	5	27	17	7	2	58
%	8.6	47.3	29.3	12.0	3.4	100.00%

The analysis showed that many of the prisoners were from population groups already at high risk of TB infection and TB disease before incarceration (Table 2).

Table 2: Risk factors present in TB cases before incarceration

Risk factors	Drug users	Drug users&smoking	Drug users&smoking&alcohol	Smoking&alcohol	Smoking	Risk factors -total
Number	5	12	3	3	14	37
%	8.62	20.68	5.17	5.17	24.13	63.79

Forty cases (70.6%) in the prisons were with pulmonary TB (PTB), 7 cases were with both localisation: PTB and extra PTB (EPTB) which means that the total number of cases with PTB was 47 (80.9%) - Table 3. Among the 11 cases with EPTB, 6 were with TB pleurisy, 2 with skin Tthe B, 1 with lymph node TB, 1 with urogenital TB and 1 with TB meningitis. EPTB among all 6 cases with PTB/EPTB were TB pleurisy.

Table 3: Localisation of TB among TB cases in the prisons

Localisation of TB	Number	%
PTB	41	70.6
PTB/EPTB	6	10.3
EPTB	11	18.9

The results from bacteriological examinations in the sputum at the time of diagnosis were as it follows: microscopy the positive (M+) were 30 TB cases, and microscopy negative (M-) was 28; culture positive (C+) were 44 and culture negative (C-) were 14. Bacteriological findings in the sputum are presented in Table 4.

Table 4: Bacteriological findings in the sputum of 58 TB cases in the prisons

Bacteriological findings	M+/C+	M+/C-	M-/C+	M-/C-
Number	29	1	15	13
%	50	1.72	25.86	22.41

The bacteriological analyses in the sputum in all 11 cases with EPTB were M- and C-. In 3 cases out of total 6 cases with TB pleurisy, there were M-, C- and GeneXthe pert- results I,n the pleural fluid. Among the other 3 cases, the results were: in the pleural fluid from one case M-, C-, GeneXpert +, in the other one M-, C+, GeneXpert-, and in the third M, C and Gene Xpert were + simultaneously. The patient with TB meningitis had negative bacteriological findings (M-/C-) in the cerebrospinal fluid.

Regarding the resistance of *Mycobacterium tuberculosis*, there were no cases with MDR-TB. In one case there was resistance to 1 drug, in another one resistance to 2 drugs and the third, there was resistance to 3 drugs (Table 5).

Table 5: Resistance to Mycobacterium tuberculosis

Resistance to Mycobacterium tuberculosis	Number of TB cases
Ethambutol	1
Streptomycin and Isoniazid	1
Streptomycin, Ethambutol and Rifampicin	1

The treatment outcome in the prisons is presented in Table 6: 17 TB cases (29.31%) were cured, and 31 (53.44%) completed the treatment, which means there were high rates of successfully treated TB cases: 48 (82.75%).

Table 6: Treatment outcome of TB cases within prisons, 2008-2017

Treatment outcomes	Number of TB cases	%
Cured	17	29.31
Treatment completed	31	53.44
Treatment interrupted	5	8.62
Loss of evidence	2	3.44
Died	1	1.72
Still on treatment	2	3.44

Discussion

All prisons in the Republic of Macedonia are under control by the Ministry of Justice - Administration for sanctioning and all procedures are regulated by the Law on the execution of sanctions. There are 13 different facilities in the different cities within the whole country: 9 prisons, 3 penitentiary homes and 1 correctional home with a total capacity to house 2,600 prisons inmates. According to the rights of the prisoners, the prisons in the RM are divided into three different types: closed type, semi-open and open type. There are possibilities for transferring the prisoners from one to another facility which is an important moment, especially in the case when the prisoner is a TB patient. Compulsory medical services are in function within all prisons. The prisoners have access to medical services all the time while their stay in the prisons. Management of TB in prisons is part of the National TB program in RM, and the Institute for Lung Diseases and Tuberculosis in Skopje is in charge of it. The “Protocol for Tuberculosis Control within Prisons in the Republic of Macedonia” is applied where the written rules for diagnosis, treatment and follow-up of the TB in the prisons exist.

Regarding the diagnosis of TB, there is a possibility for active screening and passive case findings. For active screening there are the following: at time of entry in prisons screening for TB is done with a questionnaire for TB symptoms and chest X-ray; once a year there is a screening with fluorography which is performed in all prisons, and also the questionnaire for symptoms is filled out by the prisoners, also on annual basis. With the passive case findings, every prisoner who is suspected for TB is sent for a check-up to a hospital or dispensary for lung diseases and tuberculosis. When the diagnosis of TB is confirmed, TB cases are hospitalised in a separate hospital department for prisoners located close to the Institute for lung diseases and TB where the TB cases stay and will be treated during the initial phase of treatment. After the initial phase of treatment, or after the bacteriological conversion in the sputum, the prisoners will be brought back in the prisons and will continue with the continuous phase of treatment.

During this phase, all controls are performed in the hospitals or dispensaries. The “Protocol for the Control of TB within Prisons in the Republic of Macedonia” is accepted by the Ministry of Justice and the Institute for Lung Diseases and Tuberculosis.

Even there is continuous decreasing of TB cases among the general population in the RM from 2002 till 2017, there are still few risk groups, like prisoners where the incidence rate is several times higher than in the general population or is above 100/100,000 population [8]. The analyses showed that the majority of prisoners with TB were young men with risk factors for TB infection or TB disease before incarceration. The highest prevalence of TB cases was detected in the prison of “Idrizovo”, which is the biggest prison in RM where the buildings are very old and run down to stay in. According to the “Protocol for TB Control within Prisons in the Republic of Macedonia” the prisoners should pass at time of entry in prisons screening for TB with a questionnaire for TB symptoms and chest X-ray, but usually they filled out the questionnaire only. The most TB cases are detected with annual fluorography performed, or by passive finding through the medical service in the prisons. The results have shown that the majority of detected TB cases are newly registered cases which mean reactivation from the latent TB infection or new infection from the surrounding; only 4 cases had the history of the previous TB. We have found that drug use, alcohol, and smoking were strongly associated with TB in prisoners. But, there was no association of TB and HIV infection in the prisons.

On the other hand, all detected TB cases in the prisons within the RM were regularly treated with standard TB regime, and they were followed in a regular way and treated successfully which is in concordance with good treatment results in all TB cases within prisons from 2008-2017. There were 82.75% successfully treated TB cases, and there were no registered MDR-TB cases. There is satisfactory cooperation between the medical service in the prisons and the hospitals and the dispensary for lung diseases and TB. The National TB program in RM is aware of all the weaknesses and challenges in the management of TB within prisons and of the consequences that might be a result of the TB control within the community.

According to the data from the literature, TB in prisons is a big problem worldwide: In European prisons, the prevalence of TB is estimated to be up to 17 times higher than in the general population [10]. A similar epidemiological situation has been described in low- and middle-income countries including Bangladesh, Thailand, Ethiopia, and Brazil, where TB prevalence has been reported to be almost four-, eight-, seven-, and 64-times higher, respectively, among prisoners, compared to the general population [11] [12] [13] [14] [15]. In this study we did not report any case with TB/HIV co-infection in contrast with other study were HIV prevalence increased from 20% at entry to 34% at the exit, suggesting possible disease transmission within the prison [16]. The death rate in the prisons in RM is very low, in comparison with the data from the literature where deaths due to TB are between 9 to 18 % of total deaths [17]. High levels of MDR-TB have been reported from some prisons with up to 24% of TB cases suffering from MDR forms of the disease. In the systematic review by Dara et. all, the main challenges in regard to TB control in the prisons are lack of well organized health services, quality of bacteriological services, the high turnover of the prison population between prisons and to the wider community, collaborative TB/HIV activities, political commitment [18]. In a systemic review done by Vinkeles Melchers at al. and Abrahão at al. was found that approximately 21% of all studies reporting on TB screening in prisons described the lack of a well-organized health system [19], potentially leading to the ongoing spread of TB to other prisoners, prison staff, visitors, and to the general population upon release from the prison [20]. Data from a systematic review identified that 31.2% of studies struggled with effective TB control due to loss to follow-up and a high turnover of prisoners [19].

Consequently, difficulties may be encountered in diagnosing and treating TB, leading to the further spread of infection to other inmates, prison staff, and visitors [20]. In light of the challenge of overcrowding associated with increased rates of TB in both the prison and community setting, TB infection control is a fundamental element for improved TB control [21] [22]. Globally, WHO and The International Union Against Tuberculosis and Lung Disease have recommended education on early identification of TB and early case management, screening of all inmates to prevent infection transmission, isolation of infected person (known TB patients), the right of inmates to access medical services, and to integrate TB services within prisons with the national tuberculosis programmes [9] [23]. The implementation of these measures is, however often hampered by resource constraints specific to the prison setting. Prison health services often have small budgets [24] which, in addition to the lack of skilled and motivated workforce, may jeopardise successful TB control programmes in prisons [11] [15].

In conclusion, globally there is a great concern to address TB in prisons and strategies propose directly establishing a system for early identification through a process of entry and on regular intervals. Every successful TB control program also requires effective TB control in prisons and failure to control TB in prisons has the potential to disrupt community TB control programs [25]. The requirements for enhanced TB control in prisons are good governance, clear strategies to diagnose and treat TB patients, adherence to internationally established IC policies, and the performance of cost-effectiveness analyses to evaluate screening procedures and other control strategies. The results

from our study showed that the TB control in the prisons in RM is good with satisfactory treatment outcome. On the other hand, the high incidence rate showed that the prisons in RM provide conditions for TB transmission and with other additional risk factors present place for high TB prevalence. The study findings can be used for planning more effective TB control interventions for the prison population in RM.

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Effect of Motivational Interviewing on Using Intrauterine Device in Women at High Risk for Pregnancy

Katayoon Vakilian[1], Sajedeh Molavi[1*], Ahmad Reza Zamani[2], Masoumeh Goodarzi[2]

[1]Arak University of Medical Sciences, Arak School of Nursing and Midwifery, Arak, Iran; [2]Isfahan University of Medical Sciences, Isfahan, Iran

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***Correspondence:** Sajedeh Molavi. Arak University of Medical Sciences, Arak School of Nursing and Midwifery, Arak, Iran. E-mail: s.molavi@arakmu.ac.ir

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Abstract

BACKGROUND: Reproductive health programs help women live healthier. Family planning consultation is an important component of reproductive health. A good family planning consultation can increase the use of effective birth control methods and improve the life quality of people, specifically of those at high risk. One of the most effective birth control techniques is an intrauterine device (IUD).

AIM: This study was done to investigate the effect of motivational interviewing (MI) on the use of IUD in women at high risk for pregnancy.

METHODS: This random educational trial was conducted in Isfahan on 44 women at high risk for pregnancy in 2015. Subjects were selected through random sampling. First, written informed consent of all samples was gathered, and then the intervention was made in five 45-minute motivational interviewing sessions by the researcher. For this study, a questionnaire was created to measure knowledge, attitude, acceptance, and performance. All the subjects filled this instrument prior and one month after the intervention. Data were then analysed using SPSS 20. The employed statistical tests included dependent t-test, independent t-test, Mann-Whitney, Fischer's exact test, and McNemar's test.

RESULTS: Results suggested that MI significantly improved the use of IUD in the intervention group ($p < 0.001$). Also, the mean knowledge ($p < 0.001$) and attitude ($p < 0.001$) scores significantly increased in the intervention group.

CONCLUSION: Due to the role of MI in increasing the use of IUD, embedding this type of consultation in specific programs for changing health behaviours during pregnancy can be beneficial.

Introduction

Reproductive health programs help women have a healthier life. The objective of reproductive health is the promotion of healthy behaviours to maintain health among people and society and decrease familial and marital problems. Reproductive health requires careful use of effective methods in favour of family health [1].

Non-use of contraceptives increases the rate of unwanted pregnancies and abortion, as one out of every twenty pregnancies is unwanted in the U.S. which varies depending on women's age range, race, and socioeconomic status [2]. Family planning programs help couples control the number and interval of pregnancies in a free and responsible fashion [3] and prevent many high-risk and unwanted pregnancies. From a public health perspective, the most common contraceptive used globally is an intrauterine device (IUD) whose application ranges from 2% in Sub-Saharan Africa and 80% in Asia. Clinical factors such insertion and removal costs, previous history of application, side effects, accessibility, and healthcare provider's recommendation and individual factors such as the acceptance of this method regarding cultural, economic, social, and religious considerations affect its application [4]. Inaccurate perceptions such as fear of pain, of IUDs being larger than the genitalia, and of sexual dysfunction have caused Iranian women not to be willing to use this method. Therefore, family planning counselling can contribute greatly to altering

such perceptions [5]. Family planning counselling is a two-way relationship between the client and the consultant in which the consultant encourages the client to use a safe contraception method [6]. Despite the existence of numerous family planning counselling clinics and easy access to them, the rate of unwanted pregnancies and abortions is still high [7] [8]. Of an estimated 210 million global pregnancies, 38% are unplanned, out of which 22% end in abortion. According to the statistics, every 3 minutes a woman dies of abortion complications, accounting for 13% of all maternal deaths worldwide (99% out of which occur in developing countries); whereas, 25% of maternal deaths can be controlled by preventing unwanted pregnancy and its complications [3] [9]. According to the latest statistics, the incidence of unplanned pregnancies in Iran is about 24% to 40% with 221 abortions per day. This high rate of unwanted pregnancies is a warning to the society, asking for special attention to those at high risk. Many of women at high risk with an unplanned pregnancy do abortion, mainly through unsafe methods, which are associated with the risk of mortality and maternal complications [4] [10]. Four main causes of maternal death in Iran are pregnancy under 18 years of age, pregnancy intervals of less than three years, frequent pregnancies, and pregnancy after 35-40 years of age [5] [11]. These are considered as four high-risk pregnancy factors. Making correct decisions in areas such as family size, pregnancy intervals, and other reproductive health matters in the family requires adequate knowledge and right insight, which can only be gained through proper consultation

The revision of consultation methods seems to be one of the solutions in preventing high-risk pregnancies.

Motivational interviewing (MI) is counselling approach introduced by Miller and Rollnick and was first used for alcohol abuse control. Since the first clinical description of MI by Miller in 1983, it was increasingly researched and used in treating various problems such as substance abuse, gambling problem, eating disorder, and anxiety disorders, curing and controlling chronic diseases, and improving health behaviours [9] [12]. MI is used as a client-centered, directive method for enhancing patient's intrinsic motivation to change by exploring and resolving ambivalence. This method is inspired by the client-centered technique, developed by Carl Rogers, and its most important component, i.e. empathy with the patient. MI relies on counselling techniques and attempts to change client's attitude towards the pros and cons of continuing unhealthy behaviours. This non-aggressive approach is specifically effective for people who are not ready for change or uncertain of it [10] [13].

Since opting for a specific contraception method by women depend on their perception of its mental and physical effects [6] [11] [12] [14] [15] [16]. Counselling can be very useful in raising the awareness of clients in this regard.

Therefore, this study was done to investigate the > effect of motivational interviewing (MI) on the use of IUD in women at high risk for pregnancy

Methods

This random educational trial was conducted in Isfahan on 44 women at high risk for pregnancy in 2015. After written consent were randomly divided into two equally sized groups (intervention and control) by assigning a number to the medical record of each woman and then using random number table. The intervention group received five 45-minute MI sessions once a weak. The control group received the routine training provided in clinics. Eligible patients included: (1) women at gestational age (15-49 years); (2) women with basic literacy level; (3) women with no contraindication to IUD placement (due to pregnancy, puerperal sepsis, PID or sexually transmitted disease at present or in the last three months, endometrial and cervical cancer, undiagnosed genital bleeding, fibroid tumors that change the shape of uterus, allergy to copper, and hepatolenticular degeneration); (4) women at high risk for pregnancy (due to tobacco use, pregnancy interval of than three years, substance abuse, malnutrition, aged over 35 years, having more than four pregnancies, chronic disease with health risk for pregnancy); (5) women who tend to use a reliable contraceptive method but have ambivalence towards using IUD misconceptions. Exclusion criteria were: (1) movement of mother to another city during counselling; (3) and interrupted attendance at educational sessions (e.g. more than two times absence). Data collection instruments included a researcher-made questionnaire that measured the knowledge, attitude, acceptance, and performance factors. Its first part contained demographic information. The second part was comprised of 12 questions on knowledge(true or false), 36 items addressing attitude (three-point Likert scale; "agree," "neutral," "disagree"), single question that measured acceptance ("Yes” and 'No"), and one item dealt with performance (Yes” and 'No"). The content validity method was used to assess the validity of the questionnaire. To do this, the questionnaire was reviewed by a panel comprised of 10 senior lecturers (one with PhD in reproductive health, two gynaecologists, three with masters in midwifery currently working in the clinic, and two psychologists), and their opinions were applied to the questionnaire. Finally, after the removal of problems, ambiguities, and problematic questions, the validity of the questionnaire was confirmed. To measure the reliability of it, Cronbach’s alpha with a minimum value of 0.7 was employed.

Before research initiation, the necessary authorisations were obtained, and then the researcher introduced himself to research units and explained the research objective and methodology. After collecting the written informed consent of all participants, the pre-test was completed by the subjects. Samples were then randomly divided into the intervention and control groups. The control group received routine training provided in clinics; whereas, the intervention group received five 45-minute MI sessions held individually. The main independent research variable was MI. The configuration of the MI sessions was extracted from "Motivational Interviewing in the Treatment of Psychological Problems," as well as some articles [17] [18] [19] [20] [21].

Table 1: Configuration and content of MI sessions

First	Introduction: Objectives, effective dimensions of the problem, stages of change
Second	Feelings: feelings identification practice, positive and negative dimensions of maintaining and changing behaviour, internal or external incentives for change
Third	Values: definition of values, clarification (conflict between values and current values), identification, confirmation and recognition of clients' values
Fourth	Deciding internal or external incentives, framework redevelopment, the definition of failures, clients' sense of self-failure
Fifth	Calling for clients' capabilities, perspective practice

All sessions were held by the researcher. Homework was given to the clients at the end of each session, and that of preceding session was reviewed for problem-solving. The weekly sessions were held for five weeks. One month after the completion of MI session, the questionnaire was filled again by the intervention and control groups to measure the effectiveness of MI.

The collected data were analysed with descriptive and inferential tests, dependent t-test (intergroup comparison) independent t-test (intra-group comparison), Mann-Whitney test, Fisher's exact test, and McNemar's test (to determine the effect of clients' acceptance on their performance), using SPSS 20.

Results

The mean age of the participants and their partners in the intervention and control groups was 33.18 ± 8.03 and 32.57 ± 7.89, and 38.32 ± 9.29 and 37.82 ± 11.33, respectively.

According to the statistics, three (13.6%) and one (4.5%) subjects in the intervention and control groups were employed, respectively. The remaining women were housewives. Other demographic features of the participants have been presented in Table 2.

Educational status of the intervention group versus control group was as follows: 4.5% vs 22.7% (one vs five subjects) were illiterate, 27.3% vs 22.7% (six vs five subjects) attained a primary school education, 18.2% vs 27.3% (four versus six subjects) attained a secondary school education, and 13.6% vs 4.5% (three vs one) attained an academic education.

Table 2: Demographic variable in Intervention and control groups

Variable		Intervention Group	Control Group	p-Value
		Mean ± SD	Mean ± SD	
Pregnancy Number		2.6 ± 0.4	3.2 ± 0.5	0.41
Para		2.1 ± 0.24	2.68 ± 0.45	0.29
Abortion		0.5 ± 0.18	0.5 ± 0.22	1.00
Live Child		2.14 ± 0.24	2.5 ± 0.41	0.402
Education		1 (4.5)	5 (22.7)	
Status (%)	Basic Literate			
	Primary school	6 (27.3)	5 (22.7)	
	Guidance school	8 (36.4)	5 (22.7)	0.31
	High school	4 (18.2)	6 (27.3)	
	Diploma	3 (13.6)	1 (4.5)	

The mean knowledge scores were not significantly different between the intervention and control groups before the intervention (56.81 ± 18.11 vs 54.93 ± 18.03). Other post-intervention variables are presented in Table 3. The mean post-intervention knowledge scores were 97.34 ± 3.97 and 56.72 ± 17.66 in the intervention and control groups, respectively, which was significantly higher in the former ($p < 0.001$).

The mean attitude scores were not significantly different between the intervention and control groups before the intervention (70.1 ± 18.3 vs 72.8 ± 15.9). Other post-intervention variables are presented in Table 3. The mean post-intervention attitude scores were 97.84 ± 2.8 and 73.6 ± 13.9 in the intervention and control groups, respectively, which was significantly higher in the former ($p < 0.001$). Findings from reviewing data showed that the frequency of acceptance was not significant between the two group before the intervention ($p = 0.349$), insofar as 22.7% (5 subjects) in the intervention group and 13.6% (3 subjects) in control group were positive to the use of IUD.

Findings from reviewing data suggested the significantly higher performance of intervention group in IUD placement ($p < 0.001$), insofar as 15 subjects (68.2%) in the intervention group and only one subject in the control group placed IUD. Data from McNemar's test showed that women in the intervention group had significantly higher performance than acceptance ($p = 0.006$); whereas, these factors were not significantly different in the control group ($p = 0.62$).

Table 3: The mean knowledge and attitude and performance in Intervention and control groups

Variables	Time	Control	Intervention	p-value
		Mean ± SD	Mean ± SD	
Knowledge	Before	54.9 ± 18.03	56.8 ± 18.1	0.73
	After	56.7 ± 17.7	97.3 ± 4.0	0.001
		>0.15	>0.1	p-value
Attitude	Before	72.8 ± 15.9	70.1 ± 18.3	0.61
	After	73.6 ± 13.9	97.8 ± 2.8	0.001
		>0.24	<0.001	p-value
Acceptance (%)	After	3 (13.6)	5 (22.7)	0.349
Performance (%)	After	1 (4.5)	15 (68.2)	0.001

Discussion

In this research, the effect of motivational interviewing on the use of IUDs in women with high-risk pregnancy was studied. The findings showed that IUD insertion in the motivational interviewing group was twice as that in the control group. According to the results, this performance was influenced by a significant increase in the level of awareness and attitude in the intervention group than the control group. Studies have shown that women's knowledge about intrauterine devices is low and combined with false perceptions such as fear of cancer, infection, and infertility. Therefore, education can be very helpful in this regard [22]. In a study conducted by Whitaker *et al*. titled “Knowledge and attitudes of women of childbearing age and girls around puberty about intrauterine devices”, 144 English-American women aged 14-24 received brief training for 3 minutes on how to insert and remove IUDs. The results showed an increase in the willingness of women to use IUDs by 53%. Women of childbearing age stated that the positive features of IUDs that encourage them to select them are their long-lasting effects (10 years) and their hiddenness from others. Although this three-minute training had a positive impact on the willingness of women to use IUDs, it is unclear whether this willingness will come into practice in the future. In the present study which was conducted using the motivational interviewing method, the rate of IUD insertion and removal was more than 50% one month after the intervention. The rate of adoption after the intervention also showed a significant increase in the motivational interviewing group.

Hence, raising the awareness and attitude of women was effective in adopting this method. In this study, it has been recommended that training programs be developed for women in need of long-acting reversible methods and healthcare workers to eliminate misinformation and misplaced prejudices [23]. Ferreira *et al*. studied the selection of the post-abortion contraceptive method in a family planning clinic in the northeast of Brazil and concluded that increased awareness in the post-abortion stage leads to the further adoption of various contraception methods. Accordingly, they recommended that providing individual family planning counselling and training in post-abortion, especially for people with poor economic status, can increase the adoption of contraception methods and reduce high-risk pregnancies and unsafe abortions [24].

Adoption of right decisions in families about the number of children, interval between pregnancies, and other issue related to reproductive health requires sufficient knowledge and correct insight which cannot be achieved without counselling [25]. By strengthening intrinsic motives, motivational interviewing corrects misconceptions and eliminates uncertainties of individuals and directs them towards choosing a safe method of family planning. IUS is a long-acting reversible method that prevents unwanted pregnancies. The rate of using IUD in the US increased from 1.2% in 2002 to 5.5% in 2008. The intrauterine device is a highly secure, high-performance, long-acting methods with a refractive index of 2% in 5 years. Unlike oral contraceptives that require daily and continuous consumption for maximum efficiency, IUD takes the minimum effort of the user after insertion [26].

Given that women in the study were of low economic stratum and pregnancy would jeopardise their physical and mental health, the use of a less costly method was taken into account by authors. A qualitative study titled “Wrong ideas about intrauterine device among people of Isfahan” was conducted by Manzoori *et al*., in May 2009. Women of childbearing age who had never used IUD with any contraindications for IUD insertion were interviewed in a semi-structured manner. Their findings showed that fear of side effects of IUD, religious beliefs, fear of IUD insertion process, fear of problems with sexual intercourse, and fear of damage to the fetus was the main erroneous beliefs about IUDs. Among these reasons, religious beliefs were mentioned as the most important obstacle to the use of IUDs. They recommended that providing special counselling to people who intend to use IUDs in the future can reduce false perceptions in society and increase the use of IUDs by those who need to this contraception method [6]. According to studies, the low use of IUDs can be attributed to insufficient knowledge by people and healthcare workers and false information provided by the media, including the Internet [23] [27] [28]. Peterson *et al*. conducted a study on 764 women aged 16-44 at risk of unwanted pregnancy and sexually transmitted diseases using the motivational interviewing method. Participants in the control group only received public health education in one session. The use of family planning methods (with high efficiency) was studied 2, 8, and 12 months later. At the beginning of the study, 59% and 19% of participants stated that they used the effective methods of contraception at a high and low level, respectively, and 22% of them reported no use of these methods. Two months later, the rate of contraceptive use in the intervention and control groups was reported at 72% and 66%, respectively. After 12 months, no significant difference was observed between the two groups. About 10-11% of participants in both groups had an unwanted pregnancy, and about 10% of them were infected with sexually transmitted diseases. Authors recommend the repetition of counselling sessions for decision-making about the use of contraceptives to prevent unwanted pregnancy and sexually transmitted diseases. In the present study, duration of using IUD and its rate of removal due to complication were not followed up because of time limitation [29].

According to the research findings implementation of MI increased the use of IUD. What it seems is a suitable counselling method for contraceptive counselling. Therefore, this type of counselling can be embedded in reproductive health behaviour change programs.

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Motivating behaviour change to protective risk behaviour is one of the most important duties of nurse/midwife staff, and motivational interviewing is an appropriate way to deal with uncertainties of women about the use of contraceptives.

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Evaluation of the Reliability and Validity of the Persian Version of the Fatigue Assessment Scale in Iranian Sarcoidosis Patients

Somayeh Lookzadeh[1], Arda Kiani[2], Kimia Taghavi[1], Shirin Kianersi[1], Habib Emami[3], Maryam Mirenayat[1], Atefeh Abedini[1*]

[1]Chronic Respiratory Diseases Research Center, National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran; [2]Tracheal Diseases Research Center, National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran; [3]Tobacco Prevention and Control Research Center, National Research Institute of Tuberculosis and Lung Disease (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran

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***Correspondence:** Atefeh Abedini. Chronic Respiratory Diseases Research Center, National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran. E-mail: dr.abedini110@sbmu.ac.ir

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Abstract

INTRODUCTION: Fatigue is one of the common symptoms of sarcoidosis, which occurs in about 50-70% of patients.

AIM: Considering that there are no valid Iranian questionnaires for evaluating fatigue in sarcoidosis, in the present study, for the first time, we translated Fatigue Questionnaire into Persean and evaluated its validity and reliability among Iranian patients with sarcoidosis.

MATERIAL AND METHODS: In methodological research, English version of Fatigue assessment scale (FAS) 10 items questionnaire which is designed to assess physical or mental fatigue in chronic disease patients, was translated into Persian and back-translated into English. Its validity and reliability were studied on the one hundred and thirteen confirmed sarcoidosis patients are referring to respiratory referral hospital of Iran. Reliability analysis was performed by estimation of Cronbach`s alpha test.

RESULTS: According to the cut-off point of 22.84 (74%) of the studied patients were suffering from fatigue. The internal consistency calculation revealed that the alpha value of the physical fatigue and mental fatigue was 0.945 and 0.896, respectively.

CONCLUSION: We concluded that the existence of questions number 4 and 10 in the questionnaire reduces the continuity of the questions, and therefore we suggest applying the FAS questionnaire without the two questions 4 and 10. This study showed that FAS questionnaire was very practical and can routinely be applied to assess the fatigue scale in sarcoidosis patients.

Introduction

Sarcoidosis is a chronic inflammatory disease with an unknown cause with non-caseating granulomas manifestations in different organs [1] [2]. The outbreak varies from one region to another, and the general prevalence is about 5-40 cases per 100,000 [3]. Sarcoidosis occurs at all ages, but the highest incidence in patients has been observed in the second and sixth decades of their life [4]. Clinical manifestations include systemic and general signs of fatigue, weight loss, fever, discomfort, and the involvement of specific organs such as the lungs, skin, eyes, heart, liver, joints and the nervous system [1]. The commonly involved organ is a lung.

Furthermore, additional pulmonary involvements occur in 25-30% of patients [2] [3]. Fatigue is one of the common symptoms of sarcoidosis, which occurs in about 50-70% of patients. The definitive cause of fatigue is unknown and is influenced by numerous factors [3] [5]. This symptom is associated with many chronic physical illnesses, such as multiple sclerosis, Parkinson's disease, rheumatoid arthritis, and psychiatric disorders such as depression [6]. The formation of granuloma and the release of cytokines may be factors of the beginning of fatigue in sarcoidosis [7]. Fatigue is more common

during the active period of the disease, as well as depression, cognitive impairment, exercise intolerance, and stress associated with sarcoidosis [8] [9]. In addition to the mentioned factors, it seems that changing the quality of life and the impact on occupational and social activities resulting from sarcoidosis are effective in fatigue. There is no agreement on the physical and mental contrast. Fatigue is seen as a bilateral contrast [10], but many authors still consider it a multi-dimensional structure [11]. Different dimensions of fatigue and general dissonance for fatigue definition are used to evaluate fatigue in different questionnaires. Fatigue associated scale (FAS) is the most reliable questionnaire in the study of fatigue in chronic disease patients. Fatigue assessment Scale is a self–reporting questionnaire. FAS questionnaire is a one-dimensional scale for fatigue testing and consists of 10 questions that examine five physical fatigue and five mental health issues. The whole amount of fatigue and its severity is presented by a total score is between 10 and 50 [3] [12]. This study aimed to assess the fatigue manifestations in Iranian sarcoidosis patients.

Considering that there are no valid Iranian questionnaires for the evaluation of fatigue in sarcoidosis, we evaluated the reliability and validity of the first Persian translation of fatigue associated scale, which was accomplished by the same research group.

Material and Methods

In methodological research, English version of the Fatigue assessment scale (FAS) was translated into Persian and back-translated into English. Its validity and reliability were studied.

One hundred and thirteen confirmed sarcoidosis patients are referring to respiratory referral hospital in Iran, who were over 18 years old, were included in the present study. Written consent was obtained from patients for participation in the study. The entry clause in this study was the person's ability to complete the questionnaire without the help of others. The study was approved by the University Ethics Committee.

The FAS included 10 questions with a five-point response "1 = never" to "5-always". Items were divided into two parts of physical and mental fatigue, and each section contained five questions. Therefore, the total number was between 10 and 50. Except for questions number 4 (I have enough energy for everyday life) and 10 (I can focus very well when I'm busy doing), which represent positive issues, the remaining eight questions out of 10, were negatively related issues. Thus, before the analysis of the data, the answers to items 4 and10 were reversed.

Translation and back translation was conducted based on convenient guidelines [15] [16]. Steps of the process of translating and validating the questionnaire were as followed.

Step 1: Inviting an expert committee involving two nurses, one epidemiologist and one pulmonologist. All steps of translation/back translation and validity performance of the questionnaire were supervised by this committee.

Step 2: English version of the questionnaire was simultaneously translated into Persian, by two independent native interpreters. The two versions were compared, and the translators were asked to mention the applied changes to the items during translation. The two copies were compared by the expert committee to reach agreement about the final version.

Step 3: The final Persian translation was back-translated into English, by an English-speaking expert in the United States. The expert committee confirmed the final Persian version and inserted it in the validity and reliability step.

Step 4: Face to face Content reliability

For the face to face reality of the questionnaire, a copy of the final Persian translation of it was given to 15 patients. They were asked to give their opinion on each item and to note the meaning of each question. They were also asked to declare whether they understood the concept of the questions. Finally, their opinion was evaluated to approve the principle of the questionnaire.

Reliability analysis was performed by estimation of Cronbach`s alpha. The internal consistency of the questionnaire was assessed by Cronbach`s alpha coefficient, and alpha of equal or greater than 0.70 was considered as satisfactory [17]. For the repeatability, the test was performed again, and the analysis was retested.

In qualitative reliability, the 15 patients were asked to comment on each item's grammar, the use of proper words, dictionaries, the clarity of the concept of the words and the simplicity of completing the questionnaire. Subsequently, according to their views, the phrases were reviewed and modified.

Step 5 construct validity: To determine the reliability, we distributed two copies of the questionnaire among 15 patients in two weeks interval. We determined the reliability of the questionnaire by calculating the correlation between the first and second answers. For estimating the reliability, the internal correlation of the Cronbach's alpha index was calculated. Items with results above 70% were kept in the questionnaire. The analysis of the parameters was done using the varimax rotation program.

Finally, 113 sarcoidosis patients have entered the study after signing the informed consent.

Results

One hundred and thirteen confirmed cases of sarcoidosis were admitted in the study. Of these, 70 (61%) were male, and 45 (39%) were female. Mean age of the patients was 38.77 ± 9.65. Males and females were 38.06 ± 8.95 and 39.9 ± 10.66 years old respectively. There was no ceiling or floor factor effect. Three patients (2.7%) had the lowest possible FAS score with the point of 10, and none of the patients could catch the maximum score of 50. Mean FAS score was not different in males (27.8 ± 9.57) comparing to females (27.06 ± 9.59) (t = 0.424, df = 111, P = 0.672).

None of the translators suggested any changes to the final Farsi version. Cronbach's alpha after reversing items 4 and 10 was 0.76 which is acceptable [18]. Table 1 represents item reliability results, means and standard deviation, Item-total correlation, ceiling and floor effects. Total Cronbach`s alpha was not increased by eliminating none of the items. The total correlations for the total items were positive (u > 50) and ranged from 0.52 to 0.82. We also evaluated internal consistency using Cronbach`s alpha once without reversing item four and item 10 and after eliminating these two items and the results were 0.93 and 0.927 respectively.

Table 1: Item reliability results, means and standard deviation, Item-total correlation, ceiling and floor effects

Items	N	Mean	SD	Correct item-total correlation	Alpha	Floor effect	Ceiling effect
I am bothered by fatigue	113	2.7	1.35	0.79	0.68	23.5	11.3
I get tired very quickly	113	3.06	1.4	0.82	0.67	18.3	19.1
I don't do much during the day	113	2.81	1.20	0.83	0.68	15.7	9.6
I have enough energy for everyday life	113	2.85	1.20	0.62	0.86	12.2	9.6
Physically, I feel exhausted	113	3.24	1.33	0.80	0.68	5.2	24.3
I have problems to start things	113	2.59	1.30	0.70	0.70	26.1	9.6
I have problems to think clearly	113	2.29	1.20	0.61	0.71	33.0	5.2
I feel no desire to do anything	113	2.38	1.07	0.70	0.70	24.0	3.5
Mentally, I feel exhausted	113	2.53	1.06	0.52	0.73	12.2	7.0
When I am doing something, I can concentrate quite well	113	3.20	1.03	0.55	0.84	13.2	3.5
Total scone	113	27.56	9.54				

The overall KMO for the set of the items included in the analysis was 0.80 which was above the 50% of the minimum requirement for the coefficient KMO (Table 2).

Table 2: KMO and Bartlett's Test

Kaiser-Mayer-Olkin Measures of Sampling Accuracy	0.898
Bartlett´s Test of Approx.Chi-Square Sphericity	960.746
df	45
Sig	< 0.0001

The test was correlated with the Bartlett test (0.00) and was appropriate for factor analysis. Based on the variance factor of more than one (Eigenvalue > 1), two factors were extracted which were 76.544% of the variance cover (Table 3).

Table 3: Total variance explained by the ten extracted factors of the ATTS scale

Component	Initial Eigenvalues			Extraction Sums of Squared Loadings		
	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	6.175	61.746	61.746	6.175	61.746	61.746
2	1.480	14.798	76.544	1.480	14.798	76.544
3	.716	7.158	83.701			
4	.398	3.977	87.679			
5	.309	3.088	90.767			
6	.260	2.602	93.368			
7	.231	2.314	95.683			
8	.171	1.707	97.390			
9	.156	1.564	98.954			
10	.105	1.046	100.000			

The responses to the items of FAS are shown in (Table 4).

Table 4: Subscale factors loading scores and loading of items that exceed 0.30

Items	F1	F2
Physical Fatigue		
I am bothered by fatigue	0.880	
I get tired very quickly	0.860	
I don't do much during the day	0.880	
I have enough energy for everyday life	0.717	
Physically, I feel exhausted	0.840	
I have problems to start things	0.817	
Mental Fatigue		
I have problems to think clearly		0.888
I feel no desire to do anything		0.742
Mentally, I feel exhausted		0.916
When I am doing something, I can concentrate quite well		0.696

Depending on the cut-off point identified in other studies [19], people with a mean score of 22 or above were experiencing major fatigue. According to this cutoff point, 84 (74%) of the patients were suffering from fatigue. Among males 54 (77.1%) and in females, 30 (70%) were suffering from fatigue.

Given that the presence of items 4 and 10 reduces Cronbach's alpha, we performed a separate factor analysis after removing these two items, which resulted in two subscales with a variance of 83.119. Physical fatigue subscales included the items one to six, and mental fatigue subscales consisted of items number 7, 8 and 9 (Table 5). The internal consistency calculation revealed that the alpha value of the whole instrument after dropping item 4 and 10 was 0.927. An alpha value of the physical fatigue and mental fatigue was 0.945 and 0.896, respectively.

Table 5: Subscale factor loading scores and loading of items that exceed 0.30 after eliminating item 4 and 10

Items	F1	F2
Physical Fatigue		
I am bothered by fatigue	0.891	
I get tired very quickly	0.865	
I don`t do much during the day	0.887	
Physically, I feel exhausted	0.847	
I have problems to start things	0.838	
Mental Fatigue		
I have problems to think clearly		0.911
I feel no desire to do anything		0.746
Mentally, I feel exhausted		0.921

Discussion

This was the first study that translated the FAS questionnaire into Persian and analysed its reliability and validity among sarcoidosis patients in Iran. Previous studies have suggested that FAS is reliable and reliable in measuring fatigue in patients with sarcoidosis [19]. This study showed that FAS questionnaire was very practical and because of its brief and usefulness, none of the patients refused to continue during the study. At the time of responding to the questionnaire, they answered the questionnaire completely. In the present study, the two sexes did not differ regarding fatigue, and this contradicts the results of some studies [20]. Some studies have shown a positive effect of age on fatigue [17], while others have shown the opposite [18] [19] [20]. Unlike some studies, two factors were extracted based on three creatures.

Contrary to some studies, in the present study, based on three Indicators of Scree plot, Eigenvalue and total variance, two factors resulted in [21] [22] [23] [24] [25]. In the current study, two physical and mental factors of fatigue with the coverage of over 70% and total variance of more than 70 were extracted. In this study, the Cronbach's alpha coefficient in the case of reversal of questions 4 and 10 was 0.76. Without reversing these two questions, this coefficient increased to 0.93, and when these two questions were eliminated, 0.927 was obtained as the Cronbach's alpha coefficient. The current study showed that by removing these two questions or adding them without reversing them, this questionnaire has more comprehensive capabilities and can be independently used to evaluate fatigue score in sarcoidosis patients. A previous study showed that the FAS questionnaire without questions 4 and 10 was superior to the FAS with these two questions [26]. The previous studies also showed that these two questions were unreliable [27] [28] [29]. We also concluded that the existence of these two questions in the questionnaire reduces the continuity of the questions, and therefore we propose the FAS questionnaire without the two questions 4 and 10. The reason why these two questions are not reliable could be that patients who fill out the questionnaire will rate the two questions alike. Therefore, it can be concluded that the FAS questionnaire has a high alpha coefficient without reversing or eliminating questions 4 and 10. The findings show that the Persian language FAS questionnaire with inverse questions 4 and 10, despite the low internal communication (0.75), can assess fatigue in patients with sarcoidosis.

In conclusion, we suggest that the Persian language FAS questionnaire can be included as a practical, easy and reliable method for assessing fatigue involvement in routine check-ups of patients with sarcoidosis. However, further studies with applying inversed questions four and 10 in Persian FAS questionnaire would be necessary to elucidate the exact magnitude of the prevalence of fatigue in sarcoidosis patients in Iran.

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Author contributions

Literature search: Somayeh Lookzadeh, Arda Kiani, Kimia Taghavi, Shirin Kianersi, Habib Emami, Maryam mienayat, Atefeh Abedini. Data collection: Somayeh Lookzadeh, Kimia Taghavi, Shirin Kianersi, Atefeh Abedini. Study design: Somayeh Lookzadeh, Arda Kiani, Kimia Taghavi, Shirin Kianersi, Habib Emami, Maryam mienayat, Atefeh Abedini. Data analysis: Somayeh Lookzadeh, Shirin Kianersi, Habib Emami. Manuscript preparation: Somayeh Lookzadeh, Kimia Taghavi. Manuscript review: Somayeh Lookzadeh, Arda Kiani, Kimia Taghavi, Shirin Kianersi, Habib Emami, Maryam mienayat, Atefeh Abedini.

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Public Health

On the Epidemiology and Statistical Analysis of HIV/AIDS Patients in the Insurgency Affected States of Nigeria

Patience I. Adamu, Pelumi E. Oguntunde*, Hilary I. Okagbue, Olasunmbo O. Agboola

Department of Mathematics, Covenant University, Ota, Nigeria

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***Correspondence:** Pelumi E Oguntunde. Department of Mathematics, Covenant University, Ota, Nigeria. E-mail: patience.adamu@covenantuniversity.edu.ng

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Abstract

BACKGROUND: The effect of insurgencies on a nation regarding the economy, education, health and infrastructure cannot be overemphasised.

AIM: This research is therefore focused on analysing the incidence of HIV/AIDS disease in states affected by the activities of the Boko Haram insurgency in Nigeria.

MATERIAL AND METHODS: The data collected refer to the period from 2004 to 2017, reporting information on 16,102 patients and including the age, gender, year of diagnosing and status of the patients. Descriptive, Chi-square test of independence and Correlation analyses were performed using Statistical Package for Social Sciences (SPSS) version 20.

RESULTS: It was discovered that the majority of those living with HIV/AIDS in these Boko Haram ravaged areas are females between the age group of 30 years to 39 years. Reported cases of HIV/AIDS started increasing significantly from age 20, and the highest number of reported cases of HIV/AIDS was recorded in the year 2017.

CONCLUSION: The status of the patient was found to be dependent on both the gender and age of the patients' treatment, though the strength of the linear relationship between status and age is not significantly different from zero.

Introduction

Nigeria is divided into six geopolitical zones. The North East zone consists of six (6) states namely; Adamawa, Bauchi, Borno, Gombe, Taraba and Yobe. The entire Northern region of the country is in variance with their natural endowments such as vast fertile lands, rivers and lakes for irrigation, mineral resources and abundant sunshine for renewable energy. The weak social structure of the region has resulted in excruciating poverty which often manifests as homelessness and destitution, insurgency, violence and crime [1]. The region has high poverty index, low human development index, lack of potable drinking water, electoral violence, the dearth of medical personnel, high mortality, low life expectancy, decayed infrastructure and is also an epicentre for joblessness. The region also experiences a large number of underage and teenage pregnancy, female genital mutilation, epidemics, illiteracy, malnutrition and now terrorism which comes in form of coordinated attacks on military, police formations and remote villages, guerrilla attacks, kidnappings, regicide, suicide bombings, mass killings, abduction of school girls, extra-judicial killings and summary execution, hypnotizing and forced conscriptions, indoctrination and forceful conversion to Islam.

The decadence is not limited to the region but to the entire country which is a result of corruption, tribalism, military intervention in governance, inequality, misappropriation, financial recklessness, bankrupt of ideas and dearth of developmental agendas, reduction of allocation of capital due to shortfalls of Nigeria revenue as a result of decline in crude oil price. Globally, efforts towards reducing the spread of HIV have yielded desired results except in some developing countries. Moreover, other areas have been seriously affected. For example: food security and dynamics, under five malnutrition, child and maternal mortality, escalation of cholera outbreaks, infections, sexually transmitted diseases,

worsening mental health, unsafe birth practices and abortion, child prostitution, sex for food at the displaced persons camps, increase in polio cases [2] [3] [4].

The effect of the ongoing Boko Haram insurgency is a serious health concern for Nigeria [5]. This has manifested in the child wasting [6], increasingly psychological trauma [7], intensifying hunger [8], under-five malnutrition [9] and the reemergence of polio cases [10] [11] [12]. Also, the effect has reduced the chances of Nigeria meeting the millennium and sustainable development goals as warned by [13]. Others include disruption of health activities [14], difficulty in tackling reported cases of tuberculosis [15] and increased occurrence of injury [16]. In recent times, the effects also include the adoption of school girls which might lead to raping and as a consequence, transmission of infectious diseases.

The data used in this article was obtained from one of the renowned state hospitals in Borno state which is a state from the North East region of the country that historically has high HIV/AIDS incidence [17] and now worsened by the current Boko Haram insurgency in that area. The data also contains the records of all the HIV/AIDS records of the patients in the North East region of the country.

Table 1: Similar researches on HIV/AIDS

Statistical tool	Major findings	Contributor(s)
Correlation	Children living with HIV are most likely to have Left ventricular systolic dysfunction	[33]
Correlation	The effect of herbal drugs on the HIV patients receiving antiretroviral drugs	[34]
Correlation and Chi-square tests	A significant difference in the use of a condom by sex workers	[35]
Correlation	The link between the fear of HIV AIDS and infection control practices between Nigeria and the United States	[36]
Correlation	Prevalence of HIV antibodies in patients with pulmonary tuberculosis.	[37]
Correlation	Viral correlates of neurocognitive impairment (NCI) among HIV patients	[38]
Chi-square tests	High levels of Tuberculosis among HIV patients	[39]
Correlation and Chi-square tests	HIV tests as correlates of condom use among unmarried males in Nigeria.	[40]
Chi-square tests	Knowledge of sexually transmitted infections for example HIV as a predictor of condom use for gay sexual relationships	[41]
Chi-square tests	Same sex relationships as a predictor of HIV prevalence among prison inmates.	[42]
Chi-square tests	Differences in attitudinal and knowledge of HIV/AIDS among those with hearing impairment.	[43]
Chi-square tests	Voluntary testing and awareness reduces the rate of HIV transmission	[44]
Chi-square tests	The link between some demographic factors and AIDS mortality among the youth.	[45]

Illiteracy, cultural practices and insecurity have led to the high incidence of HIV in that area. This has inadvertently resulted in high rates of mother to child transmission [18], depletion of blood bank reserves [19].

To fully understand the incidence, prevalence, epidemiology and awareness of the HIV and AIDS, several studies have been conducted, and the scope limited to some parts of North East Nigeria. The results reported the high incidence, low awareness and so on. Examples were the studies conducted in Biu, Borno state [20] [21], three senatorial districts of Borno state [22] [23] [24] [25], Adamawa state [26] [27] [28] [29] [30], Gombe state [31] and Wukari, Taraba state [32]. This research is however focused on analysing the incidence of HIV/AIDS disease in states affected by the activities of the Boko Haram insurgency in Nigeria.

Some selected contributions in the area of this research using various statistical tools are presented in Table 1.

Material and Methods

The dataset used in this research was collected as a secondary data from the University of Maiduguri Teaching Hospital, Borno State for a period of 14 years (2004 to 2017). Records of 16,102 patients were considered and information on their age at the point of diagnoses of the disease, gender, year of diagnoses and status of the patients under treatment were collected. Descriptive statistics, cross-tabulation, chi-square test of independence and correlation analysis were used to analyse the dataset using SPSS.

This particular test is used to determine if a significant relationship(s) exist between two categorical variables, in other words, it can be used to test if a variable is dependent on the other variable or not.

In general, terms, let X and Y denote the two categorical (or nominal) variables under consideration, the hypothesis to be tested would be:

H_0: Variable X is not dependent on variable Y

Versus

H_1: Variable X is dependent on variable Y

However, the specific hypotheses used are stated clearly in the next section of this paper.

The test statistic for the chi-square test of independence is:

$$\chi^2 = \sum_{i=1}^{r}\sum_{j=1}^{c}\frac{\left(o_{ij}-e_{ij}\right)^2}{e_{ij}} \square \chi^2_{(r-1)(c-1),\alpha} \qquad (1)$$

'r' is the number of rows
'c' is the number of columns
'o_{ij}' is the observed frequencies
'e_{ij}' is the expected frequencies
'α' is the level of significance which is assumed to be 0.05 in this research.

Correlation analysis, on the other hand, measures the degree or strength of linear relationship between two variables. The value of the correlation

coefficient lies between -1 and +1. So, for variables X and Y, the correlation coefficient between them is measured using:

$$r = \frac{n\left(\sum xy\right) - \left(\sum x\right)\left(\sum y\right)}{\sqrt{\left[n\sum x^2 - \left(\sum x\right)^2\right]\left[n\sum y^2 - \left(\sum y\right)^2\right]}} \quad (2)$$

This correlation coefficient can be tested for statistical significance using the test statistic:

$$t = \frac{r\sqrt{n-2}}{\sqrt{1-r^2}} \square\ t_{n-2,\alpha} \quad (3)$$

where '*n*' is the sample size and α is the level of significance (0.05).

The underlying hypothesis is:

$$H_0 : r = 0$$

Versus

$$H_1 : r \neq 0$$

For both the Chi-square test of independence and correlation analysis, the null hypothesis will be rejected if the p-value is less than or equal to the level of significance.

Results

The summary of the age of the patients is made available in Table 2.

Table 2: Summary statistics of the age of the patients

N	16102
Mean	36.11
Median	35.00
Mode	30
Minimum	6
Maximum	88
Skewness	0.583

From Table 2, we observe that 16,102 patients were considered and the mean age is 36.11 years. The minimum age is 6 years while the maximum age considered is 88 years. However, the age with the highest cases of HIV/AIDS is 30 years. This information is represented graphically in Figure 1.

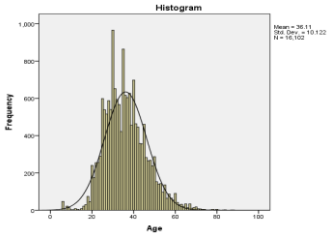

Figure 1: Distribution of age

The ages of the patients are further classified to enable us to know the incidence of HIV across age group (or bracket). The information is displayed in Table 3.

Table 3: Distribution of age according to age group (Approximated to 2decimal places)

Age Group	0-9	10-19	20-29	30-39	40-49	50-59	60-69	70-79	80-89	Total
Frequency	83	203	3,998	6,368	3,837	1,227	321	54	11	16,102
Percentage	0.52%	1.26%	24.83%	39.55%	23.83%	7.62%	1.99%	0.34%	0.06%	100%

From Table 3, we discover that the age group with the highest cases of HIV/AIDS is 30-39 followed by 20-29 while age group 80-89 has the lowest number of reported cases.

The summary of the gender of the patients is displayed in Table 4.

Table 4: Gender of the Patients

	Frequency	Per cent	Cumulative Percent
Female	9515	59.1	59.1
Male	6583	40.9	100.0
Valid Total	16098	100.0	
Missing	4		
Overall Total	16102		

From Table 4, we discover that there are more females than males with reported cases of HIV/AIDS based on the data set (or record) used. Though, information on the gender of four (4) of the patients is not known. This information is further represented in a graph in Figure 2.

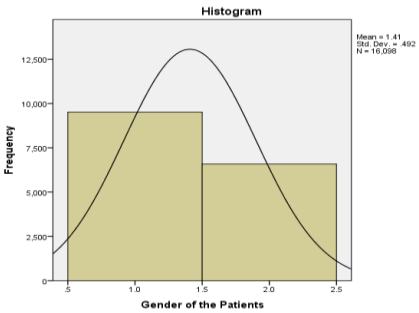

Figure 2: Gender of the patients

The summary of the information on the year of diagnosis of HIV/AIDS for each of the patients is displayed in Table 5.

Table 5: Year of diagnosis of HIV/AIDS

	Frequency	Per cent	Cumulative Percent
2004	2	0	0
2005	1760	10.9	10.9
2006	1983	12.3	23.3
2007	2179	13.5	36.8
2008	1976	12.3	49.1
2009	1756	10.9	60.0
2010	1420	8.8	68.8
2011	1133	7.0	75.8
2012	150	0.9	76.8
2013	62	0.4	77.1
2014	37	0.2	77.4
2015	28	0.2	77.5
2016	17	0.1	77.6
2017	3599	22.4	100.0
Total	16102	100.0	

From Table 5, we discover that there are more reported cases of HIV/AIDS in the year 2017 than in the previous years while the year with the lowest number of reported cases is the year 2004. The information is presented in diagrammatic form in Figure 3.

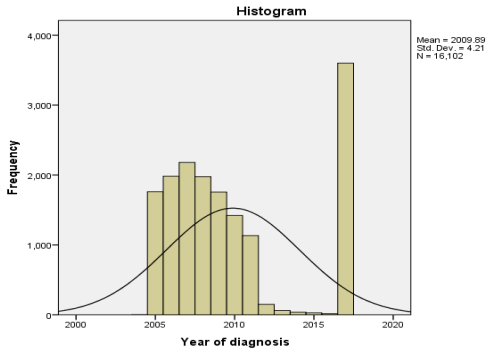

Figure 3: Chart for the year of diagnosis of HIV/AIDS

The summary of the information on the status of the patients under treatment which is classified into Active (the patient is still coming for treatment and check-up), LTFU (lost to follow up/the patient is no longer coming for treatment), Transfer and Died is displayed in Table 6.

Table 6: Status of the patients under treatment

	Frequency	Per cent
Active	6641	41.2
LTFU	194	1.2
Transfer	9054	56.2
Died	190	1.2
Valid Total	16079	100.0
Missing	23	
Overall Total	16102	

It is observed in Table 6 that the majority of the reported cases of HIV/AIDS were being transferred from one clinic to the other. Though, information on the status of 23 patients was not available. This information is represented in Figure 4.

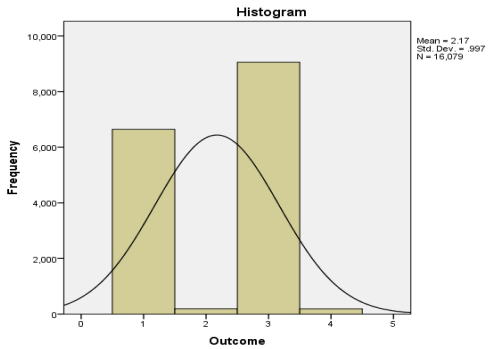

Figure 4: Chart for the status of patients under treatment

The result for the cross-tabulation between the status of the patients under treatment and gender of the patients is presented in Table 7.

Table 7: Crosstab of status and gender

Status		Female	Male	Total
Active	Count	4008	2631	6639
	% within Gender of the patients	42.2%	40.0%	41.3%
LTFU	Count	118	76	194
	% within Gender of the patients	1.2%	1.2%	1.2%
Transfer	Count	5278	3774	9052
	% within Gender of the patients	55.5%	57.4%	56.3%
Died	Count	98	92	190
	% within Gender of the patients	1.0%	1.4%	1.2%
Total	Count	9502	6573	16075
	% within Gender of the patients	100.0%	100.0%	100.0%

The information in Table 7 reveals that the majority of the patients that are still showing up in the clinic for treatment and check-up are females. Also, the majority of those that died of the sickness are females. This information is represented in the form of charts in Figure 5.

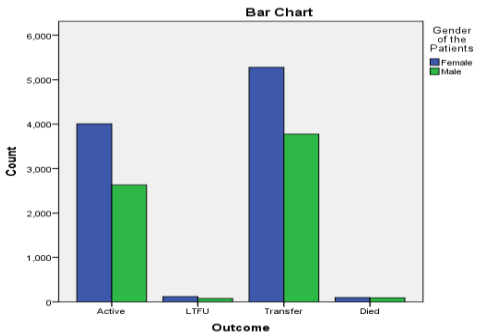

Figure 5: Chart of status and gender of patients under treatment

The result of the Chi-square test of independence between the status and gender of patients under treatment is made available in Table 7.

Table 7: Chi-square test between status and gender of patients under treatment

	Value	Degree of Freedom	P-value
Pearson Chi-Square	11.471	3	0.009
Likelihood Ratio	11.416	3	0.010
Linear-by-Linear Association	8.864	1	0.003
No. of valid cases	16075		

Hypothesis I:

H_0: The status of the patients under treatment is independent of the gender of the patient.

Versus

H_1: The status of the patients under treatment is dependent on the gender of the patient,

Decision: Reject the null hypothesis because the p-value (0.009) is less than the level of significance (0.05).

Conclusion: Based on the information contained in the dataset, the status of the patient (Active, LTFU, Transfer, and Died) is dependent on the gender of the patients. In other words, there is a significant association between the status of the patients under treatment and their gender.

The result on the strength of the relationship between the status of the patients and their gender measured by a correlation coefficient is made available in Table 8.

Table 8: Correlation analysis between status and gender of the patients

	Value	Standard Error	T- statistic	P-value
Pearson's Correlation	0.023	0.008	2.978	0.003
Spearman Correlation	0.024	0.008	3.009	0.003
No. of valid cases	16075			

The correlation coefficient between the status and gender of the patients under treatment is 0.023. This implies that the measure of the linear relationship between the status and gender is very weak. There might be some other forms of relationship (logarithmic, cubic, inverse, exponential and so on) between the status and gender of the patients since because correlation coefficient only measures the degree of **linear** relationship.

Also, the result of the Chi-square test of independence between the status of the patients and their age is made available in Table 9.

Table 9: Chi-square test for status and age

	Value	Degree of Freedom	P-value
Pearson Chi-Square	512.388	237	0.000
Likelihood Ratio	427.439	237	0.000
Linear-by-Linear Association	0.683	1	0.408
No. of valid cases	16079		

Hypothesis II:

H_0: The status of the patients under treatment is independent of the age of the patient.

Versus

H_1: The status of the patients under treatment is dependent on the age of the patient.

Decision: Reject the null hypothesis because the p-value (0.000) is less than the level of significance (0.05).

Conclusion: Based on the information contained in the dataset, the status of the patient (Active, LTFU, Transfer, and Died) is dependent on the age of the patients. That is, there is a significant association between the status and the age of the patients under treatment.

The result on the strength of the relationship between the status of the patients and their age measured by a correlation coefficient is made available in Table 10.

Table 10: Correlation between status and age of the patients

	Value	Standard Error	T- statistic	P-value
Pearson's Correlation	0.007	0.008	0.827	0.408
Spearman Correlation	0.005	0.008	0.662	0.508
No. of valid cases	16079			

The correlation coefficient between the status and age of the patients under treatment was obtained to be 0.007. This means that the measure of the linear relationship between the status and age is very weak. The p-value of 0.408 also suggests that the linear relationship between the status and age of the patients is not significantly different from zero. However, since correlation coefficient measures the strength of the **linear** relationship, we conclude that there might be some other forms of relationship (logarithmic, cubic, inverse, exponential and so on) between the status and gender of the patients.

Discussion

It can be seen that youths are more infected with HIV/AIDS because Table 1 reveals that patients at age 30 have the highest record of HIV and the disease is prominent among those in the age group 30-39, followed by 20-29. This information is contained in Table 2.

Larger percentage of the patients (59.1%) are females as shown in Table 3 and Figure 2. 22.4% of the reported cases are in the year 2017 (this is the highest based on the data set used), it can, however, mean that people are becoming more aware of the need to go for treatment and education on stigmatization has reached those areas. A significant number of cases was also recorded between years 2005 to 2011, but there is a significant drop in the number from the year 2012 (except for the year 2017). This does not mean that people are not infected with HIV/AIDS from the year 2012 to 2016 in these areas, but the activities of the insurgents were immense during that period, and we assume that people may not be able to go out and health workers were unable to capture cases of HIV/AIDS.

Only a few of the patients (194) which amounts to 1.2% of the population considered are left to follow up. This means that almost all the patients were turning up for treatments and check-ups. However, a large number of transfers was noticed (9,052 patients), this might mean that majority of the nearby clinics or health centres do not have either personnel or access to drugs that could handle this disease. Though, it is commendable that only a few of the patients (190) were recorded to be dead along the line.

The status of the patients (Active, LTFU, Transfer and Died) was found to be dependent on both gender and age of the patients. This means that age and gender were significantly associated with the status of the patients under treatment. Though this research has been able to establish that the correlation coefficient between status and age of the patients is not significantly different from zero, further

research may need to be conducted on whether age or gender would contribute to the mortality of the patients.

In conclusion, this research has been able to study the incidence and epidemiology relating to HIV in some states in Nigeria. The states considered are states that have been seriously affected by Boko Haram insurgency in the last few years. As a result of this IDP (internally displaced persons) camps had to be created in some strategic places to ensure that survivors of this Boko Haram menace are fed and taken care of. Some fellows were even kidnapped by this Boko Haram group and were raped and married forcefully; they might have been infected with this disease or other sexually transmitted diseases (STDs). As a fallout, some qualified personnel might have relocated from these targeted areas thereby depriving people of quality health care services; this might not be unconnected with the significant drop in the number of cases of HIV/AIDS reported between the year 2012 and 2016. The number of reported cases (16,102) is relatively high; this is apart from cases that are not even reported. The number of transfers from one clinic to the other is also high; this might discourage patients to continue with their treatments especially if there is a distance barrier. There are a number of missing data; this might lead to misinformation and misrepresentation. It is therefore advised that health centres should be equipped with enough and qualified personnel in this areas and security and insurance should be provided for them and their dependents.

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