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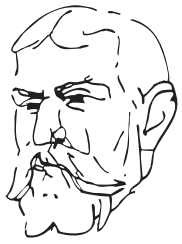

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ORIGINAL ARTICLE

MEDICATION ADHERENCE AND ITS IMPACT ON THE AVERAGE LIFE EXPECTANCY AFTER ST-SEGMENT ELEVATION MYOCARDIAL INFARCTION: THE RESULTS OF THE UKRAINIAN STIMUL REGISTRY

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ABSTRACT

The aim: The present study aimed to evaluate the adherence to medications prior and within a two-year period after ST-segment elevation myocardial infarction (STEMI) and to estimate its impact on the average lifespan of patients after STEMI.

Materials and methods: 1,103 patients with STEMI were enrolled in the prospective Ukrainian STIMUL registry with 24-month follow-up. The relationship between adherence to medical treatment and average lifespan was evaluated.

Results: The majority of prior STEMI patients were characterized with high and very high cardiovascular risk. The rate of revascularization was 29.9% (21.5% pPCI, 8.4% fibrinolytic therapy). The main reason for the low level of pPCI was late hospitalization and the inaccessibility of pPCI. This contributed greatly to in-hospital mortality (11.3%). Adherence to all medications progressively decreased ($p < 0.001$) within 24 months after STEMI. Permanent use of acetylsalicylic acid (ASA) and statins during the two-year follow-up was associated with 7.0% of the mortalities, whereas non-adherence to medications was related to a 15% risk of death (OR 4.2; 95% CI 0.2–0.9; $p < 0.05$). The average life expectancy with regular use of ASA and statins within 24 months after STEMI was 62.3 ± 1.1 years (95% CI 60.1–64.4; $p < 0.05$) and 61.2 ± 0.9 years with non-regular use of ASA and statins (95% CI 59.4–62.9; $p < 0.05$).

Conclusions: Adherence to evidence-based medicines was low in the STIMUL population both prior and after STEMI. This worsened cardiovascular prognosis and reduced average lifespan by one year within the following two years after STEMI.

KEY WORDS: ST-segment elevation myocardial infarction, STEMI, Ukraine, medication adherence, prevention, registry

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INTRODUCTION

Secondary prevention of cardiovascular diseases is strongly recommended by international guidelines [1–2]. However, several trials have shown that adherence to therapy recommended at discharge from hospital dramatically decreases among patients after acute coronary syndromes (ACS) [3–8]. To date limited data about medication adherence and ST-segment elevation myocardial infarction (STEMI) treatment in Ukraine are available.

THE AIM

The general objective of the present study was to evaluate the adherence to medications prior and within a two-year period after STEMI in STIMUL (ST-segment elevation Myocardial Infarctions in Ukraine and its Lethality) registry patients and to estimate the impact of adherence on the average lifespan of patients after STEMI.

MATERIALS AND METHODS

Details of the prospective STIMUL survey have been described previously [9]. In brief, 1,103 patients with STEMI [2] were enrolled to the registry. This study analysed a whole range of data on patients with STEMI who were admitted to three cardiology departments of the central regions of Ukraine. Informed consent was obtained from all patients at the time of enrolment. All patients with confirmed STEMI at discharge from hospital entered two-year follow-up observation with clinical assessment after 6, 12, and 24 months. The recommended post-STEMI management analysed in the present study included statins, dual antiplatelet therapy (DAPT – acetylsalicylic acid, continuously, and P2Y12 inhibitors during the first year); angiotensin-converting enzyme inhibitors (ACE-I), or angiotensin receptor blockers (ARB) in the case of ACE-I intolerance and beta-blockers. The primary end-points were cardiovascular death and non-fatal myocardial infarction.

STATISTICAL ANALYSIS

All analyses were performed using SAS software (SAS Institute Inc., Cary, NC, USA). All tests were considered statistically significant if $p < 0.05$. The use of medications was assessed as a proportion of patients who took medications to the total number of patients at the end of each follow-up period. Adherence was considered high when the number of medications was ≥ 80.0% when compared with the number of medications at discharge. The relationship between adherence and average lifespan was evaluated by the cross-tabulation analysis based on the Pearson's χ^2 (chi-squared) test. The association between two variables was calculated by the phi and Cramer's V coefficients. The Kaplan-Meier curves were constructed to graphically present crude survival estimates, with a log-rank test for the equality of survivor functions used to assess group differences.

RESULTS

BASELINE DATA AND TREATMENT BEFORE STEMI

The baseline characteristics of all patients included in the study are presented in Table I. In addition, 34.5% of the patients had a high risk of in-hospital mortality according to their GRACE score. 11.9% and 19.5% of the patients, respectively, had high and very high bleeding risk as assessed by the CRUSADE score. According to the baseline data, the STIMUL population was characterized by high or very high cardiovascular risk. However, only a few of them reported regular use of medicines prior to STEMI. The frequency of regular use of medications among patients prior to STEMI is presented in Table II. The antiplatelet therapy was used in 57.0% and 54.5% of the individuals with a history of MI and stroke, respectively. Among patients with high blood pressure at admission, 78.0% (n = 659) of the patients were aware that they had hypertension. Hypotensive therapy was used in 51.0% (n = 431) of the individuals, but only in 35.4% of the individuals on a regular basis (n = 299). Half of the patients with hypertension were treated with monotherapy. As a result, only 7.5% of them achieved target levels of blood pressure. Statins were predominantly used in patients who experienced cardiovascular events (ACS or stroke). Lipid targets were achieved only in 5.5% of patients. The main reasons for statin discontinuation or dose reduction were: a fear of side effects or perceived side-effects (25.6%) based on negative media coverage, advice from friends or family members, or lack of clinicians' guidance; the absence of clinical symptoms of dyslipidaemia making it easy to forget to take statins (forgetfulness) (18.3%), and the cost of statins (8.3%). 47.9% of patients did not take statins because of a lack of medical appointments/medical control. The main reasons for the discontinuation or episodic use of blood pressure lowering therapy were forgetfulness (22.0%), the fear of: side effects (21.6%), taking too many drugs at the same time (3.0%) and damage caused by long-lasting medication use (5.6%).

IN-HOSPITAL TREATMENT

The median time from symptom onset to hospital admission was 5.1 ± 0.3 hours. 59.8% (n = 660) of the patients were admitted to cardiology units. However, only a half of them (51.4%, 339 patients) were hospitalized in interventional units and 73.4% (n = 237) of the patients admitted to interventional units underwent primary percutaneous coronary intervention (pPCI). Fibrinolytic therapy was performed in 8.4% (n = 93) of the cases. Therefore, in the entire cohort, only 29.9% of the study population underwent reperfusion therapy. The major reasons for the non-performance of reperfusion therapy were late arrival (40.5%) and unavailability of catheterization laboratories (31.4%). Additional reasons were contraindications (7.4%), uncertain diagnosis (6.3%), patient refusal (6.1%).

In-hospital mortality rate was 11.3% (125 cases) in the STIMUL cohort and 7.0% (23 cases) among patients who underwent coronary reperfusion.

Finally, 872 (79.1%) patients were discharged from hospital with STEMI. They were included in a further two-year follow-up.

As shown in Table III, at discharge, the majority of the STIMUL population received recommendations to take the main cardiovascular medications, except for P2Y12 inhibitors. A lower level of recommendation to take P2Y12 inhibitors was related with a patient's high bleeding risk at admission.

TREATMENT DURING FOLLOW-UP AFTER ST-SEGMENT ELEVATION MYOCARDIAL INFARCTION

Post-discharge events were followed for 636 (72.9%) patients for six months, 480 (55.1%) patients for 12 months, and 278 (31.9%) patients for 24 months. During the first six months after STEMI, 64 patients (7.3%) had died and 8.0% of the patients had experienced a non-fatal myocardial infarction. After 12 months, 140 patients (16.1%) had died and 15.6% had a non-fatal myocardial infarction. After 24 months, 169 patients (19.4%) had died and 21.9% had a non-fatal myocardial infarction.

The adherence to medical treatment recommended at discharge from hospital progressively decreased ($p<0.001$) during the 24-month follow-up period (Table IV). After 24 months, regular use of ASA decreased by 35.2%, statins by 80.5%, beta-blockers by 38.1%, and ACE-I by 43.9% ($p < 0.001$), when compare with the recommendations of the clinicians at discharge from hospital.

DAPT adherence was 21.4% during the six-month follow-up period and 16.3% during the first 12 months after STEMI. The main reasons for DAPT discontinuation were the fear of side effects in 26.4% of the cases (n = 106), price in 22.4% of the cases (n = 90), and forgetfulness due to the lack of noticeable benefits in 18.4% of the cases (n = 74).

At the end of 24-month follow-up, further reduction of ASA, statin, ACE-I/ARB and beta-blockers adherence was observed (Table IV). The main reasons for medication discontinuation were similar as in the prior myocardial

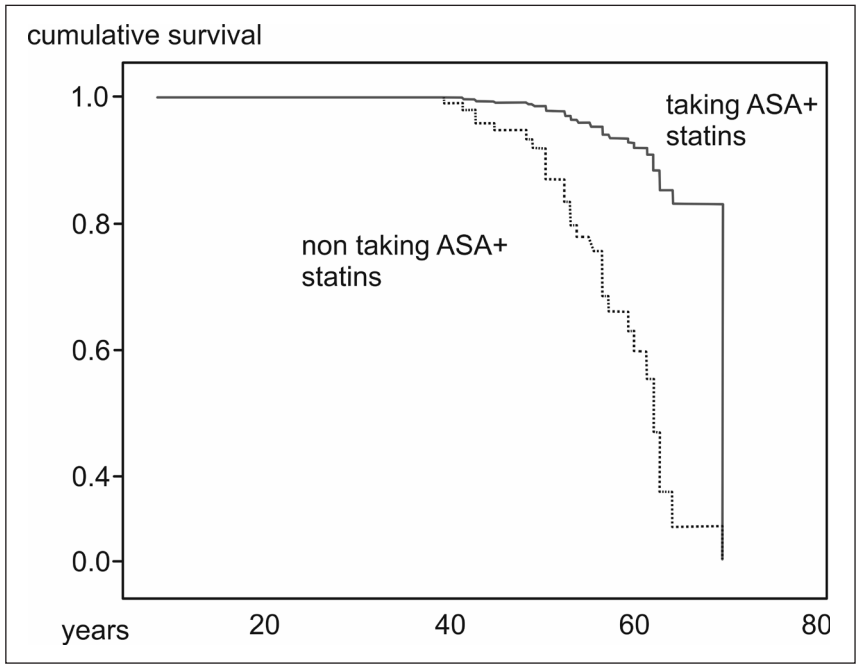

Fig. 1. Average life expectancy among patients taking and without taking acetylsalicylic acid (ASA) plus statin therapy within two-year follow-up after ST-segment elevation myocardial infarction.

Table I. Baseline, demographic and clinical characteristics of STIMUL registry population.

Characteristic	N	[%]
Age, years	63.4 ± 11.5	
Male gender	819	74.3%
Hypertension	845	76.6%
Hyperlipidemia (defined as total cholesterol ≥ 4.5 mmol/l)	565	50.7%
Body mass index (BMI) > 30 kg/m2	353	32.0%
Family history of coronary artery disease	351	31.8%
Diabetes mellitus	275	24.9%
Current smoker	300	27.2%
Past smoker	354	32.1%
Prior angina	380	34.5%
Prior myocardial infarction	267	24.2%
Prior percutaneous coronary intervention	23	2.1%
Prior coronary bypass graft surgery	3	0.3%
Prior heart failure	251	22.8%
Prior stroke/transient ischemic attack	72	6.5%
Prior renal failure	19	1.7%
Heart rate, mean bpm	83.4 ± 2.6	
Systolic blood pressure, mmHg	138.6 ± 3.6	
Killip class, ≥ II cardiogenic shock	262 39	23.8% 3.5%

infarction period: fear of side effects, especially in case of ASA (15.7%) and statins (18.4%), and forgetting to take the medication.

As shown in Table V, significant correlations were determined between two-year death risk and complete cessation of medications (ASA and statins) within 24 months after STEMI ($p<0.05$) or ASA discontinuation ($p<0.05$). We did not find any correlation between statin discontinuation and two-year death risk after STEMI ($p<0.05$) because of the small cohort of patients (n = 27) still using statins after the two-year period.

The continual use of ASA and statins for two years after STEMI was associated with a 7.0% mortality rate, while discontinuation of antiplatelet and lipid-lowering therapy

led to a 15.0% mortality rate during the two-year follow-up (Table VI). Therefore, the risk of two-year death in the case of discontinuation of the abovementioned treatment increased four times (OR 4.2; 95% CI 0.2–0.9; p<0.05). Among the patients with regular ASA intake, the rate of two-year death was 6.5%. Meanwhile, the cessation of the regular use of ASA was associated with a 15.5% mortality rate. As a result, the discontinuation of ASA during the first two years after STEMI increased the mortality risk three times (OR 3.8; 95% CI 0.2–0.9; p<0.05).

Regular statin intake was associated with a 6.9% mortality rate, whereas statin discontinuation increased this risk to 14.9%. Therefore, the two-year death risk in the case of the discontinuation of lipid-lowering therapy increased by four times (OR 4.2; 95% CI 0.1–0.9; p>0.05). However, the risk appears to be not significant statistically because of the small number of patients using statin treatment after two years.

The impact of adherence to treatment on average lifespan during the two-year follow-up period after STEMI is presented in Figure 1 and Table VII.

To conclude, the average lifespan in the case of regular use of antithrombotic and lipid-lowering therapy during the first two years after STEMI was 62.3 ± 1.1 years (95% CI 60.1–64.4; p<0.05) and 61.2 ± 0.9 years in the case of discontinuation of the recommended treatment (95% CI 59.4–62.9; p<0.05). Therefore, regular use of ASA and statins during the 24 months after STEMI prolonged lifespan by one year.

Table II. Treatment before admission to hospital.

Medication	N	[%]
ASA	295	26.8%
P2Y12 inhibitors	9	0.8%
Beta-blockers	195	17.7%
ACE-I/ARB	290	26.3%
Statins	113	10.2%

Table III. Treatment among the STIMUL population at discharge from hospital.

Medication	N	[%]
ASA	815	93.5%
P2Y12 inhibitors	674	77.3%
Beta-blockers	837	96.0%
ACE-I/ARB	778	89.2%
Statins	837	96.0%

ACE-I – angiotensin-converting enzyme inhibitor, ARB – angiotensin receptor blocker, ASA – acetylsalicylic acid

DISCUSSION

The results of our study indicate the poor level of cardiac prevention in the STIMUL registry population of Ukrainian patients before STEMI. In the present study, we observed a low and irregular rate of use of statin and hypotensive therapy prior to STEMI. This resulted in the non-achievement of target lipid and blood pressure goals and substantially increased the risk of adverse cardiovascular outcomes. The results of the STIMUL registry are much worse than those obtained in the EUROASPIRE V survey [10].

In our study, the rate of reperfusion therapy in Ukrainian patients with STEMI was dramatically low. Late hospitalization was the main barrier to invasive reperfusion treatment and had a great impact on in-hospital mortality. The importance of time in the management of STEMI was shown to directly affect both mortality and morbidity [11]. General in-hospital mortality in the STIMUL registry population was high (11.3%), when compared with international registries: 4.6% in the GRACE registry [12]; 4.0% in the second Euro Heart Survey on acute coronary syndromes [13] or 5.9% in the Polish PL-ACS registry

Table IV. Treatment among the STIMUL population at discharge from hospital and during the 6month, 12-month, and 24-month follow-up period after ST-segment elevation myocardial infarction.

Medication, %	at discharge	after 6 months	after 12 months	after 24 months
ASA	93.5%	73.3%	64.2%	58.3%
P2Y12 inhibitors	77.3%	21.4%	16.3%	–
ACE-I/ARB	96.0%	78.0%	63.3%	58.0%
Beta-blockers	89.2%	64.1%	53.1%	45.3%
Statins	96.0%	36.3%	24.2%	15.5%

ACE-I – angiotensin-converting enzyme inhibitor, ARB – angiotensin receptor blocker, ASA – acetylsalicylic acid

Table V. The impact of persistence on two-year death among STIMUL patients after ST-segment elevation myocardial infarction.

24-month medicine intake	Pearson's X^2	Degrees of freedom	p	Cramér's V	p Cramér's V	φ	p
ASA	5.9	1	< 0.05	0.2	< 0.05	–0.2	< 0.05
Statins	1.4	1	> 0.05	0.1	> 0.05	–0.1	> 0.05
ASA + statins	7.7	1	< 0.05	0.1	< 0.05	–0.1	< 0.05

ASA – acetylsalicylic acid

Table VI. Prognostic factors for two-year death risk after ST-segment elevation myocardial infarction in STIMUL population.

Variables		Two-year risk of death				OR	95% CI		p
		Yes		No			low	high	
		N	[%]	N	[%]				
ASA	Take	10	6.5%	145	93.6%	3.8	0.2	0.9	< 0.05
	No	19	15.5%	145	84.6%				
Statins	Take	2	6.9%	27	93.1%	4.2	0.1	1.9	> 0.05
	No	37	14.9%	212	85.1%				
ASA + statins	Take	11	7.0%	147	93.0%	4.2	0.2	0.9	< 0.05
	No	18	15.0%	102	85.0%				

ASA – acetylsalicylic acid

Table VII. Average lifespan among patients with different adherence to treatment within the first two years after ST-segment elevation myocardial infarction.

Adherence to medication	Average lifespan	SD	95% CI		p
			low	high	
ASA and statin adherence	62.3	1.1	60.1	64.4	< 0.05
ASA adherence	62.2	1.1	60.1	64.4	< 0.05
ASA and statin non-adherence	61.2	0.9	59.4	62.9	< 0.05

[14]. Another problem that should be emphasized when discussing the low pPCI rate in the STIMUL population is limited access to PCI procedures at the time of registry due to the lack of an organized PCI network in Ukraine. Kämpfer et al. have shown significant differences in the number of pPCI procedures for ACS in 2010 between three different socioeconomic environments [15]. In Switzerland and Poland, coronary interventions were the first choice therapy, whereas in Ukraine 30% of patients with ACS received fibrinolysis therapy and pPCI was not performed at all, as it was unavailable at this time in the study centre in Ukraine. Our results also correspond with the Euro Heart Survey 2009 Snapshot where, in comparison to western European countries, the use of pPCI for STEMI treatment in eastern European countries was low (23%), and 44% of patients called within 12 hours of symptom onset did not receive reperfusion therapy. Similarly to the our data, the low rate of reperfusion therapy resulted in a high in-hospital mortality rate (10.1%) [16].

During the first two years after STEMI, adherence to treatment of all medications recommended at the moment of discharge from hospital progressively decreased ($p<0.001$). These results correspond with data obtained by other researchers [17–23].

Data from the STIMUL registry revealed a high frequency of ASA administration at discharge. The rate of clopidogrel administration was lower, and this can be partially explained by the low rate of pPCI procedures and the high bleeding risk among the registry cohort. In the Ukrainian registry, adherence to DAPT was 16.3% at the end of the 12-month follow-up after STEMI. Despite strong evidence for a significant correlation between adherence with respect to antiplatelet and statin therapy and a decrease in mortality for patients with ACS, a reduction in adherence to DAPT and statins in patients after myocardial infarction has been commonly described [3-4, 6, 17-19, 21–22, 24]. Jackevicius et al. reported 44% of unfilled ASA prescriptions and almost 70% of unfilled prescriptions for medication other than ASA in 120 days for acute coronary syndrome [24]. According to Moalem et al., 6.5% of patients discontinued DAPT due to non-compliance at the end of a year [4]. Similar results have been shown in the international GRACE registry [19, 21], where only one third of the patients took clopidogrel after 6-month observation.

Adherence to therapy with statins decreased during the two-year follow-up period by almost 80%. In another observational study undertaken in one Ukrainian clinical centre, the rate of statin discontinuation after ACS was lower (about 64% during 3.5-year follow-up), but still higher than in western European countries or United States clinical centres [15]. In a large prospective multicentre cohort of patients with ACS, the discontinuation rate for statins was 6.7% and the main reason for discontinuation was the presence of side effects [23]. Low adherence to statins in the STIMUL population absolutely cannot be explained by the presence of side effects [6, 20]. The most common reasons for statin cessation in the Ukrainian registry were: the fear of side effects or the perceived side effects, a low disease awareness, and a lack of motivation or medical guidance. Our results correspond with the results obtained in the nationwide study in Denmark, where early statin discontinuation was significantly associated with negative statin-related news stories [25].

Among the Ukrainian population of STEMI patients, the frequency of ACE-I/ARB and beta-blocker use remained low after two years. Despite the high prevalence of hypertension among the STIMUL patients prior ACS, only 26.3% and

17.7% of the patients used these medicines regularly. After STEMI adherence to ACEI/ARB and beta-blockers decreased with time from the time of discharge and was 58.0% and 45.3%, respectively at two-year follow-up. It is a much lower rate than the current data from the EUROASPIRE V survey [10], where in the population with confirmed coronary artery disease 81% of the population were on beta-blockers and 75% were on ACE-I/ ARBs. The major reasons for discontinuation or dose reduction of blood pressure were fear of side effects, lack of self-motivation based on low disease awareness. It correspond with the results obtained by Khan et al., where strong myths responsible for the fear of antihypertensive medication use as well as for the poor acceptance of hypertension as a main risk factor of ACS and stroke have been described [26].

In our registry, we observed a high mortality rate and a high risk of recurrent myocardial infarction in a two-year period. The risk was the highest during the first year after STEMI. The high risk for adverse incidents was contributed by the low rate of reperfusion therapy and the discontinuation of evidence-based treatment with antiplatelets and statins. Discontinuation of treatment with ASA in the two-year observation period increased the risk of death three times, whereas discontinuation of both ASA and statin therapy increased the risk of death four times. In multivariable analysis, Ho et al. reported lower one-year survival after the discontinuation of both ASA and statins [27]. In our registry regular use of ASA and statins for 24 months after STEMI prolonged average lifespan by one year. This corresponds with the results of Rodriguez et al. [6].

CONCLUSIONS

1. The population of Ukrainian patients in the STIMUL registry was predominantly at a high and very high cardiovascular risk before STEMI with a poor level of cardiac prevention.
2. The level of reperfusion therapy among patients with STEMI was low, due to late hospitalization and limited access to primary PCI. This had a great impact on inhospital death. The frequency of recommendation of evidence-based therapy was high at discharge from hospital.
3. In a real-life setting, adherence to medications for secondary prevention after STEMI was low to moderate in Ukraine. The cessation of ASA and lipid-lowering therapy in two-year follow-up after STEMI led to a reduction of average lifespan by one year.
4. Our study revealed the immense need of improvement of cardiac care for patients at high and very high cardiovascular risk in Ukraine by increasing accessibility to invasive reperfusion procedures in ACS and specialist healthcare, by implementing national educational programs about the symptoms of ACS. This is required to decrease both in-hospital and long-term mortality of patients with STEMI. Currently, according to the Ukrainian Ministry of Health reports, the total number of catheterization laboratories (Cath Labs) is 42 whereas the population of Ukraine is over 44 million (1 Cath Lab for 1,048,000 people) [28]. In Switzerland it is 1 Cath Lab for 230,000 people and in Poland, a country neighbouring Ukraine, with a population of 37.97 million it is 1 Cath Lab for 239,000 people.

LIMITATIONS OF THE STUDY

Our study has several limitations that need to be addressed. Extrapolation of local results to the whole country needs to be performed with caution. The PCI results, in-hospital and general mortality since 2013 for STEMI in Ukraine could have changed. In regards to follow-up data, the reliability of the data with regards to medication is somewhat compromised by the fact that two-year follow-up was limited to 31.9% (278) of the patients of the whole registry cohort who entered the follow-up period at the time of discharge from hospital.

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ORIGINAL ARTICLE

MORPHOLOGICAL FEATURES OF TUBULAR BONES REPARATIVE REGENERATION UNDER THE INFLUENCE OF ANTITUMOR CHEMOTHERAPEUTICS

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ABSTRACT

The aim: Determination of morphological features of reparative regeneration of diaphysis defect of long tubular bones under the influence of antitumor chemotherapeutics in a model experiment.

Materials and methods: 96 white nonlinear rats after application of the perforated defect of the femur were administered the appropriate antitumor drug (doxorubicin, 5-fluorouracil, methotrexate) three times with an interval of 21 days. Morphological features of bone tissue formation and remodeling in the regenerate area were studied using histological and morphometric methods.

Results: The inhibitory effect of antitumor chemotherapeutics on the formation of regenerate, expressed by slowing down the process of bone tissue differentiation was found. This is confirmed by a decrease in the area of reticulofibrous and lamellar bone tissue, chaotic arrangement and narrowing of bone trabeculae with uneven color, slow formation of bonding lines between the maternal bone and the regenerate.

Conclusions: The revealed morphological features of reparative regeneration of the diaphysis defect of long tubular bones under the influence of antitumor chemotherapeutics doxorubicin, 5-fluorouracil and methotrexate in a model experiment indicate a slowing of reparative regeneration processes at all stages of recovery after injury.

KEY WORDS: reparative regeneration, bone, antitumor chemotherapeutics

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INTRODUCTION

The homeostatic mechanism of reparative regeneration includes the interaction of a complex of factors (osteogenic and immunocompetent cells migration,growth factors and chemotactic mediators production, an adequate vascular meshwork formation, etc.), which form the necessary proregenerative microenvironment. [1-3].

Understanding the morphological changes forming the basis of each stage of reparative bone regeneration will allow us to develop effective strategiesaimed at optimizing the conditions of regeneration, activating various sources of regenerative cells, reducing the impact of processes that slow regeneration, and, ultimately, obtaining the predicted growth of bone tissue to eliminate its deficiency caused by pathological exposure [4-6].

One of the most current issues of modern regenerative medicine is solving the problem of the negative impact of chemotherapeutics used to treat cancer, often over a long period of time. With the development of cancer in the body there are disorders of bone metabolism manifested by the development of osteoporosis and bone metastasis. In particular, bone is the most common site of metastasis in patients with breast or prostate cancer. [7-10]. By colonizing bone, tumor cells interact with different cell types (osteoblasts, osteoclasts, osteocytes, fibroblasts, immune cells), form the so-called «vicious circle» in the locus of invasion, based on the ability of metastatic cells to coax osteoclasts and osteoblasts, which further stimulate the growth of cancer cells (the concept of premetastatic niche). To date, the most characterized are the effects of VEGF (VascularEndothelianGrowthFactor) and disturbances in the system of RANKL/RANK/OPG (ReceptorActivatorofNuclearFactor-kBLigandOsteoprotegerin) [11-13].

According to a number of retrospective studies, antitumor chemotherapy, which begins immediately after diagnosis and is prescribed for a long time, is usually associated with the loss of bone mineral density and increased risk of fractures.[14,15].

Therefore, study of bone regeneration under the influence of chemotherapeutics is current in terms of understanding the structural basis of pathophysiological processes that develop in bone under such conditions. They will provide an opportunity to develop a system of measures to reduce the negative impact of antitumor chemotherapy on the bone microenvironment, which in turn will optimize the regenerative potential of bone and solve the problem of osteoporosis that accompanies cancer.

THE AIM

The aim of the study was to investigate the morphological features of reparative regeneration of the long tubular bones diaphyses defect under the influence of antitumor chemotherapeutics in a model experiment

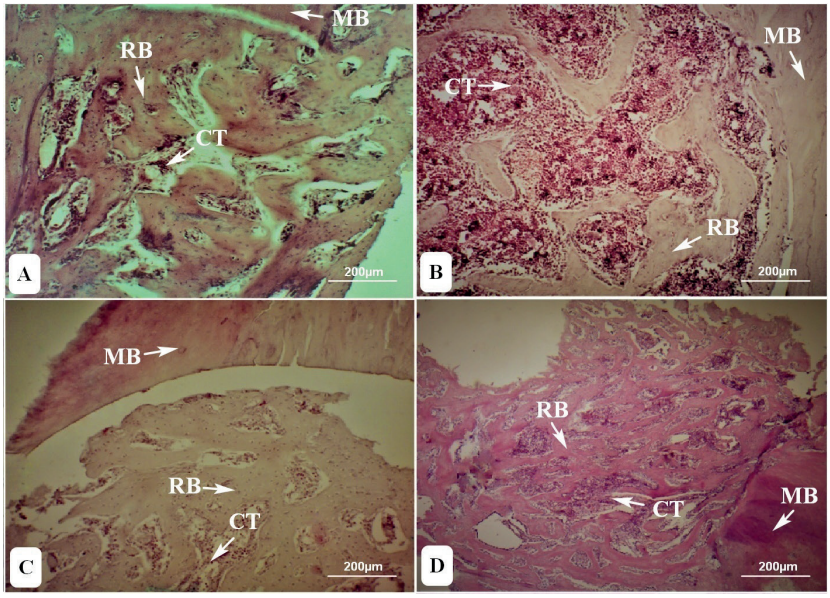

Fig. 1. Histological section of rat femur regenerate on the 15th day after the perforated defectapplication: A – control group, B – animals receiving doxorubicin, C –animals receiving 5-fluorouracil, D – animals receiving methotrexate. CT–connectivetissue, RB–reticulofibrousbonetissue, MB–maternalbone(100x)

MATERIALS AND METHODS

The research was carried out on 96 white nonlinear male rats aged 7 months at the start of the experiment (themean bodyweight was220.0 ± 10.0 g).The animals were kept innormal vivarium conditions on a standard diet and drinking regime. The study was performed in compliance with the requirements of biological ethics in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Purposes (Strasbourg, 1985) and the principles of research defined by the Commission on Bioethics in Experimental and Clinical Research of Sumy State University Medical Institute.

Experimental animals were divided into control (group 1, n = 24) and experimental (group 2, n = 72) groups by random sampling.In a sterile operating room, under ketamine anesthesia (50 mg / kg), all animals underwent a perforated defect in the middle third of the femoral diaphysis 2 mm in diameter, and depth to the medullary cavity using a sphericaldental drill cutter under cooling. Experimental animals, in turn, were subdivided into 3 groups by random sampling. Immediately after injury,the animals of group 2.1 (n = 24) were injected intraperitoneally with the pharmaceutical drug doxorubicin at a dose of 60 mg / m^2 , pre-diluted in 1.5 ml of saline, the animals of group 2.2 (n = 24) – with 5-fluorouracil at a dose of 600 mg / m^2 in 1,0 ml of saline, and the animals ofgroup 2.3 (n = 24) – with methotrexate at a dose of 40 mg / m^2 in 1,0 ml of saline.Re-administration of appropriate chemotherapeutics was performed in the same doses on the 21st and 42nd day after injury. Animals in the control group were injected intraperitoneally with a similar volume of saline according to the same scheme.

On days 15, 30, 45, and 60 after injury, animals were removed from the experiment by decapitation under ketamine anesthesia. The traumatized femur diaphyses were fixed in 10% formalin solution on phosphate buffer for 24 hours. Decalcification was performed in 5% nitric acid solution for 14 days (solution was replaced daily) at room temperature.The completeness of decalcification was determined by a needle test. Then the test material was washed with running water followed by dehydration in alcohol solutions of increasing concentration. After dehydration, the samples were embedded in paraffin. Histological sections of 6-10 μm thickness were made with a rotary ultramicrotome Shandon Finesse 325 (Thermo Scientific, USA).Preparations were stained with hematoxylin and eosin. Histological analysis was performed using a microscope «Carl Zeiss Primo Star» (Germany) at magnifications of x100 and x200.Photodetection was done by the digital camera «AxioCfm ERc 5s» (Carl Zeiss, Germany) with the image output «ZEN 2» (blue edition) (Carl Zeiss, Germany).

For morphometric analysis we applied a computer program for processing histological images «Digimizer», namely such toolsas a microgrid and a micro ruler. The following parameters were measured in the area of rats` femur defect: the area of connective tissue (Ac) , the area of reticulofibrous bone tissue (Arb), and the area of lamellar bone tissue (Alb).

Statistical analysis of the obtained data was performed using the software package Statistica v.10 ("StatSoft Inc.", USA). We calculated the mean value (M) and standard deviation (SD) by descriptive analysis of each sample. After checking the distribution of data for normality, we used Student's t-test to assess differences between independent samples. A probability level (p-value) of less than 0.05 was considered to be statistically significant.

RESULTS

15TH DAY OF THE EXPERIMENTA

In the control bone regenerate, the Arb in the defect is 43.83 ± 5.88%, the Ac– is 56.17 ± 3.87%.The reticulofibrous bone tissueis represented by trabeculae, which form large-looped mesh structures with numerous primary osteoblasts and osteocytes. Newly formed trabeculae are stained unevenly and less intensely compared to the maternal bone, indicating the beginning of ossification.The intertrabecular space is filled with connective tissue with fibroblasts, collagen fibers, and vessels.In the maternal bone, alongsidethe

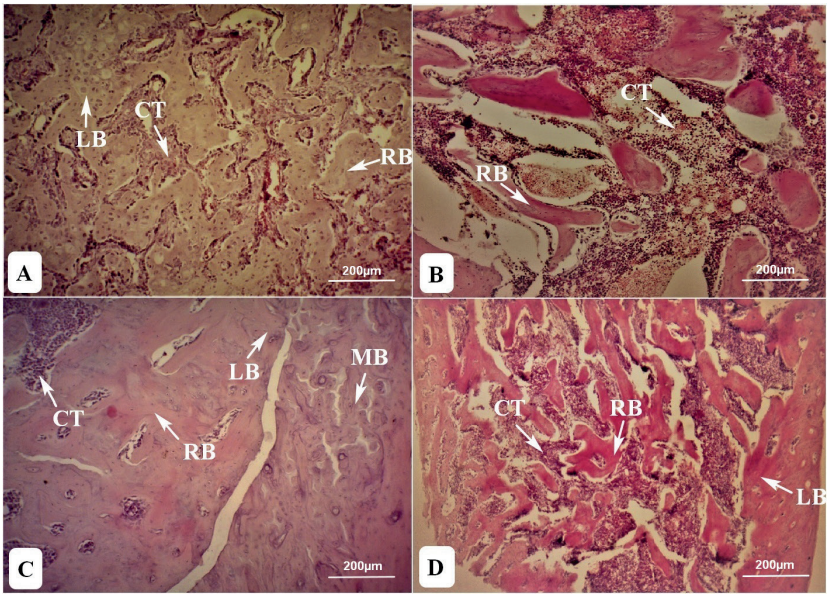

Fig. 2. Histological section of rat femur regenerate on the 30th day after the perforated defectapplication: A – control group, B – animals receiving doxorubicin, C –animals receiving 5-fluorouracil, D – animals receiving methotrexate. CT–connectivetissue, RB–reticulofibrousbonetissue,LB–lamellarbonetissue, MB–maternalbone(100x).

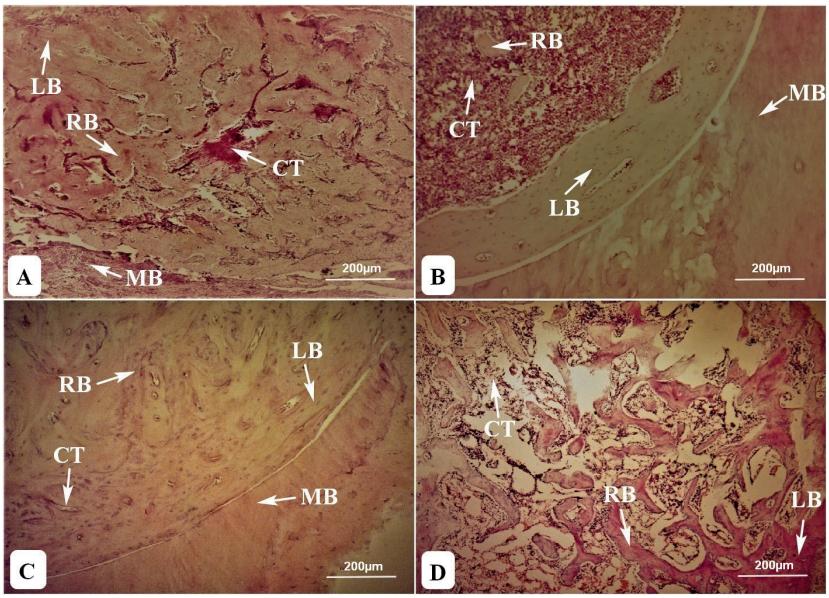

Fig. 3. Histological section of rat femur regenerate on the 45th day after the perforated defectapplication: A – control group, B – animals receiving doxorubicin, C –animals receiving 5-fluorouracil, D – animals receiving methotrexate.CT – connectivetissue, RB – reticulofibrousbonetissue,LB–lamellarbonetissue, MB – maternalbone(hematoxylin and eosin, 100x).

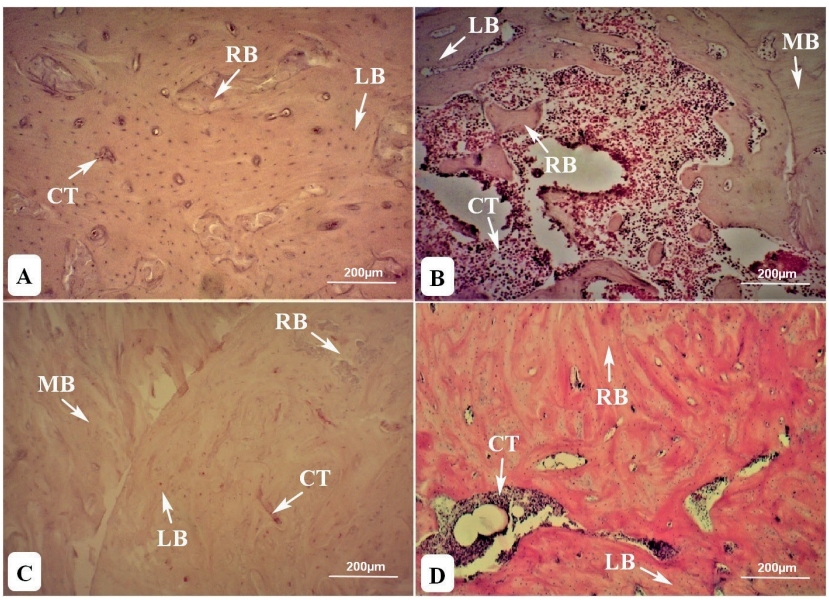

Fig. 4. Histological section of rat femur regenerate on the 60th day after the perforated defectapplication: A – control group, B – animals receiving doxorubicin, C –animals receiving 5-fluorouracil, D – animals receiving methotrexate. CT – connectivetissue, RB – reticulofibrousbonetissue, LB–lamellarbonetissue, MB – maternalbone (100x).

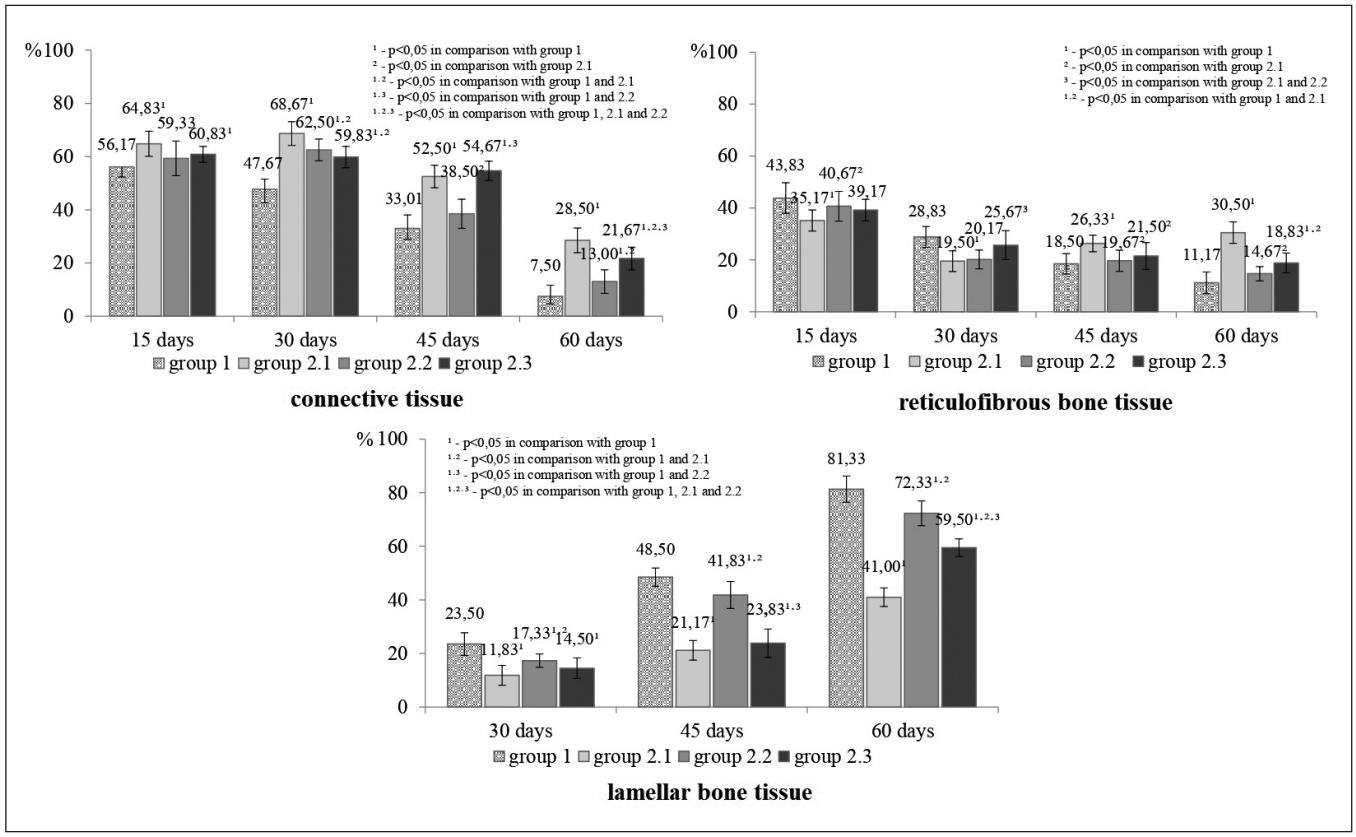

Fig. 5. Distribution of connective and bone tissue areas in bone regenerate of control and experimental groups during the experiment.

preserved cells, single empty osteocyte lacunae are located.The edges of the maternal bone are marked by signs of resorption. The boundary between the edges of the defect and the regenerate is clearly visualized (Fig.1A).

In the group 2.1 bone regenerate the Arb is less by 19.75% (p = 0.01), and the Ac, on the contrary, is greater by 15.41% (p = 0.006) compared with the bone regenerate of control group. In the defect cavity, bone tissue is represented by bone trabeculae and contains primary osteoblasts, osteocytes, and osteogenic cells. The connective tissue with areas of osteogenesis is present in the intertrabecular spaces (Fig. 1B).

In the group 2.2, reticulofibrous bone tissue and connective tissue occupy 40.67 ± 3.93% and 59.33 ± 6.50% of the total defect area, respectively. The differences between these indicators in the control group and the group 2.2 are insignificant, but their probable direction, namely, a decrease in the Arb by 7.21% (p = 0.29) and an increase in Ac by 5.62%(p = 0.33), is similar to that of the group 2.1(Fig. 1C).

In the regenerate of group 2.3, in comparison with the control group, there is an increase in the Ac by 8.29% (p = 0.04) (60.83 ± 2.99%). The indicator of the Arb(39.17 ± 4.17%) does not significantly differfromthe same indicator in control animals, but the probable direction of changes in group2.3 is towards a decrease in the indicator by 10.63% (p = 0, 14).The regenerate is represented by small-looped trabeculae mainly. The intertrabecular space is filled with connective tissue and bone marrow (Fig. 1D).

30TH DAY OF THE EXPERIMENT

In the control group the formation of complete bone regenerate is observed in the area of the defect. The regenerate is represented by lamellar and reticulofibrous bone tissues with a total area of 52.33 ± 4.15%. The basic area of the defect is formed by a small-looped mesh of trabeculae with numerous osteocytes. The coloring of trabeculae is more uniform and it approaches to the maternal bone by intensity. It indicates the normalization of osteoid ossification. The cortical plate of the regenerate is represented by lamellar bone tissue fused with the edges of the defect.In the intertrabecular spaces there are preserved areas of connective tissue with a total area of 47.67 ± 5.05%, which is 15.13% (p = 0.008) less than in the control group on the 15th day of the experiment, as well as blood vessels and bone marrow. The formation of intensely colored bone plates around blood vessels is detected. The described changes indicate the beginning of the restructuring of reticulofibrous bone tissue into lamellar (Fig.2A).

In the group 2.1 the main part of the newly formed regenerate is connective tissue (68.67 ± 4.46%), which is 41.95% (p<0.0001) more than in the control groupregenerate.Bone tissue occupies 31.33 ± 2.1% of the regenerate area. This is 40.12% (p<0.001) less than in the control group regenerate (52.33±4.11%). It is represented mainly by reticulofibrous type with an area of 19.5 ± 3.99%, in the form of single, unrelated trabeculae.The intertrabecular spaces of reticulofibrous bone tissue have the form of wide channels filled with red bone marrow with pronounced vascularization. Mainly on the regenerateperiphery, around the blood vessels, there

is the formation of concentrically arranged and intensely colored bone plates.The formation of lamellar tissue is also slowed, its area is 11.83 ± 3.71%, which is 49.65% (p = 0.003) less than in the control group.A small number of osteoblasts are located on the trabeculaesurface (Fig. 2B).

In the group 2.2, the regenerate consists of 62.50% of connective tissue and 37.50% of bone tissue. The Ac is greater by 31.10% (p = 0.0002) than in the control group, while the area of newly formed bone tissue is correspondingly less by 39.55% (p = 0.007). Bone tissue is represented mainly by reticulofibrous type (20.17 ± 3.19%) and its area does not differ significantly from the same in the control group. Lamellar bone tissue occupies 17.33 ± 2.50%. On the 30th day of the experiment the cortical layer between the maternal bone and the regenerate is not yet formed and has signs of remodelling. There is a free space between the regenerate and the edge of the maternal bone (Fig 2C).

In the group 2.3regenerate, bone tissue is represented mainly by reticulofibrous type (25.67 ± 4.13%) in the form of a small-loopedtrabecularmeshwith osteocytes. Vascular canals and structures similar to primary osteons (14.5 ± 3.83%) are formed mainly along the edges of the defect. Connective tissue occupies 59.83 ± 4.02% of the regenerate area, which is 25.50% (p = 0.0009) more than in the control group regenerate(Fig. 2D).

45TH DAY OF THE EXPERIMENT

In the regenerate area of the control group, the process of bone remodeling is marked by a further increase in the area of bone tissue, which averages 67.00 ± 4.50%. The newly formed bone tissue is represented mainly by the lamellar type (48.50 ± 3.39%). In the central part of the defect is a small area of the connective tissue, where the collagen fibers ordering and their transformation into osteoid trabeculaeis being observed (Fig.3A).

In the group 2.1, most of regenerate is represented by connective tissue with bone marrow (52.40 ± 4.23%). At the edges of the defect there is a bone tissue with an area of 47.50 ± 3.63%, main represented mainly by reticulofibrous type. However, in this locus, there is a narrowing of the space between the regenerate and the maternal bone compared to the previous term of the experiment. (Fig. 3B).

Among all components of the group 2.2 regenerate the newly formed bone tissue prevails(61.50 ± 4.56%). It is represented mainly by lamellar tissue with osteons of varying degrees of maturity. Connective tissue occupies 38.50 ± 5.47% of the regenerate. This indicator is not significantly differ fromthat in the control animals, but the probable tendency to delay the process of bone formation is noticeable, because the connective tissue occupies 16.66% (p = 0.08) more area than in the control group. In some places, there is a formation of junctions between the regenerate and the maternal bone, but it is less pronounced than in the control group (Fig. 3C).

In the group 2.3, the regenerate is represented mainly by small-looped trabeculae with an inhomogeneously colored matrix. Most trabeculae are thin, with cracks and fissures. Bone trabeculae are arranged chaotically without matching the load lines. A small number of osteoblasts are found on the outer surface of the trabeculae and cavity walls. The intertrabecular spaces are dilated and contain connective tissue, theAc is 54.67 ± 3.61%. Blood vessels and red bone marrow are also visible in the intertrabecular spaces (Fig. 3D).

60TH DAY OF THE EXPERIMENT

In the control group, the vast majority of the defect area is represented by lamellar bone tissue (Albis 81.33 ± 4.17%), with osteons of varying degrees of maturity. There are many blood vessels around which concentrically located osteons are formed. This indicates a high activity of angiogenesis in theregenerate area, which is a necessary condition for its further adequate restructuring in the area of the applied defect (Fig. 4A).

In the group 2.1, compared with the control one, the Ac (28.50 ± 4.64%) increases by 21.00% (p <0.0001) and bone area (71.50 ± 4.52%)) is less by 21.00% (p <0.0001). The lamellar bone tissue is located mainly on the periphery of the defect. There is a narrowing of the space between the regenerate and the maternal bone with a small number of junctions (Fig. 4B).

In the group 2.2, the main part of the regenerate is formed by lamellar bone tissue (72.33 ± 4.63%).It is 11.06% (p = 0.008) less than the same control indicator. There is an active process of junctions formation between the maternal bone and the defect (Fig. 4C).

In the group 2.3, the regenerate consists of connective tissue (Ac is 21.67 ± 4.27%). Bone tissue in the defect area is 78.33 ± 3.54%, which is 15.31% less than in the control group. The lamellar tissue fills 59.50 ± 3.27% of the regenerate area, which is 26.84% (p<0.0001) less than the control indicator (Fig. 4D).

Quantitative data on the area of connective and bone tissues distributionin the regenerate in the dynamics of the experiment is presented in Fig. 5.

DISCUSSION

The current study was aimed at determining the morphological features of bone tissue reparative regeneration under the influence of the most widely used antitumor chemotherapeutics.

The vast majority of patients with cancer should take long courses of antitumor chemotherapy. This is one of the causes of bone mass loss due to impaired bone microarchitecture, which reduces skeletal strength and increases the risk of fractures, usually of the spine (compression fractures of the vertebrae), thighs and wrists. Further reparative regeneration of bone tissue also occurs under the influence of chemotherapeutic drugs, which, of course, affect the speed and quality of regenerative processes[16].

Under physiological conditions, reparative regeneration occurs according to the stages of the bone defecthealing, namely through the newly formed reticulofibrous bone

tissue, which over time occupies an increasing defect area and undergoes appropriate remodeling with subsequent formation of lamellar bone tissue and its fusion with the maternal bone. [17-19].

The use of antitumor chemotherapeutics, in particular doxorubicin, 5-fluorouracil and methotrexate, in a model experiment leads to a slowing of reparative regeneration, which is manifested by a delay in the regenerateformation due to slowing tissue differentiation. This is confirmed by a slow decrease in the Ac and Arbalong with a relatively slow increase in the Albin the defect.Changes in the microarchitecture of the defect area under the influence of these antitumor chemotherapeutics are characterized by chaotic location of bone trabeculae, which are relatively smaller, by the presence of free space between the maternal bone and regenerate, by low rates of bonding lines between them. The most pronounced delay in the bone regenerateremodelingwas observed in the use of doxorubicin and methotrexate, while 5-fluorouracil showed less inhibitory effect on these processes.

A significant inhibitory effect of doxorubicin was also found in a model experiment on young mice conducted by L Straszkowski et al. In particular, it was found that doxorubicin adversely affects the longitudinal growth of bone, inhibits differentiation and reduces the volume of both spongy and cortical bone tissue, which ultimately leads to increased bone fragility[20].

According to studies by T. Rana et al, doxorubicin increases the circulating level of Transforming Growth Factor-β (TGFβ) in mice in an experiment that induces osteoclast-mediated resorption and inhibits osteoblast differentiation. This promotes the development of osteolytic bone damage and slows the reparative regeneration in bone tissue [21].

Our results are in good agreement with the data obtained by H. Fonseca et al. in an experiment on Wistar rats. The authors noted the negative impact of doxorubicin on the radial growth of the femur, the differentiation of bone tissue, its microarchitecture and mechanical properties [22].

The inhibitory effect of 5-fluorouracil on the reparative bone regeneration noted in our study correlates well with the effect of this drug described by J. Quach et al. Based on histomorphometric analysis of mouse bones, these researchers found that bone mass loss associated with using 5-fluorouracil, was caused by the inability of osteoblasts to form sufficient bone tissue mass to compensate for the increased osteoclastic bone resorption (ie, the inadequacy of the balance between bone formation and resorption). According to the authors, this is due to a change in the ratio in the system RANKL / RANK / OPG [23].The decrease in bone tissue mineralization potential under the influence of 5-fluorouracil, despite the lack of changes in osteoblasts density on the surface of the trabecular bone was also noted in the works of C. Fan et al., K. Georgiou et al. and R. McKinnon et al. As a reason, the authors noted a decrease in the osteoblastsactivity, confirming by low levels of bone alkaline phosphatase in the experimental ratsserum [24].

The negative impact of methotrexate is also confirmed by the results of an experimental study by F Robin et al., which found an increase in apoptotic osteocytes in the juvenile rats tibial metaphysis during the methotrexate chemotherapy [25]. According to K. Georgiou et al., the methotrexate causes changes in osteogenic, hematopoietic, osteoclastogenic and adipogenic bone marrow differentiation associated with impaired Wnt / β-catenin signaling.This leads to bone marrow obesity, activation of osteoclast differentiation and an increase in their number [26]. The inactivation of β-catenin in osteoblasts does not affect their activity, but causes increased osteoclastogenesis due to insufficient osteoprotegerin production. As a result, increased bone resorption causes a decrease in bone tissue mass and slows the regeneration [27].

A corresponding contribution to the development of the pathological process associated with a decrease in bone mineral density is made by increased production of proinflammatory cytokines IL-1 and IL-6 by monocytes and activation of nuclear factor kappa b (NF-kB) under long-term methotrexate therapy [28].

CONCLUSIONS

The use of antitumor chemotherapeutics doxorubicin, 5-fluorouracil, and methotrexate in a model experiment leads to a slowdown the long tubular bones reparative regeneration at all stages of healing of traumatic injury, expressing in a decrease in the rate of tissue differentiation in the regenerate area. The inhibitory impact of doxorubicin and metatrexate are relatively more pronounced than that of 5-fluorouracil.

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ORIGINAL ARTICLE

CTLA-4 POLYMORPHISM ALONG WITH PROINFLAMMATORY CYTOKINES IN AUTOIMMUNE THYROIDITIS DISEASE

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ABSTRACT

The aim: Evaluating serum concentration of IL-17 and IL-23 in autoimmune thyroiditis patient and control group along with the role of CTLA-4 rs3087243 gene polymorphism.

Materials and methods: A case control study was conducted in 30 HT (Hashimoto's thyroiditis), 30 GD (Graves' disease) who attended the consultant clinic for thyroiditis in AL-Diwaniyah teaching hospital and in 30 people as control group. Blood samples were processed for measurement of serum IL-17 and IL-23 using ELISA test. The second part used for DNA extraction then CTLA-4 polymorphism was detected by Allele – specific PCR assay.

Results: The level of IL-17, and IL23 was highest in patients with Hashimoto's thyroiditis and Graves' disease, followed by control group and the difference was highly significant ($p < 0.001$; $p < 0.001$) respectively; however, the difference between patients Hashimoto's thyroiditis and patients with Graves' disease was not significant ($p > 0.05$; $p > 0.05$) respectively. There was no significant association between rs3087243 gene polymorphism and Hashimoto's thyroiditis ($p > 0.05$), no significant association between rs3087243 gene polymorphism and Graves' disease ($p > 0.05$). Moreover, there was no significant difference in rs3087243 genotypes frequencies between Hashimoto's thyroiditis and Graves' disease ($p > 0.05$).

Conclusions: Serum IL-17 and IL-23 level have been linked with autoimmune thyroiditis disease, while CTLA-4 rs3087243 polymorphism seem to have no role in disease susceptibility in Iraqi population.

KEY WORDS: Autoimmune thyroiditis, Hashimoto's thyroiditis (HT), Graves' disease, CTLA- 4 polymorphism rs3087243 gene, IL-17, IL-23

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INTRODUCTION

When the body's immune system assaults the thyroid gland, this is known as psychoactive drugs. Graves's disease (GD) and Hashimoto's thyroiditis (HT), both AITDs, are the most frequent causes of thyroid gland failure and no endemic goiter [1,2]. Self-thyroid antigen reactivity, which manifests as inflammatory or anti-receptor autoimmune disorders [3-4], has an impact on these structures. They occur by reaction to self-thyroid antigens and are produced by a complex interaction of environmental and genetic factors [2]. One of the most well-studied and researched AITD susceptibility genes include the HLA-DR gene cluster, as well as non-MHC genes, including CTLA-4, CD40, PTPN22, thymoglobulin, and TSH receptor genes [5-15]. Iodine, medications, sickness, smoking, stress, and genetic predisposition to AITD are all important environmental triggers of thyroid autoimmune disease, pointing to novel possible routes via which genetic-environmental interactions may contribute to thyroid autoimmunity [13]. Non-MHC proteins such as CTLA-4, CD40, PTPN22, thymoglobulin, and TSH receptor genes, as well as the HLA-DR gene locus [15], have all been discovered and described as significant AITD susceptibility genes. Iodine, medicines, illness, smoking, stress, and genetic susceptibility to AITD are all significant environmental triggers of thyroid autoimmune, leading to new potential pathways by which genetic-environmental interactions may contribute to thyroid autoimmunity [16].

THE AIM

Evaluating serum concentration of IL-17 and IL-23 in autoimmune thyroiditis patient and control group along with the role of CTLA-4 rs3087243 gene polymorphism.

MATERIALS AND METHODS

A case control study was conducted in AL-Diwaniyah province. Based on 30 patients with HT, equaling 5 males and 25 females, and 30 patients with GD (9 males and 21 females), who attended the consultant clinic for thyroiditis were taking part in this study. In addition to that, about 30 people (11 males and 19 females) apparently healthy volunteers were included as a control group. Blood samples were collected by venipuncture from 60 patients (30 for HT and 30 for GD) and 30 healthy controls, five milliliters of venous blood were drawn by disposable syringe under aseptic technique. Three ml of blood were put in gel tube and allowed to clot, then the serum was separated by centrifugation (1500 rpm for 5 minute). The serum has been collected in Eppendorf tube then stored at -20°C to be used for ELISA test to determine concentration of IL-17 and IL-23 in serum. Another two ml

Table I. Primers for CTLA-4genepolymorphism

Primer		Sequence (5'-3')	PCR product size
CTLA_4 (rs3087243)	F	CACCACTATTTGGGATATACC	216 bp
	R	AGCTCTATATTTCAGGAAGGC	

Table II. The restriction enzymes were used in RFLP-PCR assay with their company and country of origin

Target gene	Polymorphism	Restriction Enzymes	Company/Country
CTLA-4 gene(rs3087243)	G/A	NcoI	New England Biolabs. UK

Table III. Polymorphism Chain Reaction (PCR) Thermo cycler Conditions

PCR step	Temp	Time	Cycle repeat
Initial denaturation	95°C	5min.	1
Denaturation	95°C	30sec.	34cycle
Annealing	55°C	30sec	
Extension	72°C	30sec	
Final extension	72°C	5min	1cycle
Stop reaction	4°C	Forever	-

Table IV. Comparison of rs3087243genotype frequencies between control group and Hashimoto's thyroiditis

rs3750920 genotypes	Control n = 30	Hashimoto's thyroiditis n = 30	p
AA	8 (26.7 %)	7 (23.3 %)	0.850 C NS
A/G	14 (46.7 %)	13 (43.3 %)	
GG	8 (26.7 %)	10 (33.3 %)	

n: number of cases; C: Chi-square test; NS: not significant at p> 0.05

Table V. Comparison of rs3087243alleles frequencies between control group and Hashimoto's thyroiditis

rs3750920 alleles	Control n = 60	Hashimoto's thyroiditis n = 60	p
A	30 (50.0 %)	27 (45.0 %)	0.583 C NS
G	30 (50.0 %)	33 (55.0 %)	

n: number of cases; C: Chi-square test; NS: not significant at p> 0.05

Table VI. Comparison of rs3087243genotypes frequencies between control group and Graves' disease

rs3750920 genotypes	Control n = 30	Graves disease n = 30	p
AA	8 (26.7 %)	10 (33.3 %)	0.410 C NS
A/G	14 (46.7 %)	9 (30.0 %)	
GG	8 (26.7 %)	11 (36.7 %)	

n: number of cases; C: Chi-square test; NS: not significant at p> 0.05

of blood were collected in ethylinditetracitic acid (EDTA) tube and stored at -20°C for DNA extraction and detection of CTLA-4 polymorphism by Allele-specific PCR study design.

GENOMIC EXTRACTION

Genomic DNA from blood samples were extracted by using G-spin DNA extraction kit (Frozen Blood) INtRON, Korea; and done according to company instructions. The extracted blood genomic DNA was checked by using Nanodrop spectrophotometer (THERMO USA), which measured DNA concentration (ng/μL) and checked the DNA purity by reading the absorbance at (260 /280 nm).

STEM LOOP RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)-PCR

Restriction Fragment Length Polymorphism-PCR was performed for detection of CTLA-4 gene polymorphism (rs3087243) (G/A) in HT and GD patients and healthy control blood samples. This method was carried out, according to described one by López-Villalobos.

PRIMERS: PRIMERS FOR THE CTLA-4 POLYMORPHISM GENE

The RFLP-PCR primer for detection and genotyping of CTLA-4 (rs3087243) (G/A) gene polymorphism were

Table VII. Comparison of rs3087243alleles frequencies between control group and Graves' disease

rs3750920 alleles	Control n = 60	Graves disease n = 60	p
A	30 (50.0 %)	29 (48.3 %)	0.855 C NS
G	30 (50.0 %)	31 (51.7 %)	

n: number of cases; C: Chi-square test; NS: not significant at p> 0.05

Table VIII. Comparison of rs3087243genotypes frequencies between Hashimoto's thyroiditis and Graves' disease

rs3750920 genotypes	Hashimoto's thyroiditis n = 30	Graves disease n = 30	p
AA	7 (23.3 %)	10 (33.3 %)	0.521 C NS
A/G	13 (43.3 %)	9 (30.0 %)	
GG	10 (33.3 %)	11 (36.7 %)	

n: number of cases; C: Chi-square test; NS: not significant at p> 0.05

Table IX. Comparison of rs3087243alleles frequencies between Hashimoto's thyroiditis and Graves' disease

rs3750920 alleles	Hashimoto's thyroiditis n = 60	Graves disease n = 60	p
A	27 (45.0 %)	29 (48.3 %)	0.714 C NS
G	33 (55.0 %)	31 (51.7 %)	

n: number of cases; C: Chi-square test; NS: not significant at p> 0.05

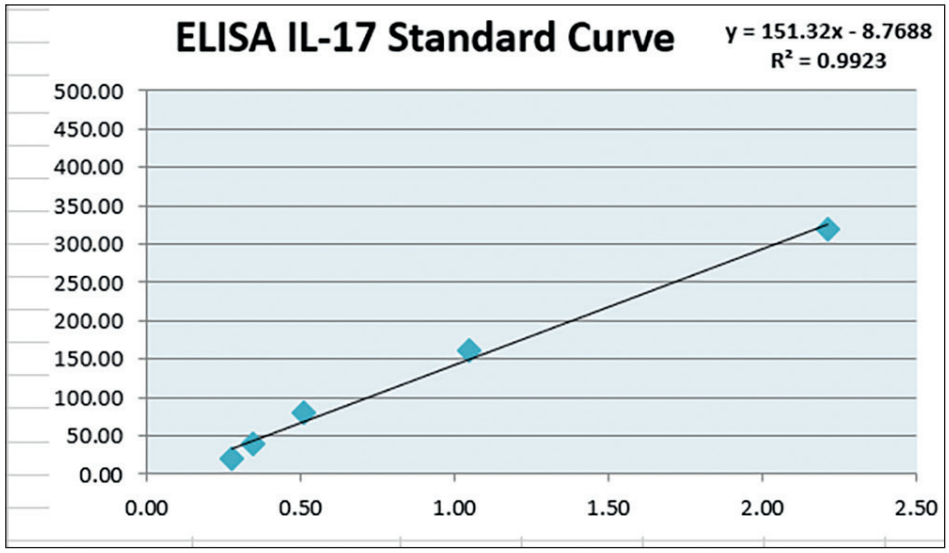

Fig. 1. Standard curve of Interleukin-17

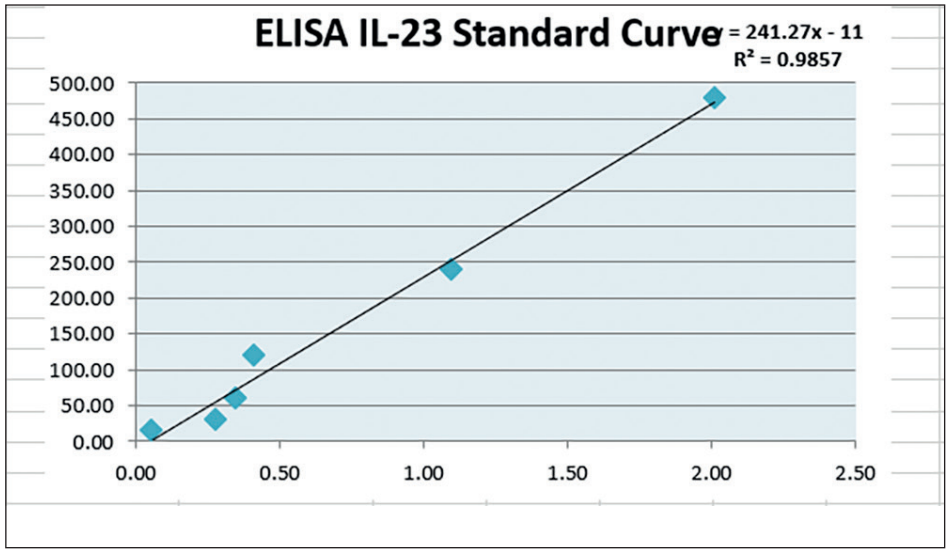

Fig. 2. Standard curve of Interleukin-23
Thermo cycler Conditions of PCR

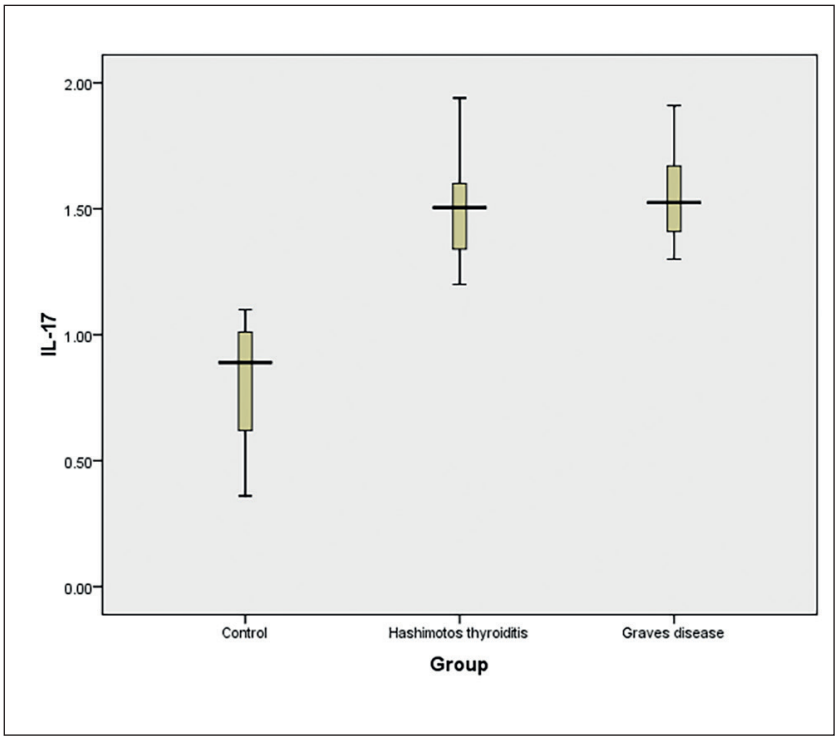

Fig. 3. Box plot showing comparison of serum interleukin-17 among patients with Hashimoto's thyroiditis, Graves' disease and control group

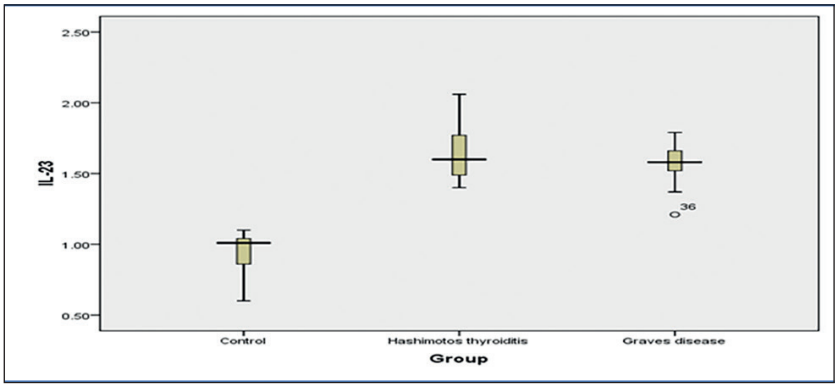

Fig. 4. Box plot showing comparison of serum interleukin-23 among patients with Hashimoto's thyroiditis, Graves' disease and control group

designed by López-Villalobos [1]. These primers were provided from (Macrogen company, Korea) as indicated in following tables (I-II).

RESTRICTION ENZYME

Table II. The restriction enzymes were used in RFLP-PCR assay with their company and country of origin

ELISA TEST

The quantitative sandwich enzyme immunoassay methods were used in this test. An antibody specific for IL-17 and IL-23 has been pre-coated on the micro-ELISA plate. The antigen is then attached to the immobilized capture antibody, and the standard and samples are pipetted into the wells, with any IL-17 and IL-23 present being bound by the immobilized antibody, following a basic washing technique to remove any loose substances. To the wells, a biotin-conjugated antibody specific for IL-17 AND IL-23 is applied. After washing, Avidin conjugated Horse radish peroxidase (HRP) was added to each microplate well, incubated, and washed to eliminate any unbound Avidin-enzyme reagent. Finally, a substrate solution specific to the enzyme in the well was added. The amount of IL-17 AND IL-23 bound in the initial stage is exactly proportional to the color intensity generated, when a stop solution is added to the enzyme-substrate reaction, the color changes to yellow. At a wavelength of 450nm, the optical density (OD) is measured spectro-photometrically. The OD value is proportional to the concentration of IL-17 and IL-23; therefore, we compared the OD of the samples to the standard curve to compute the concentration of IL-17 and IL-23 in the sample.

CALCULATION OF RESULTS

The ELISA results were: calculation depending on the average of the duplicate readings for each standard and samples optical density. Then, we created a standard curve by plotting the mean OD value for each standard on the y-axis against the concentration on the x-axis and drew a best fit curve through the points on the graph in excel office program, figures (1-2).

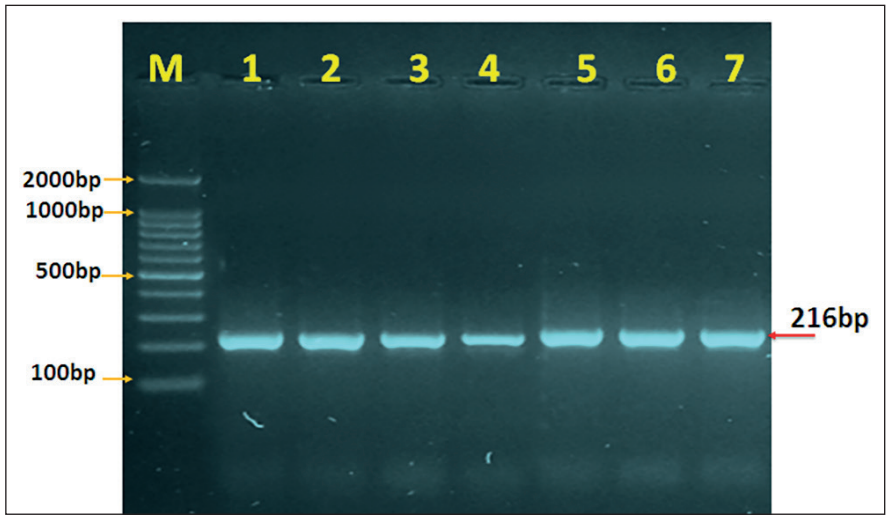

Fig. 5. Agarose gel electrophoresis image that showed the PCR product analysis of CTLA4 gene from patient and healthy control blood samples. Where M: marker (2000-100bp), lane (1-7) positive PCR amplification at 216bp PCR product size

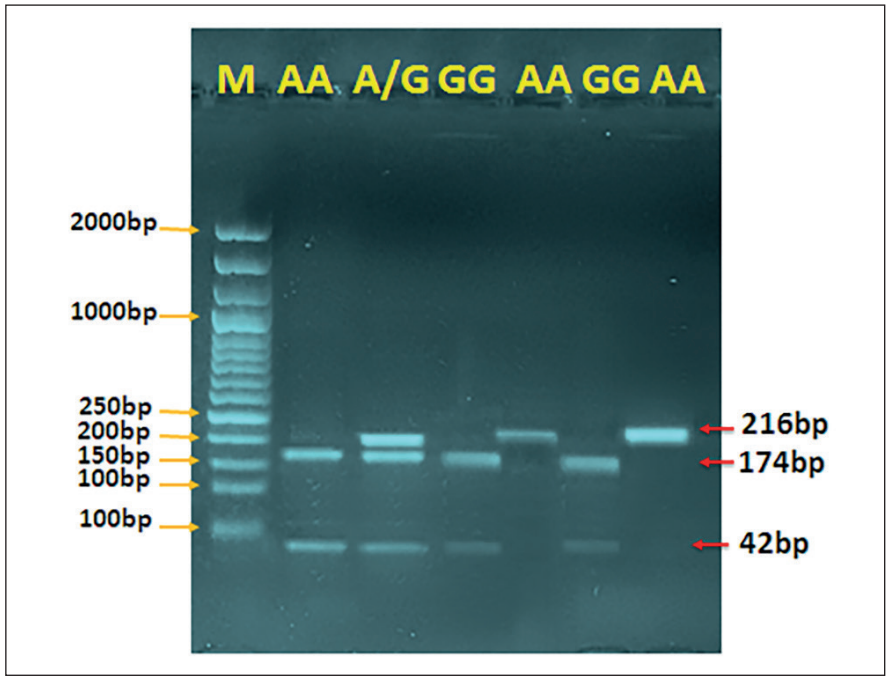

Fig. 6. Agarose gel electrophoresis image that showed the RFLP-PCR product analysis of CTLA4 (rs087243) gene polymorphism from patient and healthy control blood samples by using NcoI restriction enzyme. Where M: marker (2000-50bp), lane (AA) wild type homozygote, the PCR product was undigested by restriction enzyme and still 216bp band, the lane (GG) mutant type homozygote that showed product was digested by restriction enzyme into 174bp and 42bp band, and the lane (A/G) heterozygote, the product was digested by restriction enzyme into 216bp, 174bp, and 42bp bands.

PCR thermo cycler conditions were done for each gene in dependent as following table III.

STATISTICAL ANALYSIS

All data were normally distributed and recorded in Microsoft Excel spread sheet, statistical analysis carried out with SPSS version 0.17 software, numeric data were presented as mean, standard deviation, median and Interquartile range (IQR), while nominal data were expressed as number and percentage. Independent sample T test was used to compare mean value between two groups, while Mann Whitney U test was used to compare median value between two groups, the level of significance considered when p-value was less than 0.05.

RESULTS

The level of IL-17, and IL23 was highest in patients with Hashimoto's thyroiditis and Graves' disease and followed by control group and the difference was highly significant ($p< 0.001$; $p< 0.001$) respectively; however, the difference between patients Hashimoto's thyroiditis and patients with Graves' disease was not significant ($p > 0.05$; $p > 0.05$) respectively, figures (3-4). There was no significant association between rs3087243 gene and allele polymorphism and Hashimoto's thyroiditis ($p> 0.05$), as shown in tables (IV-V). In addition, there was no significant association between rs3087243 gene and allele polymorphism and Graves' disease ($p> 0.05$), as shown in table VI and VII. Moreover, there was no significant difference in rs3087243 genotypes and alleles frequencies between Hashimoto's thyroiditis and Graves' disease ($p> 0.05$).

GENETIC ANALYSIS

There was no significant association between rs3087243 gene and allele polymorphism and Hashimoto's thyroiditis ($p> 0.05$), as shown in tables (IV-VI). In addition, there

was no significant association between rs3087243 gene and allele polymorphism and Graves' disease (p> 0.05), as shown in tables (VII-VIII). Moreover, there was no significant difference in rs3087243 genotypes and alleles frequencies between Hashimoto's thyroiditis and Graves' disease (p> 0.05), as shown in tables IV and V.

DISCUSSION

In the present study, the level of IL-17, and IL23 was highest in patients with Hashimoto's thyroiditis and Graves' disease and followed by control group. The difference was highly significant (p< 0.001). In the study of Gerenova [2], serum levels of both cytokines, IL-17 and IL-23, were significantly higher in patients with Hashimoto's thyroiditis in comparison with healthy control subjects; therefore, the results of the current study are in line with that of [2]. Our results are also comparable to the results of Degertekin [3], who found that serum level of IL-17 was significantly higher in patients with Hashimoto's thyroiditis in comparison with healthy control subjects. According to Kim et al in 2012 [4], serum IL-23 was significantly higher in patients with Graves' disease in comparison to control subjects, thus our results are in line with [4].

Moreover, the serum level of IL-23 has been shown to be significantly higher in newly diagnosed patients with Graves' disease and patients with active Graves' disease in comparison with patients with inactive Graves' disease [5]. The significantly higher level of serum IL-17 and serum IL-23 level in the current study in patients with Hashimoto's thyroiditis and Graves' disease supports the suggestion that IL-23/IL-17 axis plays an important role in the immune pathogenesis of these thyroid autoimmune disorders. Th17 cells, a newly discovered CD4+ T cell subset, and distinguished from the Th1 and Th2 cells, mainly produce IL-17 which acts in vitro and in vivo as a potent inflammatory cytokine [6]. It's functions reflected in the ability of the collective mobilization, recruitment and activation of Neutrophils by the effectors they secreted[7,8]. Th17 cells and IL-17 play an important role in various autoimmune diseases. Previous findings[9,10] and the latest study from Nanba [11] have shown that Th17 cells and IL-17 were related to the pathogenesis of Graves' Disease, Hashimoto's thyroiditis, and Graves' ophthalmopathy. A number of studies have shown that IL-23 is required for full acquisition of the pathogenic function and maintenance of effector Th17 cells [12,13]. IL-23 promotes the secretion of inflammatory factors, cytokines and Chemokines via binding to IL-23 receptor. The combination of IL-23 and IL-23 receptor may activate STAT3 signal, induced memory T-cells to differentiate into Th17 cells and affected the expression of IL-17 by increasing the expression of RORγt, and, ultimately, promoted inflammation and autoimmune diseases. IL-23/IL-17 axis mostly is composed of IL-23, IL-23 receptor, Th17 cells and IL-17. It's a critical pathway in activation and maintenance of Th17 cells. Studies found that the IL-23/IL-17 pathway is involved in the pathogenesis of autoimmune disease including Graves' disease [14]. Thus, our findings of high serum IL-17 and IL-23 are in line with Zheng et al., 2013 in that the IL-23/IL-17 pathway is involved in the pathogenesis of Graves' disease. The exact etiology of AITDs remains unknown, but it is believed that they are caused by an interactive combination of susceptibility genes and environmental triggers.

Recently, with the advent of new genomic tools and the accomplishment of the human genome, several susceptibility genes have been identified in AITDs, including protein tyrosine Phosphatase- 22 (PTPN 22), thymoglobulin gene (TG) and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) [15-17]. These susceptibility genes implicate autoimmunity in the pathogenesis of AITDs. Among them, CTLA-4 has been recently associated with functional relevance and with susceptibility to a variety of AITDs [18,19]. Our findings are in line with the observation of several previous authors who found no significant association between CTLA- 4 rs3087243 gene and allele polymorphism and Hashimoto's thyroiditis [20] and are in controversy to the results of other authors who found significant association between CTLA- 4 rs3087243 gene and allele polymorphism and Hashimoto's thyroiditis [21]. In addition, our findings are in line with the observation of several previous authors who found no significant association between CTLA- 4 rs3087243 gene and allele polymorphism and Graves' disease [22,23] and are in controversy to the results of other authors who found significant association between CTLA- 4 rs3087243 gene and allele polymorphism and Graves' disease [20-25]. Therefore, the association between CTLA- 4 rs3087243 gene polymorphism and thyroid autoimmune diseases is still controversial and needs further research work in order to reach a clear consensus.

CONCLUSIONS

Both of the pro-inflammatory cytokines (IL-17 and IL-23) serum level have an impact on disease development in patient with autoimmune thyroiditis disease, in addition CTLA-4 rs3087243 polymorphism seem to have no role in disease susceptibility in Iraqi population.

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The Authors declare no conflict of interest.

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ORIGINAL ARTICLE

REGULATION OF ANTIOXIDANT ENZYMES IN PATIENTS AFTER PERIODONTAL TREATMENT WITH NATURAL AGENTS

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ABSTRACT

The aim: The study of the possibilities of oxidase-antioxidant system indicators regulation at patients with periodontitis under the influence of complex treatment.

Materials and methods: 36 healthy and 125 patients with chronic and exacerbated periodontitis of primary (22 and 21), I (21) and II (20) degrees were examined.Indicators of lipid peroxidation and antioxidant protection (levels of diene conjugates and malonic dialdehyde, catalase activity and transferrin iron saturation, ceruloplasmin activity) in the blood serum were studied before, 6 and 12 months after the appointed treatment. Initial periodontal therapy and a paste developed by us (spirulina microalgae powders and silica enterosorbent taken in equal amounts and 0.05% chlorhexidine bigluconate) for applications and instillations were exogenously used in the complex treatment. Spirulina tablets were prescribed per os as well.

Results: All patients exhibit elevated levels of diene conjugates and malonic dialdehyde, decreased catalase activity and transferrin iron saturation as well as an increased ceruloplasmin activity, especially pronounced at stages I and II ($p_1 \leq 0.01$-0.001). Treatment contributed to long-term and reliable ($p2 < 0.05$ - 0.001) regulation of the studied parameters: reduction of diene conjugates and malonic dialdehyde, ceruloplasmin activity and increased catalase activity and transferrin iron saturation. All indicators differed slightly from the norm during the year ($p1 > 0.05$), and complete normalization of most of them lasted six months. At the same time clinical stabilization of periodontitis was reached.

Conclusions: Indicators of the oxidase-antioxidant system in patients with periodontitis are significantly altered and indicate their participation in the pathogenesis of the disease. Complex treatment was able to almost completely normalize them within six months, but a year later the difference between the obtained indicators with data in healthy people was insignificant (except for ceruloplasmin). Clinical stabilization was achieved in all patients.

KEY WORDS: periodontitis, lipid peroxidation, antioxidant protection, blood serum, complex treatment

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INTRODUCTION

Despite numerous studies, the pathogenesis of chronic periodontitis is not fully understood and treatment is not always effective. This necessitates the search for and development of new methods and ways of its treatment [1-3]. It is known that the development of periodontitis is associated with imbalance of pro- and antioxidant (AO) system, which causes the accumulation of reactive oxygen species and toxic metabolites, in particular, diene and triene conjugates (DC) and malonic dialdehyde (MDA) [4-8]. Due to this reduces the level of AO protection: changes the activity of catalase, transferrin (TF) and ceruloplasmin (CP), etc. [5-11]. Therefore, in the complex therapy of periodontitis it is advisable to use AO and other bioregulators [12, 13], especially of natural origin with multifaceted action.

THE AIM

The study of the possibilities of oxidase-antioxidant system indicators regulation at patients with periodontitis under the influence of complex treatment.

MATERIALS AND METHODS

161 patients aged 19 - 45 years, somatically healthy, were examined, among whom 36 people had intact periodontum and 125 were diagnosed with periodontitis. 43 patients had periodontitis of initial degree (22 - with a chronic course, 21 - with an acute course), 42 - I degree (21 persons of each course) and 40 - II degree (20 persons of each course). All patients venous blood were taken in the morning hours on an empty stomach. It was settled, centrifuged and serum was collected. The level of DC was studied by a simplified spectrometric method (Havrilov VB and coauthors) [14] and MDA – by test with 2-thiobarbioturic acid [15]. In blood serum was determined: catalase activity - according to the method Bakha AN and Zubkova SV [16], iron saturation of TF and CP activity – according to the method of Babenko GO [16]. Patients were examined before, after, six months and a year after therapy.

For complex treatment we used the method developed by us, which included exogenous and endogenous use of biologically active supplement based on blue-green microalgae Spirulina platensis. Initial periodontal therapy was performed topically corresponded to the extent necessary for each case. After that, applied for 20-30 minutes on the gums and installed in the periodontal pockets of the paste, which consisted of equal parts of spirulina powders and silica enterosorbent mixed with 0.05% solution of chlorhexidine bigluconate to a gel-like consistency. Course - 6-8 procedures through 1-2 days. It was prescribed a tablets

of spirulina 2.0-4.0 g twice a day per os, course - 4 weeks. After 6 months, we carried out individual supportive therapy and topical treatment. After 12 months, the course of general therapy was repeated, and if necessary – supportive topical treatment.

We used a personal computer and a Microsoft Exsel application to process the data. The «STATISTICA 6.0» package was administrated, using descriptive statistics methods; the method of differences was administrated, using Student's t-test and correlation analysis.

The clinical study was conducted in accordance with the legislation of Ukraine and the principles of the Helsinki Declaration of Human Rights, without the participation of pharmaceutical companies.

RESULTS

According to our research (table 1), lipid peroxidation (LP) rates increase in the serum of patients with periodontitis. In particular, the level of DC in the case of chronic periodontitis initial degree was increased in 1.31 times and in exacerbated - in 1.29 (p_1<0,05). Under the influence of complex treatment, this figure decreased in 1.30, 1.29, 1.28 and 1.30, 1.26, 1.25 times at once, after 6 and 12 months, respectively (p_2<0.05) in both subgroups. The difference with the content of DC in healthy individuals in all periods of observation was not significant (p_1>0.05), however, in the case of an acute course after 12 months, this figure became significantly higher than immediately after treatment (p_3<0.05).

The amount of MDA in the serum of all patients with periodontitis of initial degree was slightly increased (p_1>0.05). Treatment contributed to a sharp decrease of its concentration at chronic course in 1.12 times (p_2<0.005), in a case of exacerbated – in 1.18 (p_2<0.001). At the same time, lower values were achieved than in healthy people. The difference with the data before treatment remained significant after six months and a year in all patients (p_2≤0.05 - 0.001), and the parameters corresponded to those in healthy people (table I).

Analysis of the state of the prooxidant system in patients with periodontitis of the I degree showed that the concentration of DC in them was increased at the chronic course in 1.45 times (p_1=0.005), and in a case of exacerbated – in 1.38 (p_1<0.01). Complex therapy in these patients was successful at all times and the decrease in the level of DC at the chronic course was 1.43, 1.40 and 1.40 раза (p_2<0.01), but in a case of exacerbated – 1.30, 1.32 and 1.29 (p_2<0.05).

A similar regularity was observed for the content of MDA in the serum of patients with periodontitis of the first degree. Its level in a case of chronic course was somewhat better regulated by treatment in all periods (p_2<0.01; p_2=0.001; p_2<0.01), than in a case of exacerbation (p_2=0.001; p_2=0.01; p_2<0.05). However, the data obtained were close and almost corresponded to the norm of six months for both variants of the disease.

According to the number of DC in the serum, the difference with healthy and in the case of chronic periodontitis of the second degree was 1.48 times (p_1<0.01). There was a decrease of it in 1.35 times as a result of complex therapy immediately (p_2<0.05) and even more – in 1.38 times (p_2<0.05) – after half of year. The difference from the original data (in 1.33 times; p_2 < 0.05) remained significant after 12 months. The level of DC in the case of exacerbated periodontitis was increased in 1.38 times (p_2=0.001) prior to treatment. The greatest decrease in this parameter was observed immediately after therapy (in 1.28 times; p_2<0.01). Subsequently, the content of DC remained significantly lower than the original data (p_2<0.05).

The amount of MDA in the serum was the highest among all examined patients with periodontitis of chronic course of the II degree and the difference with healthy people was in 1.19 times (p_1=0.001). However, treatment was able to normalize this figure immediately (p2 = 0.005) and its growth after 6 and 12 months was negligible. By the way, the difference with the data before therapy remained significant (p_2<0.01; p_2<0.001). No less successful was the treatment in the case of an exacerbated course, and patterns of reducing the level of MDA - similar (p_2<0.05). It is interesting to note the fact that the results achieved after treatment in these patients were close to those in periodontitis of the first degree and differed little from the norm (p_2>0.05).

The increased content of products of LP in the serum of patients with periodontitis was accompanied by disorders in the AO system (table 2). In particular, catalase activity in the case of chronic periodontitis initial degree decreased slightly but exacerbated - significantly (in 1.11 times; p_1<0.05). There was a sharp increase of it at once in 1.10 and 1.12 times (p_2<0.05; p_2<0.005) under the influence of complex treatment at chronic and exacerbated course respectively. There was some decrease in catalase activity subsequently, however, the data obtained were normal after 6 months at chronic periodontitis and were close to it in the case of exacerbated (p_1>0.05).

The rate of TF saturation by iron at the initial stage of periodontitis before treatment was also significantly lower than in healthy persons in both variants of the disease – in 1.08 and 1.10 times (p_1<0.01; p_1=0.001). There was an increase of it in all patients as a result of complex treatment and the patterns of these changes at the chronic course were the same as catalase activity of the same subgroup. In patients with exacerbated periodontitis, iron saturation of TF immediately increased in 1.08 times (p_2<0.05) and remained at the same level for 6 months. After 12 months, TF iron saturation decreased and the difference with the original data became insignificant (p_2>0.05).

The activity of serum CP in patients with initial periodontitis was increased in all examined: at chronic course – slightly (in 1.07 times; p_1>0.05), at exacerbated – significantly (in 1.12 times; p_1<0.05). Thanks to our measures, it has significantly decreased. The parameters were lower than in the healthy persons in both subgroups immediately and six months after treatment. A year later, the activity of CP increased in all patients, and the difference with the original data became insignificant (p_2>0.05).

Catalase activity was significantly higher immediately and six months after treatment in patients with chronic

Table I. Changes in lipid peroxidation in the serum of patients with periodontitis after complex treatment (M±m)

Exponents	Healthy	Periodontitis chronic course				Periodontitis exacerbated course			
		before treatment	after treatment	after 6 months	after 12 months	before treatment	after treatment	after 6 months	after 12 months
initial degree									
diene conjugates, (conventional units in 1 ml of plasma)	n=17 0,786±0,03	n=18 1,026±0,08 p_1<0,05	n=15 0,791±0,05 p_1>0,05 p_2<0,05	n=15 0,798±0,04 p_1>0,05 p_2<0,05 p_3>0,05	n=15 0,801±0,04 p_1>0,05 p_2<0,05 p_3>0,05 p_4>0,05	n=17 1,013±0,09 p_1<0,05	n=16 0,779±0,05 p_1>0,05 p_2<0,05	n=15 0,801±0,02 p_1>0,05 p_2<0,05 p_3<0,05	n=15 0,812±0,02 p_1>0,05 p_2<0,05 p_3<0,05 p_4>0,05
malonic dialdehyde, (nmol / ml)	n=21 3,17±0,12	n=20 3,31±0,07 p_1>0,05	n=18 2,96±0,09 p_1>0,05 p_2<0,005	n=16 3,10±0,05 p_1>0,05 p_2<0,05 p_3>0,05	n=17 3,14±0,05 p_1>0,05 p2=0,05 p_3>0,05 p_4>0,05	n=19 3,38±0,05 p_1>0,05	n=18 2,87±0,07 p_1<0,05 p_2<0,001	n=16 3,10±0,06 p_1>0,05 p_2=0,001 p_3<0,05	n=15 3,16±0,09 p_1>0,05 p_2<0,05 p_3<0,05 p_4>0,05
I degree									
diene conjugates, (conventional units in 1 ml of plasma)	n=17 0,786±0,03	n=19 1,142±0,11 p1=0,005	n=20 0,796±0,06 p_1>0,05 p_2<0,01	n=18 0,817±0,04 p_1>0,05 p_2<0,01 p_3>0,05	n=18 0,815±0,04 p_1>0,05 p_2<0,01 p_3>0,05 p_4>0,05	n=20 1,081±0,09 p_1<0,01	n=18 0,833±0,06 p_1>0,05 p_2<0,05	n=18 0,821±0,04 p_1>0,05 p_2<0,05 p_3>0,05	n=17 0,835±0,03 p_1>0,05 p_2<0,05 p_3>0,05 p_4>0,05
malonic dialdehyde, (nmol / ml)	n=21 3,17±0,12	n=20 3,57±0,07 p_1<0,01	n=20 3,11±0,12 p_1>0,05 p_2<0,01	n=18 3,18±0,08 p_1>0,05 p_2=0,001 p_3>0,05	n=16 3,22±0,10 p_1>0,05 p_2<0,01 p_3>0,05 p_4>0,05	n=19 3,47±0,08 p_1<0,05	n=18 3,15±0,05 p_1>0,05 p_2=0,001	n=16 3,18±0,07 p_1>0,05 p_2=0,01 p_3>0,05	n=16 3,25±0,07 p_1>0,05 p_2<0,05 p_3>0,05 p_4>0,05
II degree									
diene conjugates, (conventional units in 1 ml of plasma)	n=17 0,786±0,03	n=16 1,165±0,12 p_1<0,01	n=15 0,864±0,05 p_1>0,05 p_2<0,05	n=15 0,843±0,05 p_1>0,05 p_2<0,05 p_3>0,05	n=15 0,879±0,05 p_1>0,05 p_2<0,05 p_3>0,05 p_4>0,05	n=15 1,088±0,07 p_1=0,001	n=16 0,849±0,04 p_1>0,05 p_2<0,01	n=16 0,856±0,06 p_1>0,05 p_2<0,05 p_3>0,05	n=15 0,888±0,06 p_1>0,05 p_2<0,05 p_3>0,05 p_4>0,05
malonic dialdehyde, (nmol / ml)	n=21 3,17±0,12	n=20 3,76±0,12 p_1=0,001	n=16 3,18±0,15 p_1>0,05 p_2=0,005	n=16 3,22±0,15 p_1>0,05 p_2<0,01 p_3>0,05	n=15 3,26±0,13 p_1>0,05 p_2<0,001 p_3>0,05 p_4>0,05	n=20 3,68±0,16 p_1<0,05	n=17 3,19±0,15 p_1>0,05 p_2<0,05	n=16 3,24±0,14 p_1>0,05 p_2<0,05 p_3>0,05	n=15 3,29±0,11 p_1>0,05 p_2<0,05 p_3>0,05 p_4>0,05

Note. Here and in table II the probability of a difference of exponents is specified: p_1 – due to exponents of healthy; p_2 – due to data before treatment; p_3 – due to data after treatment; p_4 – due to data 6 months after treatment.

periodontitis (p_2<0.05). The result of treatment became even better in the case of an exacerbated course and after 6 months the norm was reached. After 12 months, catalase activity was in 1.12 times higher than before treatment (p_2<0.05).

The iron saturation of serum TF at periodontitis of the first degree was reduced in a case of chronic course in 1.13 times, and in the case of exacerbated - in 1.15 (p_1<0.001). It have contributed to increasing it in 1.15 times (p_2=0.001) after complex measures made by us in a case of chronic course, with exceeding the norm. Subsequently, there was some decrease in the level of iron saturation of TF, but the obtained values remained significantly different from the original data. (p_2<0.05).

Identical patterns were revealed in both subgroups of patients with periodontitis of the first degree according to the activity of CP in the serum: increasing before treatment in 1.14 and 1.16 times (p_1<0.005) and a significant decreasing immediately and 6 months after treatment in both subgroups, in particular in the case of an exacerbated course – in 1.15 and 1.14 times (p_2<0.005).

It is interesting to note that catalase activity in all patients with periodontitis of the II degree in all terms of observation was regulated successfully. Especially it grew after six months (p_2<0.01).

The effectiveness of our complex therapy was also manifested by the regulation of iron saturation of TF in serum. It was reduced in 1.20 times before treatment. (p1 < 0.001) in both subgroups of patients with periodontitis of the II degree. This figure increased immediately after therapy in 1.15 times at the chronic course (p_2<0.01). The achieved result was maintained for six months (p2 = 0.001) and changed little after a year (p_2<0,05). The iron saturation of transferrin increased immediately in 1.17 times (p_2<0,001) in the subgroup with an exacerbated course of periodontitis.

The highest level of CP activity was in the case of periodontitis of the II degree, and the difference with healthy was 1.26

Table II. Dynamics of activity of antioxidant enzymes in the serum of patients with periodontitis under the influence of complex treatment (M±m)

Exponents	Healthy	Periodontitis chronic course				Periodontitis exacerbated course			
		before treatment	after treatment	after 6 months	after 12 months	before treatment	after treatment	after 6 months	after 12 months
initial degree									
Catalase, (conventional units)	n=32 14,77± 0,48	n=21 13,65±0,44 p_1>0,05	n=22 15,01±0,29 p_1>0,05 p_2<0,05	n=20 14,77±0,33 p_1>0,05 p_2<0,05 p_3>0,05	n=19 14,67±0,42 p_1>0,05 p_2>0,05 p_3>0,05 p_4>0,05	n=20 13,31±0,41 p_1<0,05	n=20 14,96±0,32 p_1>0,05 p_2<0,005	n=19 14,47±0,31 p_1>0,05 p_2<0,05 p_3>0,05	n=18 14,47±0,29 p_1>0,05 p_2<0,05 p_3>0,05 p_4>0,05
Transferrin, (conventional units)	n=34 0,196± 0,004	n=20 0,181±0,004 p_1<0,01	n=18 0,202±0,005 p_1>0,05 p_2<0,005	n=18 0,193±0,004 p_1>0,05 p_2<0,05 p_3>0,05	n=16 0,189±0,004 p_1>0,05 p_2>0,05 p_3>0,05 p_4>0,05	n=20 0,178±0,004 p_1=0,001	n=19 0,192±0,005 p_1>0,05 p_2<0,05	n=17 0,191±0,004 p_1>0,05 p_2<0,05 p_3>0,05	n=16 0,185±0,005 p_1>0,05 p_2>0,05 p_3>0,05 p_4>0,05
Ceruloplasmin, (conventional units)	n=36 31,28± 0,92	n=22 33,40±1,35 p_1>0,05	n=19 29,07±1,43 p_1>0,05 p_2<0,05	n=18 29,13±1,51 p_1>0,05 p_2<0,05 p_3>0,05	n=16 31,58±1,64 p_1>0,05 p_2>0,05 p_3>0,05 p_4>0,05	n=21 34,91±1,48 p_1<0,05	n=18 30,92±1,22 p_1>0,05 p_2<0,05	n=17 30,90±1,04 p_1>0,05 p_2<0,05 p_3>0,05	n=16 32,80±1,59 p_1>0,05 p_2>0,05 p_3>0,05 p_4>0,05
I degree									
Catalase, (conventional units)	n=32 14,77± 0,48	n=21 13,07± 0,44 p_1=0,01	n=21 14,38±0,39 p_1>0,05 p_2<0,05	n=19 14,69±0,54 p_1>0,05 p_2<0,05 p_3>0,05	n=19 14,20±0,40 p_1>0,05 p_2>0,05 p_3>0,05 p_4>0,05	n=20 13,03±0,57 p_1<0,05	n=20 14,41±0,37 p_1>0,05 p_2=0,05	n=19 14,73±0,46 p_1>0,05 p_2<0,05 p_3>0,05	n=19 14,54±0,45 p_1>0,05 p_2<0,05 p_3>0,05 p_4>0,05
Transferrin, (conventional units)	n=34 0,196± 0,004	n=20 0,174± 0,003 p_1<0,001	n=19 0,200±0,006 p_1>0,05 p_2=0,001	n=19 0,190±0,006 p_1>0,05 p_2<0,05 p_3>0,05	n=17 0,186±0,005 p_1>0,05 p_2<0,05 p_3>0,05 p_4>0,05	n=21 0,170±0,003 p_1<0,001	n=18 0,193±0,006 p_1>0,05 p_2=0,001	n=17 0,189±0,004 p_1>0,05 p_2=0,001 p_3>0,05	n=17 0,181±0,004 p_1<0,05 p_2<0,05 p_3>0,05 p_4>0,05
Ceruloplasmin, (conventional units)	n=36 31,28±0,92	n=20 35,73±1,43 p_1<0,005	n=18 31,50±1,29 p_1>0,05 p_2<0,05	n=17 31,55±1,68 p_1>0,05 p_2<0,05 p_3>0,05	n=16 32,29±1,44 p_1>0,05 p_2>0,05 p_3>0,05 p_4>0,05	n=21 36,35±1,25 p_1<0,005	n=20 31,73±0,83 p_1>0,05 p_2<0,005	n=18 31,88±0,58 p_1>0,05 p_2<0,005 p_3>0,05	n=18 33,16±1,70 p_1>0,05 p_2>0,05 p_3>0,05 p_4>0,05
II degree									
Catalase, (conventional units)	n=32 14,77±0,48	n=20 12,06±0,65 p_1<0,005	n=20 14,09±0,50 p_1>0,05 p_2<0,05	n=19 14,79±0,67 p_1>0,05 p_2<0,01 p_3>0,05	n=19 14,00±0,63 p_1>0,05 p_2<0,05 p_3>0,05 p_4>0,05	n=20 12,25±0,67 p_1<0,005	n=20 14,42±0,39 p_1>0,05 p_2<0,01	n=19 14,86±0,59 p_1>0,05 p_2<0,01 p_3>0,05	n=19 14,06±0,40 p_1>0,05 p_2<0,05 p_3>0,05 p_4>0,05
Transferrin, (conventional units)	n=34 0,196±0,004	n=20 0,163±0,005 p_1<0,001	n=18 0,188±0,007 p_1>0,05 p_2<0,01	n=16 0,189±0,005 p_1>0,05 p_2=0,001 p_3>0,05	n=17 0,181±0,006 p_1>0,05 p_2<0,05 p_3>0,05 p_4>0,05	n=20 0,160±0,002 p_1<0,001	n=18 0,187±0,005 p_1>0,05 p_2<0,001	n=17 0,181±0,006 p_1>0,05 p_2<0,01 p_3>0,05	n=16 0,177±0,005 p_1=0,005 p_2<0,005 p_3>0,05 p_4>0,05
Ceruloplasmin, (conventional units)	n=36 31,28±0,92	n=20 39,31±2,42 p_1=0,005	n=17 32,10±2,48 p_1>0,05 p_2<0,05	n=16 32,18±2,09 p_1<0,05 p_2<0,05 p_3>0,05	n=15 34,16±2,28 p_1>0,05 p_2>0,05 p_3>0,05 p_4>0,05	n=20 41,88±1,83 p_1<0,001	n=16 33,76±2,17 p_1>0,05 p_2<0,01	n=15 34,73±2,85 p_1=0,05 p_2<0,05 p_3>0,05	n=16 34,83±2,60 p_1>0,05 p_2<0,05 p_3>0,05 p_4>0,05

Note. See table I.

and 1.34 times (p_1=0.005; p_1<0.001) at chronic and exacerbated course, respectively. Our treatment measures helped to reduce it in patients with chronic periodontitis immediately and 6 months after treatment in 1.22 times (p_2<0.05). The activity of CP increased and differed little from baseline 12 months after treatment (p_2>0.05). Somewhat different patterns are observed in the case of an exacerbated course, namely: reducing the activity of CP immediately (in 1,24 times; p_2<0.01), some growth of it 6 months after treatment and the maintenance achieved during the year.

The studied parameters were processed using correlation analysis. We found that there were strong reliable direct correlations between the content of DC and MDA in the serum in all follow-up periods before and after treatment (r>0.89-0.99; p<0.005-0.001). It was revealed a direct correlation between catalase activity and TF iron saturation before treatment (r>0.98; p<0.001) and indirect correlation between catalase and CP activity (r>-0.98; p<0.001) and TF and CP (r>-0.99; p<0.001). Only one reliable correlation remained immediately, 6 and 12 months after treatment – between parameters of TF and CP (r≥-0.83; -0.94; -0.99; p<0.005-0.001).

After analyzing the relationship between LP and AO protection, we see that before therapeutic measures they were closely related, namely: catalase – with DC (r>-0.91; p<0.005) and with MD (r>-0.98; p<0.001); TF – with DC (r>-0.86; p<0.005) and with MDA (r>-0.95; p<0.005), CP – with DC (r>0.86; p<0.005) and with MDA (r>0.95; p<0.005). The relationship between catalase and MDA held true immediately after treatment and six months later (r>-0.83-0.86; p<0.005). A year later, this relationship remained and a correlation between CP and MDA was added (r>0.85; p<0.005) (table II).

DISCUSSION

Thus, our research confirmed the significant role in the pathogenesis of periodontitis disorders in the pro- and AO system. It was found that with the deepening of dystrophic-inflammatory processes in the periodontium increases the intensification of LP and depletes the AO defense system. This is manifested by increased levels of DC and MDA, and a decrease in catalase activity, iron saturation of TF and increased activity of CP in serum. Similar were obtained by other researchers in the serum and oral fluid of patients. [5, 11-18].

It was possible to normalize the detected disorders for a long time under the influence of the developed method of complex treatment and to achieve clinical stabilization of the disease using of the drug Spirulina platensis. This is evidenced by loss of strong reliable correlations between catalase activity and TF and CP, as well as a decrease in the number of correlations between LP and AO protection from six before treatment to one immediately and six months after treatment. This effect is achieved because spirulina is a "superfood of nature" - a source of high-quality proteins, vitamins, minerals, complex carbohydrates, essential amino acids, fatty and nucleic acids. Local antibacterial and anticandidal activity of spirulina has recently been established and its local and general anti-inflammatory, antioxidant and immune effects have been confirmed [19-21].

CONCLUSIONS

Indicators of the oxidase-antioxidant system in the serum of patients with periodontitis are significantly changed and indicate their participation in the pathogenesis of the disease. The content of the DC and MDA significantly increases as well. These indicators decreased in all patients at all follow-up periods (p_2 <0.05-0.001) under the influence of complex treatment and were close to normal during the year (p_1> 0.05). Catalase activity and iron saturation of TF decrease and CP activity increases at the same time. Catalase activity and iron saturation of TF increased in all patients at all follow-up periods (p_2 <0.05-0.01; p_2 <0.05-0.001) as a result of therapy and differed slightly from data in healthy patients (p_1> 0.05). Their complete normalization lasted six months at the initial and first degrees. CP activity decreased and differed significantly from the baseline of six months in all groups (p_2 <0.05-0.005). Clinical stabilization of periodontitis was achieved in all patients.

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ORIGINAL ARTICLE

THE ROLE OF COMORBIDITY IN THE CLINICAL COURSE AND QUALITY OF LIFE OF PATIENTS WITH DIABETIC POLYNEUROPATHY

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ABSTRACT

The aim: To identify and substantiate the role of comorbidity in the clinical course and quality of life (QOL) of patients with diabetic polyneuropathy (DP).

Materials and methods: We examined 139 patients aged from 19 to 69 years with DP occured as a consequence from type I and II diabetes mellitus (DM). The examined persons were divided into two groups: DP due to type I and II DM with comorbidity (group A,n=93) and without comorbidity (group B,n=46). For the patients was done a comprehensive clinical and neurological examination, laboratory, instrumental methods of examination.

Results: We observe hypo- or areflexia much more in group A respect to reflexes on the upper and lower extremities than in group B, where the changes are more noticeable on the lower extremities. The level of QOL in group A is significantly lower than in group B. According to the McGill scale in group A, all indicators of pain characteristics are higher. Quite a high score in group A on the Pain Rating Index(PRI) – 32.17±1.57points. The lowest rates of the nerve conduction velocity (NCV) on the motor fibers were registered in group A, on the sensitive fibers of the upper extremities has got lower rates in groups A and B than in the control group, but in group A it is slightly higher.

Conclusions: Clinical manifestations of DP in group A are more pronounced than in the comparison group and a wide range of comorbidity was diagnosed, including cardiovascular, which aggravates the manifestations of DP.

KEY WORDS: diabetic polyneuropathy, comorbidity, quality of life, McGill Pain Questionnaire, electroneurography

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INTRODUCTION

Prolonged and poorly controlled diabetes mellitus (DM) leads to damage to all body systems. As a result of damage to the peripheral nerves of the lower extremities diabetic polyneuropathy (DP) is developing – the result of reduced or completely lost function of the somatosensory and autonomic vegetative nervous system [1].

Many large randomized researches have shown that early intensive glycemic control reduces the risk of developing complications of DM. However, there is other data that testifies to the long-term effect of early glycemic control on clinical results of disease, on the basis of which the concept of "hyperglycemic memory" or "metabolic memory" was put forward and proposed [2].

DP is one of the main complications of type I and II DM, significantly increases the risk of the development of ulcerative defects of the feet and amputations, and is associated with higher mortality and increasing costs of the health care system [3,4].

Amputation rates among the population with diagnosed DM are usually 10-20 times higher than among the population without DM, and in recent decades have ranged from 1.5 to 3.5 cases per 1,000 people per year among population diagnosed with DM [5].

More than 50% of patients with DP have got an asymptomatic course [6], the prevalence of DP is about 8% with newly diagnosed DM and more than 50% in patients with long-term of course [7].

Demyelinating and axonal damage to sensory and motor fibers is closely related to the age of patients, the duration of DM, but does not depend on the type of DM and has no gender characteristics. According to electroneurography (ENMG) data, the absolute majority of patients (92.6%) showed signs of DP, more than 1/2 (55.6%) of them had sensorymotor form of DP and 37% had a sensory form of DP [8].

A significant contribution to the progression and deterioration of the clinical course of DP does comorbidity, which often is in people with type II DM. The presence of DM in relatives complicates the course of DP in type I DM. The appearance of «diabetic foot» causes disability among people of working age, «plunges» the patient into depression, deprives him of motivation for further treatment and control of glycemia, as it occurs in the early stages of DP, and sometimes is a peculiar kind of «indicator» for testing of the presence of DM.

In type II DM, the frequency of development of cardiovascular pathology is 3-4 times higher than in patients who do not suffer from it. The risk of cardiovascular mortality in patients with type II DM without coronary heart disease (CHD) is identical to that in individuals who have suffered

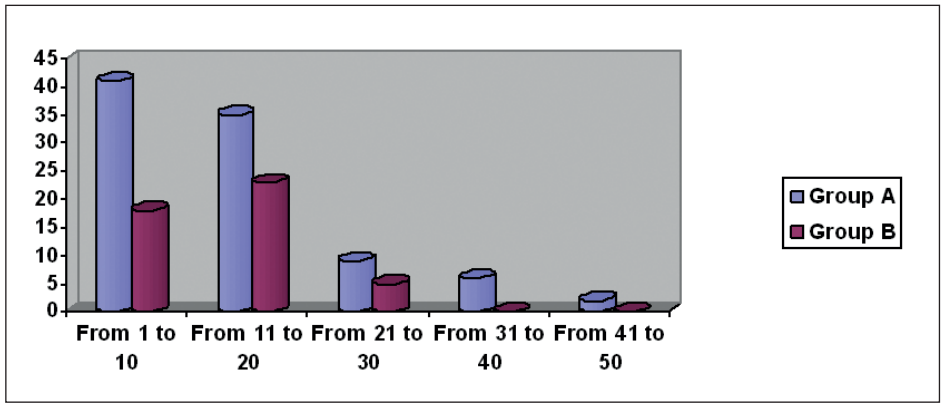

Fig. 1. Time interval of DM in the studied groups

a myocardial infarction but did not have carbohydrate metabolism disorders [9].

Polyneuritic disorders of sensitivity and autonomic-trophic disorders are more common in people with DP on the background of type I and II DM without thyroid pathology, they have a higher frequency of comorbidity and longer duration of DM. The influence of thyroid pathology on the manifestations of DP is reflected in the intensification of neuropathic pain syndrome [10].

The level of quality of life (QOL) in persons with DP on the background of type I and II DM with multimorbidity is generally low. Patients with DP due to type I DM, concomitant cardiovascular pathology (CVP) and gastrointestinal pathology have higher QOL rates than those with DP and type II DM with the same diseases, as they are persons that receive insulin therapy and, respectively, are less vulnerables to complications of DM and have a better course of nosology [11].

Neuroendocrinology in modern clinical practice attracts interest in physicians of various specialities because such patients have some concomitant nosologies. Actual focusing on the peculiar clinical features of DP in comorbidity, identifying specific areas of poor patient function will facilitate rapid diagnosis of DP and active involvement of psychotherapists who will help the family and the patient to modify the perception of the disease and to take care of their own health.

THE AIM

The aim of the research is to identify and substantiate the role of comorbidity in the clinical course and QOL of patients with DP.

MATERIALS AND METHODS

We examined 139 patients (67 women (48%), 72 men (52%) aged from 19 to 69 years with DP occured as a consequence from type I DM - 74 (53%) and type II - 65 (47%). The average age of patients is 48.87 ± 1.28 years. The examined persons were divided into two groups: DP due to type I and II DM with comorbidity (group A, n = 93) and DP due to type I and II DM without comorbidity (group B, n = 46).

For the patients was done a comprehensive clinical and neurological examination, laboratory (general analysis of blood, urine, biochemical analysis of blood, glycated hemoglobin - HbA1c), instrumental methods of examination (stimulation ENMG, performed by a computer multifunctional complex «Neuro-MVP-4»). QOL was determined using a non-specific questionnaire «SF-36 Health Status Survey» (The Short Form). The intensity of the pain syndrome was assessed by the Visual Analog Scale (VAS). The characteristics of pain were studied using the McGill Pain Questionnaire (MPQ), which demonstrates the quantitative component of the pain syndrome. Static data processing was done in Microsoft Office Excel 2003.

RESULTS

In group A, the average age of the examined persons was 53.47 ± 1.25 years, in group B – 39.56 ± 2.43 years. Women predominate in group A 48 (52%), men in group B – 27 (59%). Type I DM in group A was diagnosed in 38 (41%), in group B in 36 (78%), type II in group A – in 55 (59%), in group B – 10 (22%). According to the duration of DM (Fig. 1) in group A more patients have got DM for up to 10 years (41%), in group B from 11 to 20 years (23%). The average duration of DM in group A – 14.47 ± 1.04 years, in group B – 12.73 ± 0.97 years.

The average rate HbA1c in group A was 9.07 ± 0.18%, in group B – 9.06 ± 0.27%.

Trophic disorders were detected in both groups, in particular in group A was dominated hyperkeratosis – 51 (55%), changes of the nail plate – 49 (53%), foot fissure – 48 (52%), in group B – changes of the nail plate – 20 (43%), hyperkeratosis – 19 (41%), hypohidrosis – 19 (41%). White dermographism predominates in patients of groups A and B, which indicates the dominance of the sympathetic division of the vegetative nervous system with spasm of the arterial system on the periphery and disorder of blood flow. The prevalence of vegetative disorders indicates the presence of primarily neuropathy of small nervous fibers in DM.

Changes in the reflex system are given in Table I. We observe hypo- or areflexia much more in group A respect to reflexes on the upper and lower extremities than in group B, where the changes are more noticeable on the lower extremities. Movement disorders in the form of mild

peripheral paresis of the hands and feet were found in 2 (2%) patients of group A and 1 (2%) of group B. Significant damage to the distal extremities is due to the involvement in the process first of the longest distal axons (Yakhno NN, Shtulman DR, 2001). In fact, the comorbidity (group A) enhances the course of DP with the formation of a clear clinical picture.

Polyneuritic sensitivity disorders were detected in 85 (91%) persons of group A and 40 (87%) of group B with a predominance of hypoesthesia. The decrease in vibration sensitivity in group A on the upper extremities is 11.07 ± 0.40 s, on the lower extremities – 6.84 ± 0.29 s, in group B on the upper extremities – 13.15 ± 0.52 s, on the lower extremities - 7.69 ± 0.36 s.

"Diabetic foot" in group A was verified in 13 (14%) patients, of which 7 had amputations of the phalanges of the fingers. In group B in 1 (2%) patients had such a terrible complication of DM.

DP, as a variant of axonopathy leads to the inevitable permanent damage to peripheral nervous fibers, and in combination with comorbidity has a devastating effect on the vascular network of the body as a whole, in particular the lower extremities, which is reflected in a significant number of diagnosed cases of "diabetic foot" in group A.

For a clinical example, we observed a patient V., 78 years old, with type II DM (sick since 2010), who in 2019 sought medical care with complaints of headache, dizziness, difficulty reading, memory impairment, pain in the left leg from the level of the knee and down. It is known that in 2010 the patient suffered an ischemic stroke in the left middle cerebral artery and in 2017 an ischemic stroke in the vertebrobasilar basin. Manifestations of DP previously in the patient were not observed. In addition, he has got hypertension of the III grade and gastritis. Medically take aspirin 75 mg at night, metformin 500 mg twice, enalaprilum 10 mg in the morning. Examination of the neurological status revealed the following changes: easily smoothed nasolabial fold on the right. Dyslexia. Proboscis reflex (+). Muscle strength in the right extremities is 4 points. Tendom and periosteal reflexes from the upper extremities: d>s, medium liveliness; from the lower extremities: knee d>s, medium liveliness, Achilles d=s, torpid, plantar are not caused. Superficial sense is normal. Vibration sensation on the arms 16-17 s, on the legs – knee level 6-7 s, foot – 3-4 s. In the area of the plantar surface of the first toe of the left foot there is a rounded wound formation (the patient did not notice it), the skin of the left foot is slightly hot to the touch.

Clinical diagnosis: Dyscirculatory encephalopathy of the II gr. (ischemic stroke in the left middle cerebral artery in 2010 and in the vertebrobasilar basin 24.03.2017) with the presence of dyslexia, mild right-sided hemiparesis, right-sided cerebellar insufficiency, moderate vestibulo-atactic syndrome. Diabetic polyneuropathy with lesions of the lower extremities, mixed form, as the vibration sensitivity reduction, trophic defect of the left foot.

The level of HbA1c is 7.12%, fasting blood glucose is 9.22 mmol/l. Duplex scanning of the arteries of the lower extremities was performed (13.02.19): multiple atherosclerotic plaques of heterogeneous structure, radiography of the first toe of the left foot (15.02.19): deforming osteoarthritis of the first metatarsophalangeal joint, obsolete fracture of the nail phalanx I toe of the left foot was not excluded. The patient was consulted by a surgeon (15.02.19): type II DM. Diabetic angiopathy of the lower extremities. Trophic ulcer

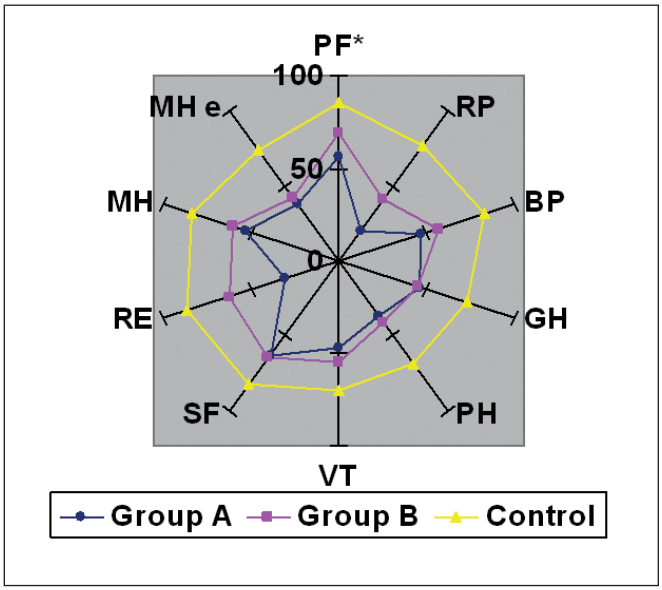

Fig. 2. Level of QOL on the SF-36 scale in groups (average data)
Note. PF* – Physical Functioning, RP – Role-Physical Functioning, BP – Bodily pain, GH – General Health, PH – Physical Health, VT – Vitality, SF – Social Functioning, RE – Role-Emotional, MH – Mental Health, MHe – Mental Health component.

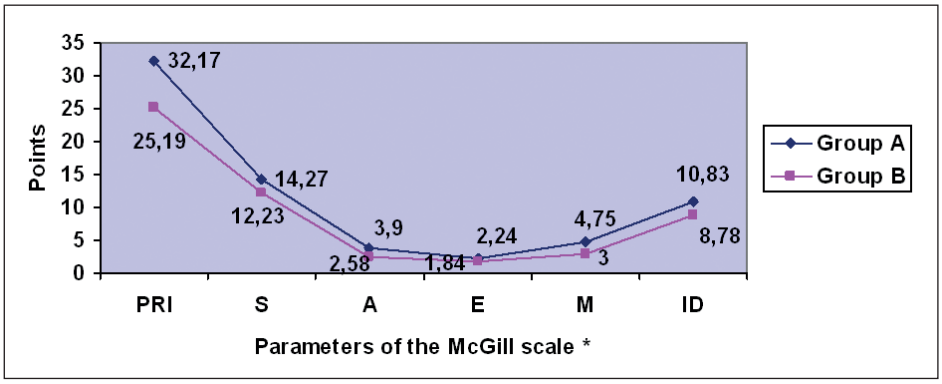

Fig. 3. Quantitative characteristics of pain on the McGill scale
* Note: affective (A), evaluative (E), miscellaneous (M) subscale.

Table I. Evaluation of the reflex sphere in the examined groups

Name of reflexes		Group A n=93	Group B n=46
Carpo radial	areflexia	25 (27%)	2 (4%)
	hyporeflexia	19 (20%)	11(24%)
Biceps	areflexia	15 (16%)	1 (2%)
	hyporeflexia	23 (25%)	6 (13%)
Triceps	areflexia	3 (3%)	0 (0%)
	hyporeflexia	18 (19%)	3 (7%)
Knee	areflexia	9 (10%)	2 (4%)
	hyporeflexia	59 (63%)	20 (43%)
Achilles	areflexia	43 (46%)	8 (17%)
	hyporeflexia	40 (43%)	25 (54%)
Plantar	areflexia	49 (53%)	19 (41%)
	hyporeflexia	35 (38%)	16 (35%)

Table II. Deviations on the ECG in both groups

Changes on the ECG	Group A n=93	Group B n=46
Lengthening of the QT interval	2 (2%)	0 (0%)
Shortening of the PQ interval	0 (0%)	3 (7%)
Blockade of the legs of the His bundle	19 (20%)	9 (20%)
Early repolarization syndrome	7 (8%)	4 (9%)
Sinus bradycardia	4 (4%)	2 (4%)
Sinus tachycardia	14 (15%)	4 (9%)
Myocardial scarring	5 (5%)	0 (0%)

of the first toe of the left foot. Recommended antibiotic therapy (after preliminary-sowing of the content of the wound on the sensitivity were allocated St. haemolyticus of the IV gr. and Ent.faecalis of the II gr.) with Clindamycin 150 mg 2 tab. twice a day for 7-10 days.

Thus, the onset of DP occurred in a patient with manifestations of "diabetic foot" without a previous history of the existence of DP, but with the presence of cardiovascular comorbidity, which is complicated by ischemic stroke. It is nessesary that patients with DM examine their feet and toes daily for the identification of trophic defects and seeking medical care to prevent secondary infection and limb amputations, which ultimately causes a material burden on the patient.

The examined persons of group A have got more disorders detected during electrocardiography (ECG) (Tab. II), in particular the blockade of the legs of the His bundle and sinus tachycardia, in addition, 5% of patients suffered a myocardial infarction. In our opinion, this is due to damage to the autonomic nervous system (parasympathetic division), as tachycardia is often the first manifestation of autonomic cardioneuropathy.

The level of functioning according to the scale QOL SF-36 (Fig. 2) in patients of groups A and B is low compared with the rates of healthy individuals (control). The level of QOL in group A is significantly lower than in group B, especially in the domain "Role-Physical Functioning (RP)" – 20.55 ± 3.53 points, "Role-Emotional (RE)" – 30.35 ± 4.0 points, "Physical Health (PH)" – 36.92 ± 0.91 points, "Mental Health component (MHe)" – 37.75 ± 0.98 points. QOL in group A in the domains "Physical Functioning (PF)" – 56.83 ± 2.57 points, "Social Functioning (SF)" – 63.19 ± 2.12 points and "Mental Health (MH)" – 52.71 ± 1.58 points, higher than other indicators.

In group B, the lowest rates of QOL in the domain "Physical health (PH)" 40.8 ± 1.31 points, "Role-Physical Functioning (RP)" 41.27±6.42 points, "General Health (GH)" – 45.39 ± 2.45 points," "Mental Health component (MHe)" 43.03 ± 1.33 points.

The intensity of pain in the studied groups on VAS was: 4.09 ± 0.18 points in group A and 3.30 ± 0.29 points in group B, which corresponds to the indicator "moderate pain".

According to the McGill scale (Fig.3) in group A, all indicators of pain characteristics are higher. Quite a high score in group A on the Pain Rating Index (PRI) - 32.17 ± 1.57 points. The variety of pain sensations (pulsating, cutting, expanding, etc.) was widely demonstrated in group A of Sensory Pain Rating (S) – 14.27 points, the Index of the number of selected descriptors (ID) in group A is – 10.83 ± 0.47.

Table III. ENMG rates in the examined (motor fibers)

ENMG rates on the motor fibers of the peripheral nerves		Healthy persons n=30	Group A n=93	Group B n=46
Abductor pollicis brevis, Medianus on the left	Amplitude of the M-response in the wrist area, mV	11,21±0,43	8,32±0,32	8,98±0,65
	Amplitude of the M-response of the elbow flexion, mV	10,22±0,50	6,49±0,36	7,04±0,70
	Residual latency, m/s	1,56±0,06	2,49±0,12	2,46±0,19
	NCV average according to the F-wave, m/s	59,91±1,02	53,62±0,64	53,92±1,14
	NCV, m/s	54,6±0,48	45,22±0,85	45,01±0,94
Abductor pollicis brevis, Medianus on the right	Amplitude of the M-response in the wrist area, mV	12,53±0,58	8,78±0,34	9,2±0,50
	Amplitude of the M-response of the elbow flexion, mV	10,11±0,70	6,35±0,37	7,47±0,56
	Residual latency, m/s	1,62±0,07	2,60±0,12	2,54±0,18
	NCV average according to the F-wave, m/s	58,46±1,09	50,50±1,14	50,76±0,97
	NCV, m/s	54,76±0,76	45,97±0,79	46,98±0,85
Abductor digiti minimi, Ulnaris on the left	Amplitude of the M-response in the wrist area, mV	10,40±0,22	8,38±0,35	8,65±0,43
	Amplitude of the M-response of the elbow flexion, mV	9,05±0,52	6,30±0,33	6,03±0,48
	Residual latency, m/s	1,22±0,06	3,41±0,98	1,53±0,13
	NCV average according to the F-wave, m/s	55,77±1,08	49,46±0,77	47,84±0,86
	NCV, m/s	58,50±0,83	49,0±0,96	48,06±1,02
Abductor digiti minimi, Ulnaris on the right	Amplitude of the M-response in the wrist area, mV	10,60±0,23	8,29±0,29	8,90±0,34
	Amplitude of the M-response of the elbow flexion, mV	9,38±0,38	6,84±0,34	7,24±0,38
	Residual latency, m/s	1,19±0,07	1,43±0,05	1,41±0,10
	NCV average according to the F-wave, m/s	55,31±0,99	50,40±0,75	47,85±0,68
	NCV, m/s	60,45±0,76	47,95±0,88	50,2±0,72
Abductor hallucis, Tibialis, on the left	Amplitude of the M-response in the medial bone area, mV	14,14±0,61	5,92±0,51	8,17±0,82
	Amplitude of the M-response in the fossa poplitea, mV	8,3±0,67	3,26±0,35	4,8±0,57
	Residual latency, m/s	1,87±0,11	2,32±0,10	2,24±0,11
	NCV average according to the F-wave, m/s	44,80±0,75	38,38±0,87	37,81±0,79
	NCV, m/s	47,68±1,00	38,98±0,57	40,36±0,89
Abductor hallucis, Tibialis, on the right	Amplitude of the M-response in the medial bone area, mV	14,01±0,66	6,05±0,55	8,57±0,94
	Amplitude of the M-response in the fossa poplitea, mV	7,40±0,80	3,17±0,33	5,47±0,64
	Residual latency, m/s	1,9±0,1	2,61±0,11	2,51±0,16
	NCV average according to the F-wave, m/s	45,34±1,03	38,29±0,61	37,98±1,07
	NCV, m/s	55,47±0,90	42,87±4,12	50,45±10,57

Extensor digitorum brevis, Peroneus, on the left	Amplitude of the M-response in the area of the metatarsus, mV	6,19±0,25	3,17±0,25	4,32±0,41
	Amplitude of the M-response in the area of the tibial plateau, mV	5,27±0,30	2,65±0,23	3,50±0,35
	Residual latency, m/s	1,90±0,11	2,65±0,23	2,44±0,16
	NCV, m/s	48,21±0,85	38,85±0,70	40,19±0,95
Extensor digitorum brevis, Peroneus, on the right	Amplitude of the M-response in the area of the metatarsus, mV	6,44±0,31	3,50±0,30	4,21±0,49
	Amplitude of the M-response in the area of the tibial plateau, mV	5,68±0,34	2,92±0,26	3,79±0,49
	Residual latency, m/s	1,9±0,1	2,82±0,13	2,57±0,20
	NCV, m/s	46,75±0,66	38,81±0,62	39,87±0,98

Table IV. ENMG rates in the examined (sensitive fibers)

ENMG rates on the sensitive fibers of the peripheral nerves		Healthy persons n=30	Group A n=93	Group B n=46
Peroneus superficialis on the left	Amplitude of the M-response in the middle third of the tibia, mV	4,14±0,42	2,68±0,36	2,65±0,61
	NCV, m/s	55,18±1,08	36,56±0,83	38,48±1,06
Peroneus superficialis on the right	Amplitude of the M-response in the middle third of the tibia, mV	4,62±0,62	2,31±0,24	1,84±0,27
	NCV, m/s	87,99±23,82	36,32±0,77	37,97±1,10
n.Suralis on the left	Amplitude of the M-response in the middle third of the tibia, mV	4,56±0,44	4,14±0,38	3,87±0,59
	NCV, m/s	52,06±0,66	37,31±0,60	39,13±0,75
n.Suralis on the right	Amplitude of the M-response in the middle third of the tibia, mV	6,61±0,65	3,92±0,40	3,97±0,52
	NCV, m/s	53,68±1,02	38,23±0,59	37,53±1,02
n. Medianus on the left	Amplitude of the M-response in the wrist area, mV	35,1±4,55	7,98±0,93	9,31±1,79
	NCV, m/s	58,60±0,86	42,57±0,63	41,73±1,19
n. Medianus on the right	Amplitude of the M-response in the wrist area, mV	32,84±5,18	6,77±0,61	7,08±0,91
	NCV, m/s	57,50±1,27	42,01±0,78	40,74±1,77
n. Ulnaris V dig. on the left	Amplitude of the M-response in the wrist area, mV	23,91±3,66	8,95±1,42	4,81±0,66
	NCV, m/s	57,45±0,62	42,03±0,70	40,17±1,35
n. Ulnaris V dig. on the right	Amplitude of the M-response in the wrist area, mV	21,66±3,06	7,15±0,78	6,13±0,80
	NCV, m/s	60,72±1,10	40,48±1,16	39,95±1,25

In group A there were different types of comorbidity in various combinations, in particular quite often and widely represented diseases of the cardiovascular system (hypertension 67(72%), CHD 29(31%), cardiosclerosis 11(12%), atrial fibrillation 4(4%), angina pectoris 4(4%), myocardial infarction in history 2(2%), patent foramen ovale 1(1%), varicose veins of the lower extremities 8(8%), suffered acute vascular thrombosis 1(1%), gastroenterological system (chronic cholecystitis 6(6%), gallstone disease 5(5%), chronic pancreatitis 5(5%), gastroduodenitis 5(5%), hepatitis 5(5%), gastritis 2(2%), biliary dyskinesia 1(1%), steatohepatosis 1(1%), duodenal ulcer 1(1%) and thyroid lesions (multinodular goiter 13(14%), hypothyroidism 6(6%), postoperative hypothyroidism 4(4%), autoimmune thyroiditis 4(4%), thyrotoxicosis 2(2%).

Diseases of the urinary system (urolithiasis 6(6%), chronic pyelonephritis 6(6%), respiratory (bronchial asthma 1(1%) were diagnosed with a lower frequency.

The presence of cardiovascular pathology in group A, in particular hypertension and CHD, explains the low QOL and reduced tolerance to pain, which is clinically demonstrated by changes in the McGill scale.

Diagnosed a large number of cases of «diabetic foot» indicates a combined vascular and neural lesion of the

lower extremities. According to ECG in 5 (5%) of examined group A were revealed scarring miocardial changes, in the anamnesis in 2 (2%) people - clinically suffered miocardial infarction, which allows us assume the presence of «mute» myocardial ischemia in the remaining 3 (3%) patients with DM.

Disorders of lipid metabolism in the form of obesity in group A were recorded in 13(14%) patients, in group B – in 2(4%).

ENMG rates show a significant decrease in all parameters in the examined groups, compared with the control group (almost healthy individuals). The lowest rates of the nerve conduction velocity (NCV) on the motor fibers (Tab. III) were registered in group A on Abductor pollicis brevis, Medianus on the right – 45.97 ± 0.79 m/s, for comparison in group B – 46.98 ± 0.85 m/s, according to Abductor digiti minimi, Ulnaris on the right in group A – 47.95 ± 0.88 m/s, Abductor hallucis, Tibialis, on the left 38.98 ± 0.57 m/s, on the right – 42.87 ± 4.12 m/s, Extensor digitorum brevis, Peroneus, on the left – 38.85 ± 0.70 m/s, on the right – 38.81 ± 0.62 m/s.

The amplitude of the M-response in the area of the medial bone on Abductor hallucis, Tibialis on the left in group A is very low – 5.92 ± 0.51 mV, on Abductor hallucis, Tibialis on the right – 6.05 ± 0.55 mV, the amplitude of the M-response in the fossa poplitea - 3.17 ± 0.33 mV.

Residual latency (RL) is quite prolonged on Abductor digiti minimi, Ulnaris on the left 3.41 ± 0.98 ms in group A.

Evaluation of ENMG characteristics of sensitive fibers (Tab. IV) of the lower extremities shows significantly lower rates of NCV in group A, in particular on Peroneus superficialis on the left 36.56 ± 0.83 m/s, on the right – 36.32 ± 0.77 m/s, n.Suralis on the left – 37.31 ± 0.60 m/s. The NCV of the sensitive fibers of the upper extremities has got lower rates in groups A and B than in the control group, but in group A it is slightly higher.

DISCUSSION

A number of clinical forms of peripheral nervous system lesions in DM have been described, ranging from cranial mononeuropathies, polyneuropathy, ending with lumbosacral radiculoplexopathies (so-called diabetic amyotrophy). We should not forget about the involvement in the pathological process of various parts of the vegetative nervous system, which threatens the appearance of vegetative neuropathy.

There are differences in the types of nervous fiber damage in DP (Takahashi O., 2020) actually, in mononeuropathy demyelination is observed, while in cases of DP there is a tendency to combine demyelination and axon damage.

The presence of DP sometimes leads to the appearance of «diabetic foot». Martínez Delgado M.M. (2018) described a clinical case of a 61-year-old patient diagnosed with type II DM who was diagnosed fourteen years ago [12]. The clinical case indicates that in the patient was developed DP quite quickly and, despite relatively compensated DM (HbA1c data), trophic disorders of the lower extremities were developed, which are closely related to vegetative disorders of DP.

An urgent problem of modern medicine is comorbidity. According to a study by Fahad A.S.Aleidan et al. (2020), hypertension was common in patients with DP ($p = 0.005$), as well as peripheral vascular disease ($p<0.001$), cerebrovascular accidents ($p = 0.027$), chronic kidney disease ($p<0.001$) and dyslipidemia ($p = 0.002$).

There is evidence of a negative effect of hypertension on the appearance of signs of «diabetic foot», especially due to the effect on the stiffness of the arterial walls (Magalhães et al, 2011). Sharp fluctuations in blood pressure may lead to the need for amputation (Gardner and Afaq, 2008; Lambert and Belch, 2013). Thus, in our study, this is confirmed by the appearance of «diabetic foot» in 13 (14%) people in group A.

In the journal Neurology (2015) E. Matthew Hoffman, Nathan P. Staff, in their study indicate that DP is an independent factor in a variety of functional disorders, namely: difficulty walking, tendency to fall, amputation of the lower extremities.

DP without (21.16 [21.31 - 21.01]) and with existing neuropathic pain (NP) (20.85 [21.04 - 20.67]) is independently associated with low QOL and higher indicators of depression levels (DP without: 4.18 [3.53-4.84] and with NP: 3.35 [2.51-4.18]), unsatisfactory sleep (DP without: 4.65 [4.04-5.27] and with NP: 2.22 [1.44-3.00]) and anxiety (DP without: 3.97 [3.31-4.64] and with NP: 2.73 [1.89- 3,58]) after monitoring the age, sex, duration of DM [13].

ENMG reflects the primary damage with a greater extent of motor and sensory fibers of the lower extremities, especially in group A. In group B of the NCV is within the norm for Abductor digiti minimi, Ulnaris on the right is 50.2 ± 0.72 m/s and Abductor hallucis, Tibialis on the right is 50.45 ± 10.57 m/s.

Data from current studies (2019) indicate satisfactory motor function of the median and ulnar nerves in 18.6% of patients with DP, tibial in 2.33% and peroneus nerves in 2.33% of patients [14].

CONCLUSIONS

Clinical manifestations of DP in group A are more pronounced than in the comparison group, because the average age of the examined persons is higher, dominated by type II DM and a longer course of DM. With a higher frequency in group A, scarring miocardial changes were detected as a consequence of a heart attack, and a wide range of comorbidity was diagnosed, including cardiovascular, which aggravates the manifestations of DP.

In general, the rates «Physical Health (PH)» and «Mental Health component (MHe)» in patients of group A are low (36.92 ± 0.91 points and 37.75 ± 0.98 points), combined with high results on the parameters of the McGill scale in this sample.

ENMG data indicate impaired conduction along motor and sensory fibers to a large extent in the lower extremities in both groups, but with an advantage in group A, where

there is a significant number of patients (14%) with «diabetic foot».

DP – the «cornerstone» of further vascular and ischemic disorders, increased pain, increased incidence of disability and catastrophically low QOL in all areas of functioning. This is a problem not only for neurologists, but also for doctors of related specialities, because a patient with DP has got an extremely large number of complications of DM and comorbidity. It is necessary to determine such patients with a certain frequency of QOL, to assess their level of pain, to pay attention to the initial, minimal sensitivity disorders in the onset of DP to prevent the future occurrence of a number of disabling consequences for the patient.

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ORIGINAL ARTICLE

PRODUCTION OF INTERLEUKINS 1B, 2, 4, 10 AND C-REACTIVE PROTEIN IN ISCHEMIC STROKE

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ABSTRACT

The aim: The aim of this study was to determine the content of interleukins (IL) 1β, 2, 4, and 10, as well as the generally accepted marker of inflammation - C-reactive protein (CRP) - in the peripheral blood on the first and tenth days of ischemic stroke (IS).

Materials and methods: The study involved 25 patients with IS (including 8 people with mild case of neurological disorders, 9 – moderate case and 8 – severe case) and 14 people of the control group. The levels of IL-1β, IL-2, IL-4 and IL-10 in the blood were determined by the immunoenzyme method.

Results: It was found that on the first day in patients with IS an increase in the concentration of CRP and all the studied cytokines, especially pro-inflammatory cytokines IL-1β and IL-2, is marked. On the tenth day, the content of pro-inflammatory cytokines and CRP significantly decreases compared to the first day, but remains higher than in the control, but the concentration of anti-inflammatory cytokines (IL-4 and IL-10) continues to increase.

Conclusions: The results obtained on the first day of IS indicate the development of neuroinflammation. On the tenth day the severity of the inflammatory process is significantly reduced, but it still occurs. It was also shown that the outcome of IS depends on the concentration of cytokines in the blood: the higher the level of pro-inflammatory interleukins on the first day, the lower the content of anti-inflammatory interleukins and the higher the amount of pro-inflammatory interleukins on the tenth day, the more pronounced the neurological deficit.

KEY WORDS: C-reactive protein, cytokines, ischemic stroke, neuroinflammation, innate and adaptive immunity

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INTRODUCTION

Ischemic cerebrovascular accidents (ICA) are an important medical and social problem. They make up 13-15% of all causes of death and disability in these diseases consists of 20%. Acute and chronic ICA lead to severe neurological deficit, which greatly affects the quality of life. In the future, according to the conclusion of WHO experts, the number of patients with this pathology will increase, as recently, there has been a tendency to the spread in the population of the causes leading to ICA, namely: arterial hypertension, obesity, atherosclerosis, diabetes mellitus, metabolic syndrome, etc. [1].

At present, inflammatory and neuroimmune processes are of great importance in the pathogenesis of ICA [2]. First of all, the aforementioned diseases are the causes of ICA, as well as the pre-stroke hypoxic brain damage arising on their background are characterized by the so-called chronic low-grade inflammation. It is believed that this inflammation is the basis of the majority of the chronic non-infectious diseases [3]. By itself, it may not manifest itself clinically for a long time (the so-called "silent" inflammation), persists for years, gradually damaging organs and tissues, but is characterized by an increase in the level of cytokines in the blood and infiltration of peripheral tissues by macrophages [4, 5].

With the development of ischemic stroke (IS) as well as any other tissue damage acute inflammation occurs, which among other common inflammatory manifestations [or so-called systemic inflammatory response syndrome (SIRS)] also manifested by increased level of cytokines in the blood [6].

Thus, inflammation in IS appears to be a combination of pre-stroke chronic low-grade inflammation and post-stroke acute inflammation.

It is known that in the early period after having IS the increase in the level of cytokines in the blood, in particular IL-1β and IL-10, is determined [7, 8]. The experiment has shown that during cerebral hypoxia an increase in the concentration of IL-1β in the blood is determined, and, with its prolonged presence in high doses in blood plasma and cerebrospinal fluid, more pronounced neurological symptoms are observed [9].

Of considerable interest is a comprehensive study of the cytokines production - markers of various cells of the immune response and inflammation – monocytes/macrophages, T-helper lymphocytes of the 1st and 2nd types (Th1 and Th2) - IL-1β, IL-2, IL-4, IL-10 - and analysis of the relative role of the phenomena mediated by them with IS.

IL-1β is produced mainly by macrophages, that is, it is a marker of within named cells and innate (nonspecific) *innate immunity* and the major pro-inflammatory cytokine. Being one of the first mediators of inflammation, in partic-

ular, in the ischemic zone, it also stimulates the synthesis of other cytokines, including IL-2 [10, 11].

IL-2 is produced mainly by Th1, is a marker of Th1 and adaptive (specific) cellular immunity, plays a key role in the development of a rapid immune response, induces T-lymphocyte proliferation and activates cytotoxic T-lymphocytes, and also belongs to pro-inflammatory cytokines [11, 12].

IL-4 is produced mainly by Th2, that is, it is a marker of Th2 and adaptive humoral immunity, regulates the growth and differentiation of B-lymphocytes, biosynthesis and secretion of antibodies [13]. IL-10 is produced mainly by monocytes and Th2 and increases the survival rate of B-lymphocytes, their proliferation and antibody production [14].

IL-4 and IL-10 are anti-inflammatory cytokines that control the strength and shape of the immune response and inflammation. IL-4 prevents Th1 differentiation, arrests most functions of macrophages; IL-10 suppresses antigen presentation by macrophages and Th1 activation; both of them inhibit the production of pro-inflammatory cytokines by Th1 cells and macrophages [14].

THE AIM

The aim of this study was to determine the content of interleukins (IL) 1β, 2, 4, and 10, as well as the generally accepted marker of inflammation - C-reactive protein (CRP) - in the peripheral blood on the first and tenth days of ischemic stroke (IS).

MATERIALS AND METHODS

All procedures were performed with the informed consent of the patients and in compliance with the principles of medical bioethics and deontology.

The study did not participate persons with acute inflammatory, autoimmune, neurodegenerative diseases, diabetes mellitus, neoplastic processes and repeated acute ischemic cerebrovascular accidents.

25 patients with IS (12 women and 13 men) were examined in the age range 41-73 years, and 14 people from the control group (7 women and 7 men). The patients were divided into 3 groups, depending on the clinical symptoms' severity. 1st group - 8 people (32%) - with minor case of neurological disorders, 2nd group - 9 people (36%) - with moderate case and 3rd group - 8 people (32%) - with severe case. Patients with neurological disorders of extreme severity and being in coma did not participate in the study. The severity of the course was assessed by the National Institutes of Health Stroke Scale (NIHSS). This scale assesses the level of consciousness, answers to questions, commands execution, eye movements, visual field, facial paralysis, movements of the upper and lower limbs, limb ataxia, sensitivity, aphasia, dysarthria, agnosia. The severity level is estimated within the range from 0 to 5 scores. The sum of scores is determined after evaluating individual functions, and the higher it is, the worse the patient's condition is. The diagnosis of IS was made on the basis of clinical neurological and *instrumental apparatus* studies. From the moment of admission to in-patient department all patients took medicamentous therapy in accordance with the protocols of the Ministry of Health of Ukraine, aimed at combating cerebral edema, improving cerebral circulation and correcting the work of the respiratory and cardiovascular systems.

The level of IL-1β, IL-2, IL-4 and IL-10 in the blood was determined using ELISA-BEST reagent kits A-8766, A-8772, A-8754 and A-8774, respectively, designed to determine in serum blood and urine cytokine concentrations by the immunoenzyme method. To investigate the venous blood it was taken on the first and tenth days of the disease and centrifuged at 3000 rpm for 10-15 minutes. The obtaining serum samples results were frozen at a temperature of -20 ° C and thawed immediately before analysis. The results were recorded by measuring the values of the optical density of liquids in the wells on a vertical-type scanning spectrophotometer in a dual wavelength mode. Outcome evaluation was carried out by such method as constructing in a linear calibration curve graph of optical density on the concentration of interleukins in the calibration samples and determining the content of cytokines in the control sample and analyzed samples according to the calibration graph. The level of CRP in the blood was also studied on the 1st and 10th days of the disease by the method of determining the highly sensitive hsCRP, allowing to estimate the degree of risk of the onset and outcome time of acute ischemic stroke [15].

The data obtained in this study was validated using Student`s t-criterion, a posteriori test of the Bonferroni correction, Pearson's correlation.

RESULTS

In the control, in the blood IL-1β and IL-10 far outweigh, as products of monocytes-macrophages and mediators of innate immunity, over products of lymphocytes and mediators of adaptive immunity IL-2 and IL-4, and between IL-1β and IL-10 - anti-inflammatory IL-10 over pro-inflammatory IL-1β (Table I). This is probably due to the fact that normally adaptive immunity is not yet involved and inflammation is absent.

With IS the content of all investigated cytokines in blood considerably increases: IL-1β - in 8,9 times, IL-2 - in 26,5 times, IL-4 - in 1,5 times, IL-10 - also in 1,5 times. As one can see, the content of IL-2 increases the most, which indicates the greatest activation of Th1, in other words adaptive cellular immunity, as well as IL-1β - a marker of macrophages and innate cellular immunity. Th2, that is adaptive humoral immunity, is less involved.

It is also seen that the production of pro-inflammatory cytokines increases rather, than anti-inflammatory, which indicates the development of inflammation. This is confirmed by a significant increase of the content of CRP in the blood - 3.8 times.

On the 10th day of treatment the content of IL-1β and IL-2 is significantly reduced compared to that one that

Table I. The content of cytokines and CRP in the blood of patients with ischemic stroke both in pretreatment and posttreatment time

Value	Control	Ischemic stroke	
		pretreatment time (the 1st day)	posttreatment time (the 10th day)
IL-1β, pg/ml	1,445 ±0,010	12,917 ±0,453 p_1< 0,001	3,797 ±0,433 p_1< 0,001 p_2< 0,001
IL-2, pg/ml	0,074±0,014	1,964±0,138 p_1< 0,001	0,266±0,065 p_1< 0,001 p_2< 0,05
IL-4, pg/ml	0,110±0,023	0,167±0,010 p_1< 0,05	0,357±0,023 p_1< 0,05 p_2< 0,001
IL-10, pg/ml	3,235±0,224	4,851±0,150 p_1< 0,001	16,459±0,517 p_1< 0,001 p_2< 0,001
CRP, pg/ml	1,354±0,068	5,081±0,176 p_1< 0,001	4,363±0,142 p_1< 0,001 p_2< 0,001

Note. p_1 - compared with control, p_2 - compared with pretreatment value.

Table II. The content of cytokines and CRP in the blood of patients with ischemic stroke in pretreatment time (on the 1st day) depending on the severity evaluation scale

Value	Stroke scale		
	minor	moderate	severe
IL-1β,pg/ml	10,449 ± 0,436 p_1< 0,001	13,065 ± 0,444 p_1< 0,001 p_2< 0,001	15,219 ± 0,353 p_1< 0,001 p_2< 0,001 p_3< 0,01
IL-2, pg/ml	1,575 ± 0,106 p_1< 0,001	1,740 ± 0,170 p_1< 0,001 p_2>0,05< 0,1	2,604 ± 0,262 p_1< 0,001 p_2< 0,01 p_3< 0,05
IL-4, pg/ml	0,193 ± 0,012 p_1< 0,01	0,174 ± 0,013 p_1< 0,05 p_2>0,05< 0,1	0,133 ± 0,020 p_1>0,05 p_2< 0,05 p_3>0,05
IL-10, pg/ml	4,998 ± 0,135 p_1< 0,001	5,278 ± 0,205 p_1< 0,001 p_2>0,05< 0,1	4,222 ± 0,283 p_1< 0,05 p_2>0,05< 0,1 p_3< 0,01
CRP, mg/l	4,306 ± 0,159 p_1< 0,001	5,013 ± 0,192 p_1< 0,001 p_2>0,05< 0,1	5,931 ± 0,276 p_1< 0,001 p_2< 0,001 p_3< 0,05

Note. p_1 - compared with the control (see table 1), p_2 - compared with minor case, p_3 – compared with moderate case.

was at pretreatment time - 3.4 times and 7.4 times respectively. At the same time, it still remains significantly larger than the control - 2.6 times and 3.6 times, respectively. In contrast, the content of IL-4 and IL-10 continues to increase. It significantly increases compared to that one that was at pretreatment time - 2.1 times and 3.4 times, respectively - and becomes greater than the control by 3.2 times and 5.1 times, respectively. Thus, on the 10th day of treatment, the production of pro-inflammatory cytokines is already significantly reduced compared to that that was at pretreatment time, but has not yet returned to the control, and the production of anti-inflammatory cytokines continues to increase. These data indicate that on the 10th day of treatment for IS, the inflammatory process subsides significantly, but is still quite pronounced. This is confirmed by the fact that the content of CRP in the blood on the 10th day of treatment, although significantly is reduced compared to that one that was at pretreatment

Table III. The content of cytokines and CRP in the blood of patients with ischemic stroke in posttreatment time (on the 10th day) depending on the severity evaluation scale

Value	Stroke scale		
	minor	moderate	severe
IL-1β, pg/ml	2,275 ± 0,184 p_1< 0,01	3,669 ± 0,470 p_1< 0,001 p_2 >0,05	5,463±0,979 p_1<0,01 p_2<0,01 p_3>0,05
IL-2, pg/ml	0,126 ± 0,036 p_1>0,05	0,210±0,098 p_1>0,05 p_2>0,05	0,468±0,151 p_1<0,05 p_2>0,05 p_3>0,05
IL-4, pg/ml	0,288 ± 0,005 p_1< 0,001	0,296±0,023 p_1< 0,001 p_2>0,05	0,496±0,029 p_1<0,001 p_2<0,001 p_3<0,001
IL-10, pg/ml	16,585 ± 0,579 p_1< 0,001	18,731±0,545 p_1< 0,001 p_2<0,05	13,777±0,536 p_1<0,001 p_2<0,01 p_3<0,001
CRP, mg/l	3,863 ± 0,141 p_1< 0,001	4,374±0,216 p_1< 0,001 p_2>0,05	4,850±0,258 p_1<0,001 p_2<0,05 p_3>0,05

Note. p_1 - compared with control (see table 1), p_2 - compared with minor level, p_3 – compared with moderate level.

time - 1.2 times, but remains much higher than the control (3.2 times).

Analyzing the content of cytokines in the blood depending on the Stroke scale it is seen that at minor severity the content of all studied cytokines is significantly higher than the control: IL-1β - 7.2 times, IL-2 - 21.3 times, IL-4 - 1.8 times, IL-10 - 1.5 times (Table II).

At the moderate case the content of IL-1β is significantly higher than at the mild one and at the severe case is higher than at the moderate one. Thereafter, in both cases, it becomes higher than the control. The content of IL-2 at the moderate case tends to increase compared to the minor case, but at the severe case is significantly higher than at the moderate one. Accordingly, in both cases, it is also becoming more than the control. The content of IL-4 at the moderate case tends to decrease compared to the minor case, but it remains significantly higher than the control, and at the severe case decreases even more and, although in adequately compared to the moderate case, but adequately compared to the minor case and so it significantly has no difference from the control. The content of IL-10 at the moderate case tends to increase compared to the minor case, and at the severe case is significantly decreasing compared to the moderate case, so that it significantly does not exceed that one at the minor case, but still remains significantly higher than the control.

Thus, the level of pro-inflammatory cytokines in the blood is increasing with increasing the severity of IS and the content of anti-inflammatory cytokines is decreasing. Apparently, this is due to the increased activity of the inflammatory process, as evidenced by changes in CRP production. At the minor severity of IS the level of CRP in the blood is significantly increasing compared with the control - 3.2 times (see Table II). At the moderate severity it tends to increase further but at the severe case it is increasing significantly compared to the moderate case, so it is significantly higher than one that was at the minor case. Of course that at the last two levels it remains significantly higher than the control (3.7 times and 4.4 times, respectively).

On the 10th day of treatment at the minor severity of IS the content of IL-1β, IL-4 and IL-10 in the blood increases significantly (1.6 times, 3.9 times and 5.1 times, respectively) and IL-2 does not differ with assurance from the control (Table III).

At moderate case the level of IL-1β has significantly no difference from that one at the minor case, and at the severe case - from that one at the moderate case, but becomes significantly higher than at the minor case. At the moderate and severe case it remains significantly greater than control.

The content of IL-2 at the moderate case does not differ with assurance from that one at the minor level and control, and at the severe case - from that one at the moderate and minor case, but becomes significantly higher than control.

The level of IL-4 at the moderate case is not significantly different from that one at the mild case, and at the severe case is significantly higher than both at the moderate and mild cases. At the moderate and severe cases, it remains significantly greater than the control.

The content of IL-10 at the moderate case is significantly higher than at the minor one and, accordingly, remains significantly higher than the control, and at the severe case

- significantly less than at the both moderate and minor case, but remains significantly higher than the control (4.3 times).

Thus, on the 10th day of treatment with increasing severity of IS the levels of IL-1β and IL-4 are increasing slightly, IL-2 - does not change significantly, and IL-10 - first is increasing and then decreasing, but remains significantly higher than the control.

Compared with the first day of IS, there is a less pronounced in terms of control increase in the production of pro-inflammatory cytokines and decrease - anti-inflammatory. Apparently, this is due to a decrease in activity of the inflammatory process, which is confirmed by changes in CRP production. Thus, on the 10th day of IS at its minor level, the level of CRP in the blood is 2.9 times higher than the control. At the moderate case it does not significantly differ from that one at the minor level, and at the severe level - significantly does not differ from that one at the moderate case, but becomes significantly bigger than at the minor level. Both at the moderate and severe cases it remains significantly bigger than the control.

In calculating the Pearson correlation coefficient on the first day of disease was found a moderate positive correlation between the concentrations of IL-1β and IL-2 (r = 0.613), IL-1β and CRP (r = 0.643), and a negative correlation - between the content of IL-2 and IL -10 (r = -0.611); on the tenth day - a moderate positive correlation between the concentrations of IL-1β and IL-2 (r = 0.667), IL-1β and IL-4 (r = 0.463), and negative - between the levels of IL-1β and IL-10 (r = -0.408), IL-4 and IL-10 (r = -0.687). The positive relationship between the content of IL-1β and IL-2 confirms the above mentioned that IL-1β stimulates the synthesis of IL-2. More broadly, there is a synergistic effect between IL-1β and IL-2, macrophages and lymphocytes in the pathogenesis of inflammation: they stimulate each other's production or activity [16]. The negative correlation between the levels of IL-2 and IL-10 and IL-1β and IL-10 confirms the above about the antagonism between pro- and anti-inflammatory mediators. The positive relationship between IL-1β and CRP confirms that pro-inflammatory cytokines may be, along with acute-phase proteins, markers of inflammation [17]. The decrease in the strength of the negative link between IL-1β and IL-10 after treatment, the appearance of a positive correlation between IL-1β and IL-4 and a negative correlation between IL-4 and IL-10 show that in inflammatory processes treatment the correlations between the indicators begin to change to the opposite, in particular, in this case the strength of the negative relationships decreases and there are appearing positive links between pro- and anti-inflammatory cytokines, as well as negative links between anti-inflammatory cytokines.

DISCUSSION

Analyzing the mechanisms of the results obtained, it should be noted that with IS, neurons and neuroglia cells - astrocytes - are primarily involved in the pathological process. Within the first minutes after carrying out the hypoxic damage of neurocytes their cytotoxic edema develops, which occurs as a result of stopping the work of the sodium-potassium pump, leading to sodium retention. Raised hyperosmolarity promotes the flow of water into the brain cells and causes their osmotic death. Hypoxia also contributes to the inhibition of mitochondrial oxidation, which leads to progressive ATP deficiency. This deficiency is partially compensated by glycolysis, but its activation causes the rapid development of acidosis. Due to the dysfunction of the membrane during energy deficiency Ca^{2+} accumulates in the cell. This leads to the activation of defense mechanisms, consisting in the capture of Ca^{2+} by the energy stations of the cell. Mitochondria, which are deficient in ATP under conditions of hypoxia, have to work actively to maintain a constant inner mitochondrial membrane charge, which in turn further disrupts energy metabolism [18]. In parallel with cytotoxic edema ionic edema occurs, where there is a flow of Na^+ and Cl^- from the vascular bed followed by water. The development of vasogenic edema aggravates the pathological process. At this stage the intercellular spaces increase due to the contraction of endothelial cells, but still do not let the blood corpuscle. The reason for the contraction of endotheliocytes, apparently, is the action of the formed and released in increased amounts of biologically active substances which are mediators, when inflammation happens, they are histamine, bradykinin, derivatives of arachidonic acid, free radicals, thrombin, etc. [19]. The next phase in the pathogenesis of cerebral edema is observed with the progression of endothelial dysfunction. Here, necrosis of endotheliocytes occurs, that means the complete destruction of contacts between them, which contributes to the passage of the blood corpuscle, above all of erythrocytes, and the development of diapedesis. Hemorrhagic transformation leads to severe disruption of homeostasis and, as a consequence, neuronal necrosis develops [20].

All of the above factors lead to the development of inflammation. In the nervous system, microglia, represented by resident macrophages, have protective functions [21]. In addition to microglia, astrocytes are actively involved in the process of neuroinflammation, regulating the functional activity of neurons and the blood-brain barrier permeability. They possess pro- and anti-inflammatory effects, producing gliotransmitters and cytokines [22].

It is known that the brain is "behind a barrier" body, i.e. the blood cells are unable to penetrate the hematoencephalic, hematoliquor and hematoleptomeningeal barriers [23]. However, modern imaging methods have made it possible to detect the migration of monocytes from the circulating blood into the central nervous system through these barriers in pathology [24, 25]. This, in turn, leads to the accumulation of immunocompetent blood cells in the inflammation focus, which, along with resident macrophages, can release and initiate the formation of inflammatory mediators. Thus, the appearance of interleukins not only in the cerebrospinal fluid, but also in the peripheral blood is the result of the activation of both local cells and those that have emigrated from the blood. It should also be kept

in mind that the increase in the content of mediators in the blood occurs not only due to their incoming from the focus of inflammation, but also due to the activation of blood leukocytes [26].

The obtained results are consistent with a number of literature data that in ischemic stroke there is an increase in the level of circulating IL-1β [27]. At the same time, literature data on the production and role of IL-2 in ischemic stroke are ambiguous and contradictory [28]. In addition, the literature data in general indicate mainly a deficiency of anti-inflammatory cytokines – IL4 and IL10 – in ischemic stroke [29, 30].

CONCLUSIONS

1. At an ischemic stroke on the 1st day/in pretreatment time the content in blood of all investigated cytokines - IL-1β, IL-2 IL-4, IL-10, and also CRP considerably increases. At the same time the level of pro-inflammatory cytokines increases rather than anti-inflammatory. All this indicates the development of inflammation. The most activated is the production of IL-2, a marker of Th1 lymphocytes - effectors of adaptive cellular immunity.
2. On the 10th day of treatment for ischemic stroke the production of pro-inflammatory cytokines and CRP is significantly reduced compared to that before treatment, but still significantly exceeds the control, and the production of anti-inflammatory cytokines continues to increase. This indicates that at this time the inflammatory process is significantly reduced, but it is still quite pronounced.
3. On the 1st day of ischemic stroke with increasing severity of the disease the level of pro-inflammatory cytokines and CRP in the blood increases and the content of anti-inflammatory cytokines decreases, which is associated with an increase in the severity of the inflammatory process.
4. On the 10th day of treatment with an increased severity of ischemic stroke compared with the first day there is less pronounced in terms of control of increased production of pro-inflammatory cytokines and CRP and decrease - anti-inflammatory cytokines, which is associated with reduced severity of inflammation.

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ORIGINAL ARTICLE

USING THE POSITIVE REAPPRAISAL COPING INTERVENTION TO CHANGE STUDENTS APPRAISAL AND ATTITUDES TOWARD NURSING

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ABSTRACT

The aim: This study aimed to evaluate the effect of positive reappraisal intervention model in changing nursing students' attitudes toward nursing profession.

Materials and methods: A quasi-experimental study design (one group pretest-posttest) to achieve the study objectives.The emotional regulation questionnaire (ERQ-10) and the students' attitudes scale were used pre and post applying the positive reappraisal intervention in a random sample of 165 undergraduate nursing students, male and female, in their sophomore level to senior level. The process of collecting student's data took place in July 2020 – February, 2021.

Results: A statically significant difference in students' appraisal ($t = -26.320$, $p < .0005$) and their attitudes towards nursing ($t = -15.460$, $p < .0001$) were registered after applying (compared to the results before) the positive reappraisal intervention.

Conclusions: The positive reappraisal coping intervention is proved as an easy model to apply and is highly effective in terms of changing students' cognitive appraisal, which in turns changes their attitudes toward nursing. This assumption is concluded, basing on the significant increase in the level of appraisal and attitudes of nursing students after applying the intervention model; their levels are increased to about (96%) positive appraisal and about (94%) positive attitude level.

KEY WORDS: Cognitive appraisal, intervention model, students' attitudes, positive reappraisal, nursing students

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INTRODUCTION

Human behaviors are determined by their emotions that are formed and extracted by their evaluation (appraisals) to the experienced situations [1]. Two dimensions of cognitive appraisal have been identified in researchers' studies which are positive appraisal and negative appraisal. Positive appraisal describes the action, when a person looking at the bright side of an event or situation; whereas, negative appraisal is described as perceiving and thinking negatively, which can impose stress on the individual, who experience the situation. Thus, stressful life events can be results of cognitive appraisal specifically negative appraisal [2-3] . Cognitive appraisal in general has direct effects on the psychological and the physical health of individuals as it is approved by empirical studies. For instance, when the appraisal is positive, stress, anxiety, and depression can be avoided [4]. Furthermore, positive appraisal leads to positive emotions and better emotional response to the situation and that leads to positive affect and action [5]. Attitudes are learned tendencies for positive or negative reaction toward a specific situation, object, institution, concept, or other people [6]. Nurses attitudes toward their profession, work, organization, and administration can serve as a valid predictor to their behaviors in the practice settings; therefore, changing students` attitudes in a positive way can have great influence on the quality of healthcare services [7]. The quality of healthcare services has found to be linked to professionals' mental elements including, but not limited to appraisal level, since that appraisal and performance significantly correlated [8]. Negative appraisal and students' attitudes toward nursing also have undesirable effects on students' academic performance and the future of the nursing profession as it results in job dissatisfaction and poor patient health outcome. Globally, there is gap in nursing research concerning the use of positive reappraisal model to manipulate the attitudes of individuals [9], which means that professionals have lost the benefits of an essential element for positive change. Therefore, this research is designed to measure the effectiveness of the positive reappraisal model in changing students' appraisal level and their attitudes towards nursing as their future career. Two main research questions were developed in this study as follow: Is positive reappraisal intervention model effective in changing student appraisal level and attitudes toward nursing? And is there a gender difference in people, when we are talking about respect to their appraisal and attitudes level?

One of the major challenges, facing students among the university period is stress, which has negative consequences on students' academic performance; it , mostly, shows up as decreasing. In addition, negative attitudes toward their future profession inhibit student's motiva-

tion to develop knowledge or optimize their academic performance. All these challenges can be the result of their cognitive appraisal [10-11]. In some countries, over 45% of nursing students reported depression symptoms associated with levels of stress they experience as a result of seeing various health conditions during their clinical practice [12]. Depression leads to consequences affecting their social functioning as it effects their ability of emotional regulation [13]. Positive reappraisal is considered one of the most suitable way to manipulate individuals' response to stress and change their thoughts and emotions in a positive way [14]. Studies have found a link between academic satisfaction among nursing students and the coping strategies they use. Hirsch et al. (2015) stated that "students, satisfied with the course, used positive coping strategies targeting the problem, whereas dissatisfied students used negative strategies focusing on the emotion" [15]. Therefore, it has become necessary to design effective intervention programs and to stop stress and depression in college students, because of the fact that they are the most risk age groups that could be affected negatively by stress from their early point in their school [16].

THE AIM

This study aimed to measure the effectiveness of the positive reappraisal model in changing students' appraisal level and their attitudes towards nursing.

MATERIALS AND METHODS

HYPOTHESES

H_1. Positive reappraisal intervention would have direct effect improving students' appraisal level.

H_2. Positive reappraisal intervention would have indirect effect changing students' attitudes toward nursing positively.

DESIGN

A quasi-experimental study design (one group pretest-posttest) was carried out to answer the study questions. One group retest-posttest design is one of the most common used designs in nursing research in which that pretest serves as control or comparison group.

SAMPLE, SAMPLING, AND SETTING

Probability sample, simple random sampling techniques, were used to enhance the representation of the target group (nursing students) and minimize the sampling error. The adequate sample size was determined, based on the 10 % condition, by which the study sample is recommended to be 90 participants out of the total population, which are 900 undergraduate nursing students. To increase the capacity of the recent study to discover the differences in the attitudes of nursing students between pretest and posttest, a total of 165 consenting male and female nursing students were included in the study. The study was conducted at the University of Babylon, College of Nursing from July 2020 – February 2021.

STUDY INSTRUMENTS

The emotion regulation questionnaire (ERQ) and the student attitudes scale (SAS) were used to serve the study objectives. The emotion regulation questionnaire (ERQ) is a 10 items self-reported scale that is used to measure the cognitive appraisal of individuals. It has two parts: cognitive reappraisal (6 items) and the expressive suppression (4 items). This tool is originally developed in English by Gross & John in 2003 [17]. The questionnaire was translated to Arabic and tested for validity and reliability. The minimum content validity ratio (CVR) for each item was 0.80, and the content validity index (CVI) for the total items was 0.86. The Arabic version of (ERQ) was reliable at Cronbach's Alpha value 0.85 and the scale items were measured on a 7-point Likert scale, ranging from absolutely disagree - "1" to absolutely agree - "7". The higher scores indicating higher level of appraisal or emotion control. The second scale was the students' attitudes scale. It is a 55 items scale and originally developed in Arabic by Shakora in 2002 to measure the attitudes of nursing students toward nursing profession[18]. The attitudes scale was also reliable at Cronbach's Alpha value (0.94). Items are measures on a 5-point Likert scale, ranging from absolutely disagree - "1" to absolutely agree - "5", scoring of some items were reversed for statistical purposes. The higher scores indicate higher level of students' attitudes.

DESIGNING THE INTERVENTION

The positive reappraisal intervention is an effective strategy that is used widely to manipulate individual's thinking positively. In fact, there are no standardized guidelines to be used in all research studies that use the reappraisal intervention; therefore, the intervention guidelines can be tailored according to objectives of the programs. For this research, the appraisal theory of Lazarus and the strategies of cognitive appraisal and positive reappraisal were used as a guide to construct the scenario that helps applying the intervention in a simple way. The scenario was constructed and then reviewed by experts for modification and enhancements. The constructed scenario was recorded as video considering it would be short, clear, relevant, and easy to follow. The intervention video was sent to some students to verify its clarity, simplicity, and understandability before final use. Usually, the intervention is applied directly to target populations using various methods of application. However, the COVID-19 pandemic and the restriction order for social distance had limited the direct communication between the researchers and the target population; therefore, communication and applying the intervention were done online using various social media platforms.

DATA COLLECTION

Data collection (pre-test): Questionnaire was transformed to an electronic form (Google forms) and the link of the questionnaire was shared in students with one week to fulfill it. Students, who completed the questionnaire were 172; however, 7 students did not fill all scale items and some demographic information were missing. Therefore, only 165 questionnaires were valid for pre-test analysis.

Data collection (post- test): After students completed the pre-test questionnaire, the link for the intervention video was shared with students and students were asked to review the video in order to fill the post-test questionnaire. Students were also given one week to review the video and try the new ways of thinking about events. Then, the link for the post-test questionnaire was shared with students to complete after watching the video. Two questions were added to the post-test questionnaire to assure that same students who completed the pre-test survey also completed the post-test survey and watch the intervention video. The added questions were: "Have you watched the video? if you don't, please watch it then back to fill the questionnaire." "Have you filled this questionnaire before?" Number of students who completed the posttest survey were 169, one student did not watch the video, two students did not fill the pretest questionnaire, and one questionnaire was missing some information. Finally, 165 valid questionnaires were collected.

ETHICAL CONSIDERATIONS

All participants were informed about the aim and contribution study could have on nursing. Students were also informed that taking part in the study is voluntary. Confidentiality of students' information was also taken into account through using of anonymous questionnaires. Students also informed that they could leave anytime during the study phases. Furthermore, all of the aforementioned steps were included in the informed consent form.

DATA ANALYSIS

The SPSS® version 27.0 was used to analyze the collected information. Variety of statistical tests was used to create best understanding about study hypothesis and the effectiveness of the reappraisal intervention. Frequency and percentages were computed to describe the socio-demographic variable, appraisal levels, and attitudes levels of nursing students who participated in this study. The dependent t-test (paired-samples t-test) was used to compare the results between pretest and posttest in respect to students' appraisal and attitudes. Independent t-test was also run to compare the results between male and female students.

RESULTS

Female students who participated in the study constitute more than three quarters (79.4%) of the total study participants. Also, more than three quarters (75.2%) reported that they were living in urban areas. About (73%) of the nursing students reported no other nurses in their families (table I). In terms of the household income, nurses reported that their family income is ranged between rather enough and enough (47.9%; 46.1%), about two third (60%) of nursing students enrolled in the nursing school out of their interest.

A paired t-test analysis was used on a sample of (165) nursing students to determine whether there was a statistically significant difference between the appraisal level of nursing students before and after applying the positive reappraisal intervention (table II). A statistically significant difference was found in the appraisal scores before and after applying the intervention ($t = -26.320$, $p < .0005$). Nursing students show higher mean score on the appraisal measure (post-test) ($6.44 \pm .51$) as opposed to the appraisal measure (pre-test) ($4.25 \pm .93$); with significant increase in the mean score (2.198).

A paired t-test was also used to determine whether there was a statistically significant mean difference in the attitude measures before and after applying the reappraisal coping. Nursing students showed more positive attitudes after being exposed to the intervention strategy ($4.43 \pm .41$), as opposed to the pre-test ($3.62 \pm .48$), with a statistically significant increase in the mean scores (2.198). These results indicate a statistically significant difference in the score of students' attitudes toward nursing before and after applying the positive reappraisal intervention ($t = -15.460$, $p < .0005$).

Independent t-test was used to analyze the differences between male and female students in respect to their appraisal scores before applying the intervention. The findings show that there was no statistically significantly difference between male and female in respect to their appraisal scores with a low mean difference (0.132).

DISCUSSION

This quasi-experimental study aimed to measure the effectiveness of positive reappraisal coping in changing the appraisal level of nursing students and their attitudes toward nursing. According to Koushali et al. (2012) and Mai et al. (2018), nursing students are expected to have a good appraisal level and attitudes toward nursing [19-20] . However, the findings of the recent study showed that before applying the positive reappraisal intervention, there were only 25% of nursing students had positive level of cognitive appraisal and 75% had neutral level. The findings also indicate that more than 50% of the nursing students had levels of attitudes ranged between neutral and negative toward nursing profession, which highlight the need for meaningful strategy to enhance their attitudes toward their future career. There are several factors could influence student's cognitive appraisal. One of the possible factors is that nursing students have the passion toward nursing profession as thinking of helping others in need. However, students may also think that nursing is a highly stressful profession as a result of witnessing stressful situations through clinical practices, and this could influence their ways of thinking. Alhurani et al., (2018) highlighted

Table I. The descriptive statistics of the socio-demographic variables of the nursing students

Demographic Data		f.	%
Gender	Male	34	20.6
	Female	131	79.4
	Total	165	100.0
Residency	Rural	41	24.8
	Urban	124	75.2
	Total	165	100.0
Academic Years	First	23	13.9
	Second	30	18.2
	Third	49	29.7
	Fourth	63	38.2
	Total	165	100.0
Have a nurse in the family	Yes	45	27.3
	No	120	72.7
	Total	165	100.0
Income	Not Enough	10	6.1
	Rather Enough	79	47.9
	Enough	76	46.1
	Total	165	100.0
Interest in nursing	Yes	66	40
	No	99	60
	Total	165	100.0

Table II. Difference in the students' appraisal levels before and after applying the intervention

Paired Differences					t	df	Sig.
Mean difference	SD	Std. Error Mean	95% Confidence Interval of the Difference				
			Lower	Upper			
-2.198	1.072	.083	-2.363	-2.03	-26.320	164	.0001

Table III. Difference in the students' attitudes before and after applying the intervention

Paired Differences					t	df	Sig. (2-tailed)
Mean Difference	SD	Std. Error Mean	95% Confidence Interval of the Difference				
			Lower	Upper			
-.8128	.6754	.0525	-.916	-.709	-15.460	164	.0001

that when the level of stress is higher than individuals' tolerance level, their ability to use more rational coping is diminished [21].

Another study also mentioned that "experience of stress differs significantly between individuals, depending on how they interpret an event and the outcome of a specific sequence of thinking patterns, called appraisals" [22]. Furthermore, students' cognitive appraisal could be related to their personal experiences. Studies have highlighted those personal experiences shape the way that they react or think; in other words, it shapes their cognitive appraisal or psychological response. Therefore, it is highly recommended that effective strategies should be used to enhance individuals' appraisal in a positive way [23]following a right shoulder dislocation. The 22 page narrative account provided by the athlete offered a holistic and integrated account of his experiences from the onset to return to play. A six step narrative analysis was analyzed by two qualified psychologists and two medical practitioners. Results: The themes are extracted to understand what was important to the participant. The cognitive appraisal and lived experiences are discussed within three dominant themes: 1. Students' attitude toward nursing, as their future profession, is influenced by several factors including, but not limited to social factors, economic factors, and student's ability of handling the change from the environment of secondary

Table IV. Gender difference in respect to student's appraisal level (pre-test)

Appraisal Scores	Levine's Test for Equality of Variances		t-test for Equality of Means				
	F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference
Equal variances assumed	1.621	.205	.731	163	.466	.132	.180
Equal variances not assumed			.674	46.773	.504	.132	.195

school teaching to the environment of college learning. Social factors are considered to be the one of the most and common affecting factors on nursing students' attitude. In some cultures, nursing is a suitable profession for females only; while, others think that nursing needs hard work, therefore, it is suitable for males. Self-esteem also plays a significant role in determining individual attitudes; level of self-efficacy contributes in a low or high level of student's attitude [10], [24].

Moreover, some studies indicate that nursing profession has little autonomy in their work environment, which negatively influences their attitudes. In other words, autonomy is essential in any profession to take decisions work freely and working the knowledge base, so lack of these authorities create low self-efficacy among professional nurses and among nursing students as they think and realize that concept and, in turn, resulting in low level of attitude [25]. After applying the positive reappraisal intervention, the findings of the recent study showed a significant increase in the students' cognitive appraisal from neutral level (75.2%) to positive level about (96%), which reflects the great effect of the coping intervention in manipulating students thinking [26-27] . Therefore, it has become necessary to use coping intervention when there is high level of stress and low level of appraisal; since, it has been proven by the results of the recent study that the intervention strategy was effective in changing the way students think. Positive reappraisal is considered to be one of the best models that is simple to use and cost effective [28]. The changes that the intervention made in students' appraisal level was expected, because of the intervention was originally designed to reduce stress through the way of changing individuals' cognitive appraisal in positive way. The recent findings also showed a significant increase in the level of student's attitude after applying the positive reappraisal intervention by effecting the cognitive appraisal of students. The significant improvement in the student attitudes toward nursing profession is moved from 49.7% to about 94% after applying the intervention. Also, posttest results showed no negative attitudes at all after applying the intervention; whereas, negative attitudes were reported in the pretest results. This improvement concludes the ability of the positive reappraisal intervention in changing individuals' attitudes positively. Studies have shown that students stress creates negative appraisal, which leads to negative emotions and attitude; therefore, when stress is eliminated by reappraisal strategies, positive attitude level will result [32-33]. After applying reappraisal intervention student's appraisal level is improved, and because cognitive appraisal and attitude are linked together, attitude has changed too.

CONCLUSIONS

The positive reappraisal coping intervention is proved as an easy model to apply and highly effective in terms of changing students' cognitive appraisal, which, in turns, changes their attitudes toward nursing. This assumption is concluded based on the significant increase in the level of appraisal and attitudes of nursing students after applying the intervention model; their levels are increased to about (96%) positive appraisal and about (94%) positive attitude level.

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ORIGINAL ARTICLE

FEATURES OF INTESTINAL MICROBIOTA IN PATIENTS WITH NONALCOHOLIC FATTY LIVER DISEASE: EFFECTS ON MARKERS OF INFLAMMATION AND HEPATIC STEATOSIS

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ABSTRACT

The aim: To study the state of the intestinal microbiota (IM) in patients with Nonalcoholic fatty liver disease (NAFLD) and to determine changes in its composition at the level of basic phylotypes.

Materials and methods: The study included 114 patients with NAFLD with metabolic disorders and 64 patients of control group. Determination of the composition of the IM at the level of major phylotypes was performed by identifying total bacterial DNA and DNA of *Bacteroidetes, Firmicutes* and *Actinobacteria* by quantitative polymerase chain reaction (PCR) in real time (qRT-PCR) using universal primers for the 16S rRNA gene and taxon-specific primers of production (Thermo Fisher Scientific).

Results: It was defined the weak correlation between the content of *Firmicutes* and proinflammatory markers (C-reactive protein (CRP) and Tumor necrosis factor (TNF) alpha) ($p < 0.05$) and inverse correlation of CRP with the content of *Bacteroidetes* ($p < 0.001$). Also have been observed significant changes in the main intestinal phyla in the direction of increasing the content of *Firmicute*s in patients with NAFLD with a high degree of steatosis and elevated levels of proinflammatory cytokines ($p < 0.05$).

Conclusions: IM imbalance leads to excessive synthesis of pro-inflammatory cytokines, promotes the activation of cellular mechanisms, which increases the flow of fatty acids into hepatocytes and increases the degree of hepatic steatosis.

KEY WORDS: Nonalcoholic fatty liver disease, intestinal microbiota, proinflammatory markers, hepatic steatosis

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a spectrum of disease that is caused by various mechanisms, including dietary, metabolic, genetic, environmental and microbiotic factors. A number of experimental and clinical studies have demonstrated evidence of a close relationship between NAFLD and intestinal dysbacteriosis [1, 2]. Nowadays, the study of the microbiota and its role in the pathogenesis of NAFLD have become extremely relevant.

Trillions of microbes that colonize the human body, including bacteria, archaea, viruses and eukaryotic microbes, spread along the length of the gastrointestinal tract (GIT) [3].

The combination of a small number of pathogens and a large number of key genera of bacteria characterizes the healthy state of the IM [4]. Factors influencing the state of the microbiota include: genetics, diet, method of childbirth, geographical location, the impact of drug treatment and others [5, 6]. As a result, the IM is unique to each person and at the same time under the influence of various factors changes throughout life. In it's turn, the IM affects the metabolic phenotype of the host, participates in food and drug metabolism and improves the immune system [7, 9-11].

The strategy to demonstrate the causal role of the IM in the pathogenesis of NAFLD is to investigate the association of the whole microbiome and to analyze potential key intestinal microbial phylotypes that may be associated with the etiology or development of a particular chronic disease. Basically, there are six different phylotypes in the IM: *Firmicutes, Bacteroidetes, Proteobacteria, Verrucomicrobia, Actinobacteria* and *Fusobacteria* [12]. These bacteria may be involved in a variety of important metabolic processes, including regulation of polysaccharide levels, bile acid production, choline metabolism, energy intake, stimulation of endogenous ethanol production, and protection against pathogens [13-15].

The integration of current available data supports the hypothesis that mild, systemic, and chronic inflammation caused by the IM may initiate and exacerbate the development of metabolic diseases such as obesity, diabetes, and NAFLD in humans [16-18].

Despite recent studies on human beings and animals have shown the connection between intestinal dysbacteriosis and NAFLD many questions remain open.

THE AIM

The aim of the stage of our research is to study the state of the IM in patients with NAFLD and associations of the main phylotypes with risk factors for the development and progression of NAFLD.

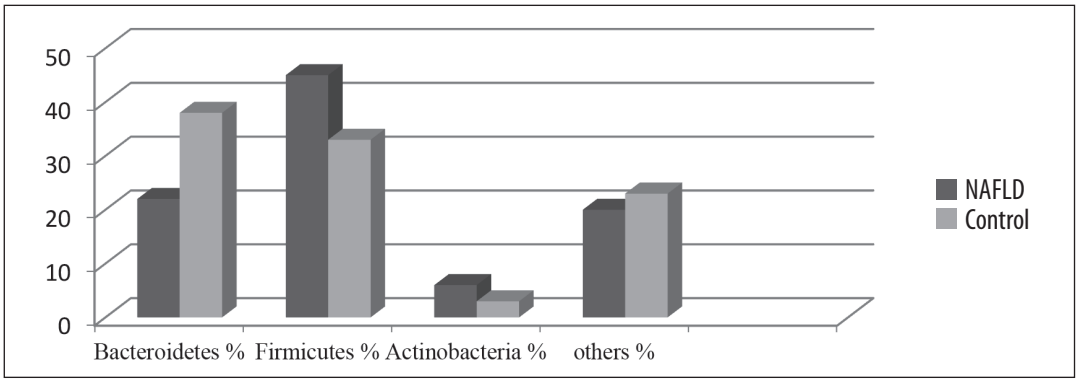

Fig. 1. Relative composition of IM at the level of basic phylotypes in patients with NAFLD and control group.

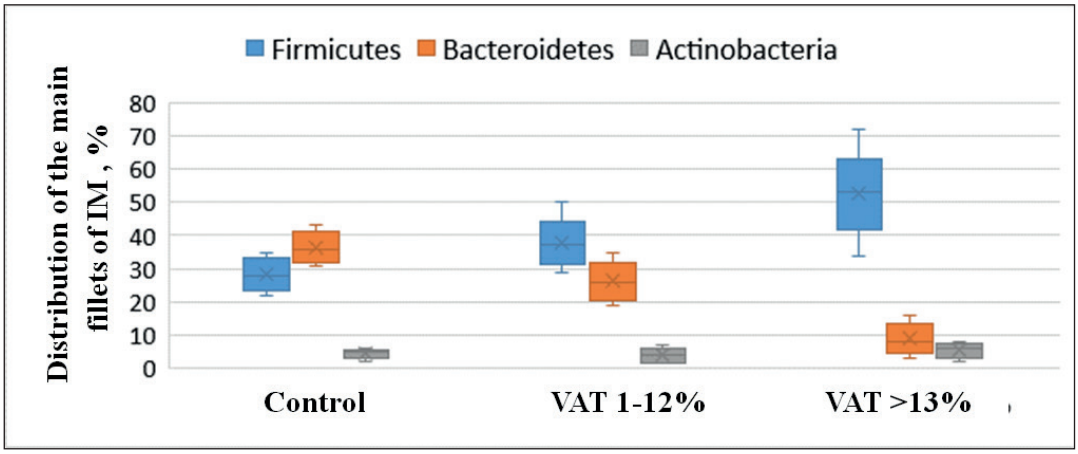

Fig. 2. Distribution of the main fillets of IM in the examined patients with NAFLD depending on the percentage of VAT.

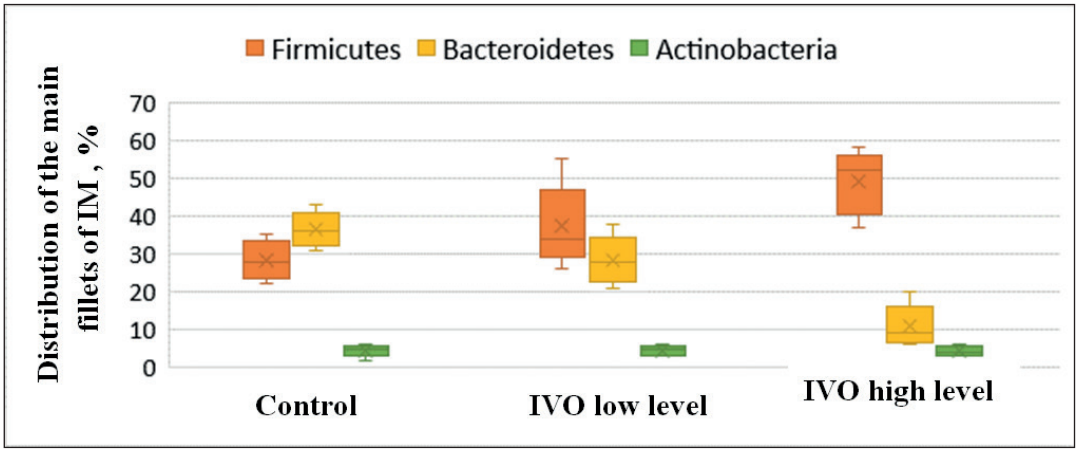

Fig. 3. Changes in the content of IM of patients with NAFLD depending on the activity of VAT.

MATERIALS AND METHODS

The research was conducted in the department of studying diseases of the digestive system and their comorbidity with non-infectious diseases of the Government Institution "L.T. Malaya Therapy National Institute of the National Academy of Medical Sciences of Ukraine" (certified license № AE 197294 dated 06.06.2013, Ministry of Health of Ukraine).

The study included 114 patients with NAFLD with metabolic disorders, 64 patients of control group, who were examined on the basis of the Department of Gastroenterology and Therapy and the outpatient department of the Government Institution "L.T. Malaya Therapy National Institute of the National Academy of Medical Sciences of Ukraine". Gender distribution was reciprocal. The mean age of the examined patients with NAFLD was (52.56 ± 11.7) years.

Estimation of anthropometric parameters included measurement of growth and determination of body weight with calculation of body mass index (BMI). All patients were evaluated for the function of liver, carbohydrate metabolism and lipid metabolism.

To study the body composition of patients (determination of total % of body fat, % visceral adipose tissue (VAT) it was used an electronic device - body weight monitor OMRON BF-511 (Japan, 2011). To determine the dysfunction of VAT the index of visceral obesity was calculated (IVO) by the method of Amato M.C. [1].

Determination of serum C-reactive protein (CRP) levels was performed by enzyme-linked immunosorbent assay

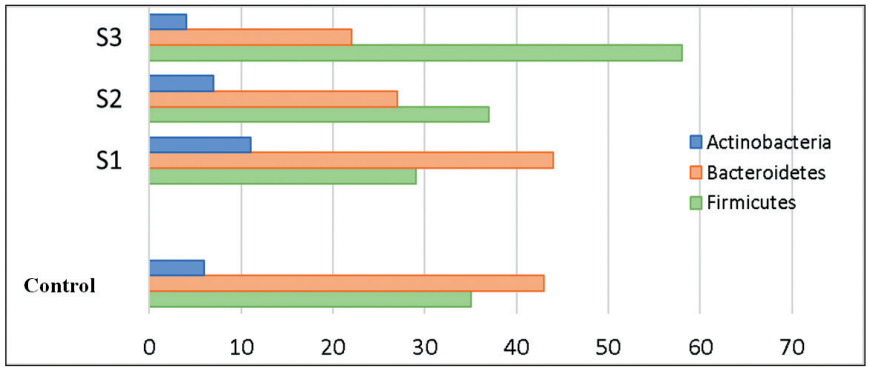

Fig. 4. The content of the main fillets of IM (%) of patients with NAFLD depending on the degree of hepatic steatosis.

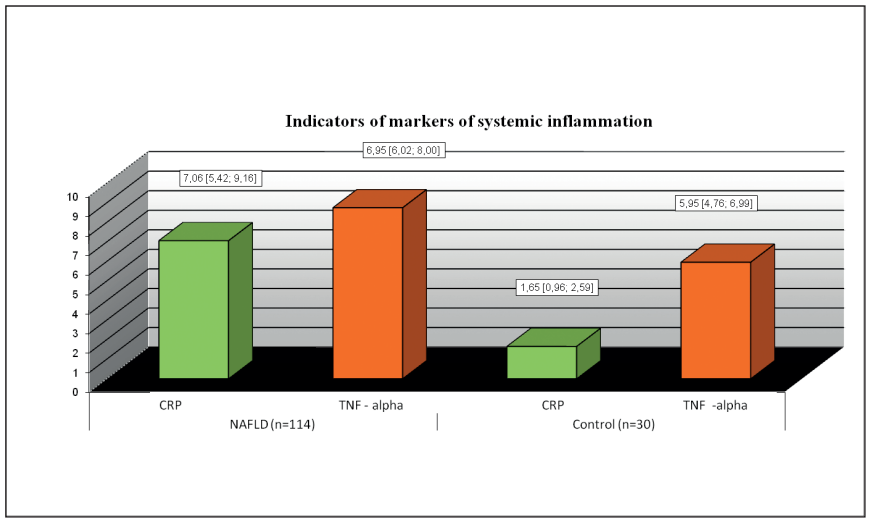

Fig. 5. Indicators of markers of systemic inflammation in patients with NAFLD.

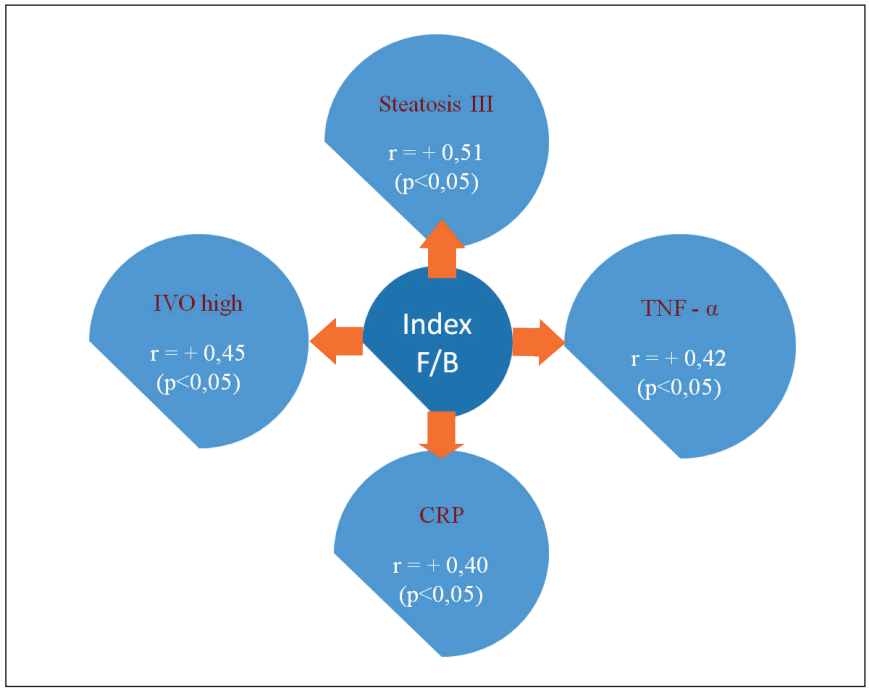

Fig. 6. Dependence of the ratio of major intestinal fillets (Firmicutes / Bacteroidetes) on markers of systemic inflammation (CRP and TNF-alpha), steatosis and activity of visceral obesity (IVO) of patients with NAFLD.

using hs-CRP ELISA KIT reagent kits - DRG International Inc. (USA) according to the manufacturer's instructions.

The level of Tumor necrosis factor (TNF) alpha in the serum was determined by using a set of reagents «ELISA-TNF-alpha», series 154 for the quantification of tumor necrosis factor in serum by the ELISA method.

The degree of steatosis levels was assessed by determining the wave attenuation coefficient (WAC) and performing shear wave elastometry (SWE), respectively.

Determination of the relative composition of the main phylotypes of the IM was performed by the method of molecular genetic research. The DNA concentration in the

Table I. Dependence of the main fillets of IM on markers of systemic inflammation.

	N	Spearman coefficient	t(N-2)	p
CRP & % *Bacteroidetes*	114	-0,298117	-3,66890	0,000347*
CRP & % *Firmicutes*	114	0,0249397	3,02535	0,002963*
CRP & % *Actinobacteria*	114	0,061222	0,72054	0,472409
CRP & % other	114	-0,118557	-1,40262	0,162974
TNF & % *Bacteroidetes*	114	-0,074736	-0,88041	0,380167
TNF & % *Firmicutes*	114	0,178312	2,12880	0,035047*
TNF & % *Actinobacteria*	114	-0,070311	-0,82802	0,409089
TNF & % other	114	-0,086554	-1,02061	0,309224

* - <0.05 relationships are valid

Table II. The dependence of the main fillets of IM on markers of systemic inflammation of patients with overweight and NAFLD.

	N	Spearman coefficient	t(N-2)	p
CRP & % *Bacteroidetes*	114	-0,298117	-3,66890	0,000347*
CRP & % *Firmicutes*	114	0,0249397	3,02535	0,002963*
CRP & % *Actinobacteria*	114	0,061222	0,72054	0,472409
CRP & % other	114	-0,118557	-1,40262	0,162974
TNF & % *Bacteroidetes*	114	-0,074736	-0,88041	0,380167
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TNF & % other	114	-0,086554	-1,02061	0,309224

* - <0.05 relationships are valid

extracts was measured using a Qubit 3 fluorometer and with a set of Qubit dsDNA HS Assay Kits (Thermo Fisher Scientific) and adjusted to ~ 10 ng / μl. Determination of the composition of the IM at the level of major phylotypes was performed by identifying total bacterial DNA and DNA of *Bacteroidetes, Firmicutes* and *Actinobacteria* by quantitative polymerase chain reaction (PCR) in real time (qRT-PCR) using universal primers for the 16S rRNA gene and taxon-specific primers of production (Thermo Fisher Scientific) [19].

In addition to the standard examination, all patients were quantified by the composition of the colon microbiota by polymerase chain reaction (PCR) with hybridization-fluorescence detection of results in real time using a test system "Colonoflor-16", manufactured by "Alphalab".

STATISTICAL ANALYSIS

Statistical processing was performed using the package 'STATISTICA 13.1'. According to the Kolmogorov-Smirnov criterion, the distribution of all studied indicators was different from normal (Gaussian), so data processing was performed using non-parametric statistics. Further data are presented in the form of Me [LQ; UQ], where Me is the median (50 quartiles), and LQ and UQ are the lower and upper quartiles (25 and 75 percentiles, respectively). The dependence of the indicator on the group was investigated using the Kraskel-Wallace test and the Spearman correlation coefficient.

RESULTS

THE COMPOSITION OF INTESTINAL MICROBIOTA, THE RATIO OF THE MAIN FILES OF PATIENTS WITH NAFLD AND HEALTHY PEOPLE

To assess the state of the intestinal microbiota of patients with NAFLD, the composition of the IM was determined at the level of basic phylotypes by identifying total bacterial DNA and DNA of *Bacteroidetes*, *Firmicutes* and *Actinobacteria* and the data were compared to the obtained control indicators.

The obtained results are presented in fig 1.

In addition, significant changes were observed in the index of the *Firmicutes* / *Bacteroidetes*. This indicator was significantly higher than in the control group of patients with NAFLD and was 6.43 and 1.26 ($p < 0.05$), respectively.

THE COMPOSITION OF INTESTINAL MICROBIOTIA, THE RATIO OF MAIN FILES OF PATIENTS WITH NAFLD DEPENDING ON THE QUANTITY AND DISTRIBUTION OF ADIPOSE TISSUE

A detailed analysis of the obtained data showed that in patients with varying degrees of obesity there was a significant decrease in *Bacteroidetes* to 16.6 [8,3; 22.4]%, with moderate obesity and up to 10.6 [6.5; 19.1]% in morbid obesity with a simultaneous increase in the ratio of *Firmicutes* / *Bacteroidetes* to 3.1 [1.7; 6.2] and 5.4 [2.8; 7.5], respectively.

At the same time in patients with overweight these changes were a trend. The relative amount of *Actinobacteri*a did not differ in any of the examined groups. As the weight increases, there could be seen deeper changes in the ratio of the main bacterial fillets of IM.

In the future, we studied the dependence of IM changes on the amount and activity of VAT. The distribution of the main IM branches from the percentage of VAT are presented in fig 2.

In patients with NAFLD, as the percentage of VAT increased, there was a redistribution of IM in the direction of increasing *Firmicutes* compared with the control group and patients with NAFLD with a normal amount of VAT. At the same time, the number of *Bacteroidetes* decreased in the group of NAFLD with visceral obesity, while the content of *Actinobacteria* did not significantly change.

Taking into account the pathogenetic role of visceral fat in the formation of NAFLD, we studied the associations between indicators of VAT activity and the quantitative composition of IM. The data are shown in Fig 3.

The obtained data demonstrate a similar dependence of the distribution of the main IM cells on the activity of VAT. In patients with a high index of visceral obesity (IVO) there was a probable increase in the content of *Firmicutes bacteria*, in contrast, the number of *Bacteroidetes decreased* in these patients. In the group of patients with NAFLD with a low level of IVO, high variability in the composition of *Firmicutes* bacteria was found, although in general the group showed a tendency to increase the content of bacteria of this class.

These changes indicate the probable participation of bacteria of the *Firmicutes* genus in the formation of VAT, increasing its activity with the further development and progression of NAFLD.

We analyzed the dependence of the main IM fillets in patients with NAFLD on the degree of hepatic steatosis. The obtained data are presented in Fig 4.

The study revealed an imbalance of IM of patients with NAFLD with varying degrees of fatty infiltration of the liver compared with the control group. Namely, in patients with third grade of hepatic steatosis, maximal changes were observed, which was accompanied by inhibition of the growth of bacteria of the *Bacteroidetes* class, with a simultaneous increase in the content of *Firmicutes* ($p < 0.05$). Similar changes were observed in the group of patients with low and moderate steatosis, but the differences were tendentious.

THE RELATIONSHIPS BETWEEN INTESTINAL MICROBIOTA AND SYSTEMIC INFLAMMATION IN PATIENTS WITH NAFLD AGAINST COMORBID CONDITIONS

Markers of systemic inflammation were identified in all examined patients, namely tumor necrosis factor (TNF) -alpha, highly sensitive C-reactive protein (CRP), the data are shown in Fig 5.

When analyzing the concentrations of markers of systemic inflammation in the serum of patients with NAFLD compared with the control group significantly elevated levels of CRP and TNF-alpha ($p < 0.05$) were obtained indicating the pathogenetic role of inflammation in the development and progression of the disease. We analyzed the dependence of the main IM fillets from markers of systemic inflammation. The obtained data are shown in table I.

When comparing the levels of proinflammatory markers with the main IM phyla, a direct correlation dependence of weak degree of CRP and TNF alpha with *Firmicutes* content ($p < 0.05$) and an inverse correlation dependence of CRP with *Bacteroidetes* content ($p < 0.001$) were revealed.

In order to determine the influence of metabolic factors on the composition of IM and the activity of inflammation, the relationship of IM with markers of systemic inflammation in groups of patients with NAFLD with overweight and obesity was evaluated. The results are presented in table II.

We studied the dependence of the ratio of major intestinal fillets (*Firmicutes / Bacteroidetes*) from markers of systemic inflammation of patients with NAFLD with varying degrees of steatosis. It has been shown that there is a direct correlation between the presence of adipose tissue and the level of hepatic steatosis and pro-inflammatory markers (CRP and TNF-alpha), and one can indicate the development of IM in the process of accumulation of systemic chronic adipose of the liver tissue, which is more accordant with ailments with a high level of visceral obesity activity (fig 6).

It has been shown that in patients with NAFLD and a high degree of steatosis there were more significant changes in the main intestinal fillets in the direction of increasing the content of *Firmicutes*. High levels of TNF and CRP also affected the composition of IM, namely in these groups of patients an imbalance of the IM was detected, which was manifested by an increase in the *Firmicutes / Bacteroidetes* index. However, the identified changes were a trend.

DISCUSSION

Numerous studies have shown that altered IM can affect liver function in some way, causing inflammation, insulin resistance, and fat accumulation, which is also related to NAFLD [20].

Differences at the taxonomic level between IM of healthy people and patients with NAFLD can be multidirectional: conducive for increased intestinal microbiota resistance, or if continuous external influences are stressful and destructive, they can lead to unstructured microbiome, and dysbacteriosis in it's turn can contribute to disease progression [21].

The two largest phylotypes that make up the human intestinal microbiota are *Firmicutes* and *Bacteroidetes*, and to a less extent other phylotypes are represented: *actinobacteria, proteobacteria, fusobacteria* and *verrucomicrobial* medications [22]. The *Firmicutes / Bacteroidetes* ratio is associated with a number of pathological conditions. In particular, obesity was specifically associated with a greater number of *Firmicutes* and / or a decrease in *Bacteroidetes* (ie, an increase in the ratio); however, some studies did not show

any changes or even increases in bacteroid content [21, 23].

We consider the ratio *Firmicutes* / *Bacteroidetes* as an integral indicator, which best characterizes the violation of the relative composition of the microbiota at the level of basic phylotypes. However, the assessment of this ratio in some cases may require additional research, because the IM is not constant and shows significant heterogeneity within one phylotype [24, 25]. It should be noted that the *Firmicutes* / *Bacteroidetes* ratio increases from birth to adulthood and subsequently changes with aging. This ratio varies significantly between infants, adults and the elderly. This may be due to general changes in bacterial profiles at different stages of life [26].

Analysis of the literature shows a close relationship between the growth of the *Firmicutes* / *Bacteroidetes* index and the presence of metabolic disorders. Significantly higher values of this indicator are observed in obese people and obese mice (ob / ob) compared with the control group of lean people [27, 28]. The microbiota of obese people also had less bacterial diversity than in lean people [29]. In addition, when obese people lost weight using a low-fat or low-carbohydrate, low-calorie diet, the *Firmicutes* / *Bacteroidetes* ratio decreased due to a percentage reduction in body weight [23].

However, other studies on humans and rodents have not showed differences in this index in obese individuals compared to lean people and under the influence of weight loss, or even demonstrated its reduction in obese individuals [30]. The reason for these conflicting observations regarding the *Firmicutes* / *Bacteroidetes* index and obesity is currently unclear.

This may be an indication that the change in IM may occur unevenly for each taxon in the same phylotype. In addition, the results which were obtained are not consistent in different studies due to the small number of people and differences in age, gender, ethnicity, geographical location and medication use [31-43].

Undoubtedly, the IM plays a significant role in the pathogenesis and progression of NAFLD, which makes it extremely important to study the mechanisms of its influence on the homeostasis of the intestine and liver.

According to the modern literature, there are ambiguous research results on changes in the IM of patients with NAFLD in overweight and varying degrees of obesity. We analyzed the two largest phylotypes that make up the human IM - *Firmicutes* and *Bacteroidete*s, depending on BMI.

During the study of the relative quantitative composition of IM there were revealed significant differences in patients of examined group with NAFLD with overweight and varying degrees of obesity compared with the control group and the group of patients with NAFLD with normal weight. Thus, in all patients with NAFLD with normal weight, the distribution of the main microbial fillets did not differ from the control group. At the same time, in patients with NAFLD with overweight and obesity, there was a shift in the ratio of the main fillets towards an increase in *Firmicutes* and a decrease in *Bacteroidetes*, which led to an increase in the index of *Firmicutes* / *Bacteroidetes*.

The obtained data indicate the involvement of IM in the processes of systemic chronic inflammation, molecular mechanisms of ectopic fat deposition, in particular the formation of fatty degeneration of the liver, which is more evident in patients with a high degree of visceral obesity. IM imbalance leads to excessive synthesis of pro-inflammatory cytokines, promotes the activation of cellular mechanisms, which increases the flow of fatty acids into hepatocytes and increases the degree of hepatic steatosis.

CONCLUSIONS

1. In comparison with healthy individuals, patients with NAFLD showed a significant decrease in the relative content of *Bacteroidetes* with a simultaneous increase in the content of *Firmicutes* and an increase in the index of *Firmicutes* / *Bacteroidetes* ($p < 0.05$).
2. Overweight and obese NAFLD patients had a more significant IM imbalance in the form of an increase in the *Firmicutes* / *Bacteroidetes* index, due to inhibition of *Bacteroidetes* growth, compared with patients with normal BMI.
3. The revealed changes of the main IM fillets of the examined patients were observed not only with the increase of body weight, but also depended on the number and activity of VAT. The most significant changes were detected at high activity of VAT.
4. Deviations in the composition of IM, namely inhibition of the growth of bacteria of the class *Bacteroidetes* and the growth of the microflora *Firmicutes*, had an impact on the formation and severity of fatty infiltration of hepatocytes. Maximum changes in IM were observed in patients with a high degree of hepatic steatosis.
5. It was defined the weak direct correlation between the content of *Firmicutes* and proinflammatory markers (CRP and TNF alpha) and inverse correlation of CRP with the content of *Bacteroidetes*.
6. No associations were found between the composition of IM and inflammatory activity of patients with NAFLD with overweight and obesity, which indicates the absence of a significant effect of abdominal obesity on the state of IM.
7. The dependence of the ratio of the main intestinal phyla (*Firmicutes* / *Bacteroidetes*) on the markers of systemic inflammation and the activity of VAT was detected. Maximum index values (*Firmicutes* / *Bacteroidetes*) were observed in patients with NAFLD with a high degree of the activity of visceral obesity and elevated levels of CRP and TNF alpha.
8. Significant changes in the main intestinal phyla in the direction of increasing the content of *Firmicute*s were observed in patients with NAFLD with a high degree of IVO and elevated levels of proinflammatory cytokines.

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The work was carried out within the framework of scientific research "Developing individual ways to correct metabolic disorders in patients with nonalcoholic fatty liver disease by studying intestinal mikrobiom, regulatory molecules and markers of systemic inflammation" (№ state. registration 017U003030).
The research was performed in accordance with international standards for the coordinated participation of respondents, the ethical component of research and biomaterial collection. All patients provided informed consent.

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Conflict of interest:
The Author declare no conflict of interest.

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A - Work concept and design, **B** – Data collection and analysis, **C** – Responsibility for statistical analysis, **D** – Writing the article, **E** – Critical review, **F** – Final approval of the article

ORIGINAL ARTICLE

MORPHOLOGICAL CHARACTERISTICS OF DIABETIC GLOSSITIS

DOI: 10.36740/WLek202203109

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ABSTRACT

The aim: To identify characteristic features of structural change of the dorsal part of the mucous membrane of the tongue (MMT) in experimental streptozotocin-induced diabetes (ESID).

Materials and methods: The study included 20 adult white male rats of Vistar line (body weight 180-200 g), which were equally divided into 2 groups: experimental (simulated streptozotocin diabetes mellitus) and control ones

Results: 8 weeks after the beginning of ESID modeling, the changes in MMT are particularly pronounced. A large number of lamellar structures and keratin conglomerates are found on the surface of MMT. This phenomenon is closely correlated (r=0.70) with a decrease in the absorption capacity of superficial epitheliocytes and an increase in the number of heterogeneous microflora on the impression smear with low activity of leukocyte elements. The number of epitheliocytes of differentiation stages I-III continues to increase, and the number of epitheliocytes of differentiation stages IV-VI diminishes, which leads to a significant decrease in the index of cell differentiation and an increase in the nuclear-cytoplasmic ratio. Such changes in MMT impression smears indicate active processes of epithelial desquamation with increasing duration of ESID.

Conclusions: Thus, the morphological changes of MMT in ESID are characterized by a diverse combination of atrophic and hyperplastic processes, resulting in uneven thickening of multilayered squamous epithelium. There are pronounced dystrophic changes in the epitheliocytes of the stratum corneum (dyskeratosis, parakeratosis) in the area of the taste buds. All areas of MMT are inflamed which indicates the development of diabetic glossitis.

KEY WORDS: tongue, mucosa, microcirculatory flow, streptozotocin-induced diabetes

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INTRODUCTION

The mucous membrane of the tongue (MMT) is one of the important structures of the body. It performs multifaceted functions throughout life. Clinical changes of MMT are determined by morphofunctional features and caused by its localization in the first division of the digestive system. Due to the general neuroreflex regulation, chronic diseases, in particular diabetes mellitus (DM), have a special effect on the tissues of the tongue [1, 2]. The neurotrophic component in diabetes plays an important role in pathogenesis of destructive and inflammatory lesions of the tongue, but the mechanism of development of these lesions and morphological manifestations have not been studied enough. The issue of the state of the tongue in diabetes has been covered repeatedly in the scientific literature, but researchers described only the clinical manifestations of diabetic glossitis (DG) [3 4], as well as some features of laboratory diagnosis [5].

THE AIM

The aim of the study was to identify characteristic features of the structural change of the dorsal part of the mucous membrane of the tongue (MMT) in experimental streptozotocin-induced diabetes (ESID).

MATERIALS AND METHODS

The study included 20 adult white male rats of Vistar line (body weight 180-200 g), which were equally divided into 2 groups: experimental and control ones. The experimental group (10 rats) received intraperitoneally streptozotocin "SIGMA" (USA) which was diluted in 0.1 M citrate buffer with a pH of 4.5 (at the rate of 6 mg per 100 g of body weight), (Patent of Ukraine No. 62966; 11.02.2011, published on 20.09.2011, Bulletin No. 18). The control group (10 rats) in an equivalent dose received intraperitoneally 0.1 M citrate buffer with a pH of 4.5. The development of ESID was monitored by blood glucose levels, which were measured daily on an empty stomach in a drop of blood from the tail vein on an Assu-Chec Active (Germany) glucometer. The material was collected 8 weeks after the start of ESID modelling, after previous euthanasia, under thiopental anesthesia.

Animal experiments were carried out in accordance with the provisions of the European Convention for the Protection of Vertebrate Animals Used for Research and Other Scientific Purposes (Strasbourg, 1986), Council of Europe Directive 86/609/EEC (1986), the Law of Ukraine from December 15, 2009 and orders of the Ministry of Health of Ukraine No. 690 from 23.09.2009, No. 616 from 03.08.2012 (expert opinion of the commission on ethics

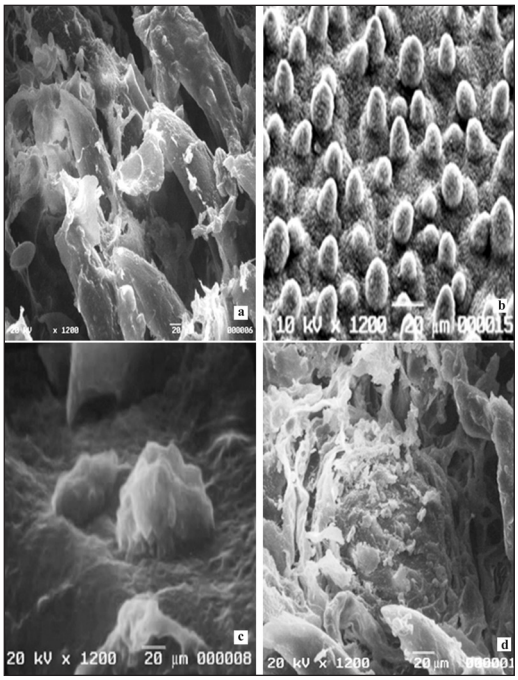

Fig. 1. Deposition of horny scales on the surface of filiform (a), cone-shaped (b), fungiform (c) and circumvallate (d) papillae of the mucous membrane of the tongue 8 weeks after the start of ESID modeling. Scanning electron microscopy. Electronic microphotographs. Magn.: x 1200.

of SHEI "Ivano-Frankivsk National Medical University", protocol No. 88/16 from 02.03 .2016).

For histological examination, pieces of the tongue were fixed in 10% neutral formalin, embedded in paraffin blocks; sections 5-7 μm thick were made with their following staining with hematoxylin and eosin.

To study the cytological characteristics of MMT, the impression smears on sterile slides were stained according to Romanowski-Gimza, viewed under a binocular light microscope MS 300 (THR) and photographed using a Digital camera for microscope DCM 900, installed in its tube with a resolution of 1200x1600 and saved in TIF format. The degree of destruction of epithelial cells was evaluated on the tissue specimen, the nuclear-cytoplasmic ratio (NCR) of epithelial cells was measured, on its basis the stages of differentiation of each epitheliocyte were evaluated. Then, the index of cell differentiation (ICD) was calculated by the formula ICD = 1a + 2b + 3c + 4d + 5d + 6e, where 1-6 is the numerical designation of the differentiation stages, a, b, c, d, e, e – the percentage of cells of the corresponding differentiation stage [6]. The keratinization index (KI) was determined by calculating the percentage of non-nuclear cells on a cytological preparation.

For transmission electron microscopic examination, pieces of the tongue were fixed in 2% osmium tetroxide solution, performed and contrasted according to the conventional method. The investigation of the material was performed using an electron microscope PEM-125 K, at an accelerating voltage of 75 kV, followed by photography. For scanning electron microscopy of the mucous membrane, the tongue was fixed in 10% neutral formalin, dehydrated in series of ethanol and acetone of increasing concentration. After that, it was dried by the critical point transition method. The samples were sprayed with carbon (at an angle of 90°), shaded with aluminum (at an angle of 15°) and an electrically conductive layer of silver was created (15 nm). The samples were viewed under the scanning electron microscope REMMA-102E ("SELMI", Ukraine) with an accelerating voltage of 10 and 20 kV.

Microphotographs were used for morphometric studies. Morphometry was performed using NIH USA "Image J" and "Bio Vision 4.01" programs in manual mode, taking into account magnifications. Computer data processing was performed using the statistical package Stat. Soft. Inc; Tulsa, OK, USA; Statistica 6. Statistical changes were considered significant when the level of statistical significance was $p<0.05$.

RESULTS

In the course of ESID, there is a gradual probable increase in the level of glucose and glycosylated hemoglobin, which 8 weeks after the beginning of the experiment, respectively, are: 18.6±0.36 mmol/l (control – 5.2±0.56 mmol/l, $p<0.05$) and 9.8±0.31% (control – 2.2±0.34%, $p<0.05$); it indicates the development of persistent decompensated diabetes. 8 weeks after the start of ESID modeling, the changes in MMT are particularly pronounced. A large number of lamellar structures and keratin conglomerates are found on the surface of MMT. These structures are distinctly seen on the filiform and circumvallate papillae. In some areas, the entire surface of the papillae is covered with small globular structures, which look like mulberry fruit (Fig. 1). This phenomenon is closely correlated ($r=0.70$) with a decrease in the absorption capacity of surface epitheliocytes and an increase in the number of heterogeneous microflora on the impression smear with low activity of leukocyte elements. Compared with the control group of animals, 8 weeks after the start of ESID modeling on preparations taken from the mucous membrane of the tongue, cell complexes were often observed. Their number exceeds 4-5 cells in each layer. Their edges have a sharp contour which is in some areas scalloped. The number of microorganisms is significantly increased and the number of lymphocytes is increased by 23.4% (20-30 in the field of view).

It should be noted that 8 weeks after the beginning of ESID modeling, a variegated cytological picture of MMT is observed, which is characterized by a combination of atrophic and hyperplastic processes. There is an increase in cells of the 1st and 2nd class of the destruction, respectively, to 30.7±2.23% (control – 10.8±2.02%, $p<0.01$) and 27.6±2.38% (control – 1.6±0.32%, $p<0.001$), with a decrease in cells from 0th class of destruction to 41.7±3.23% (control – 88.2±4.46%, $p<0.01$).

During the experiment, a significant recalibration in the relative content of epitheliocytes of different stages of differentiation was observed due to a decrease in the number of cells of the stages V and VI by 14.5% and 18.9%, respec-

Table I. Results of cytological examination of impression smears of MMT at different times from the beginning of modeling of experimental streptozotocin-induced diabetes mellitus (M±m, n=20)

Groups of Animals	Stage of Cell Differentiation (%)						ICD NCR
	I	II	III	IV	V	VI	
Control Group	0	0	0	4.2± 0.24	78.7± 1.15	16.2± 1.18	512.8±9.48
							0.16±0.004
ESID	6.0± 1.14 *	9.5± 1.18 *	27.5± 1.16**	20.2± 1.61**	30.2± 1.33**	6.6± 1.41**	378.9±8.33*
							0.29±0.004*

Note: probable difference with control *p<0.05, **p<0.01;

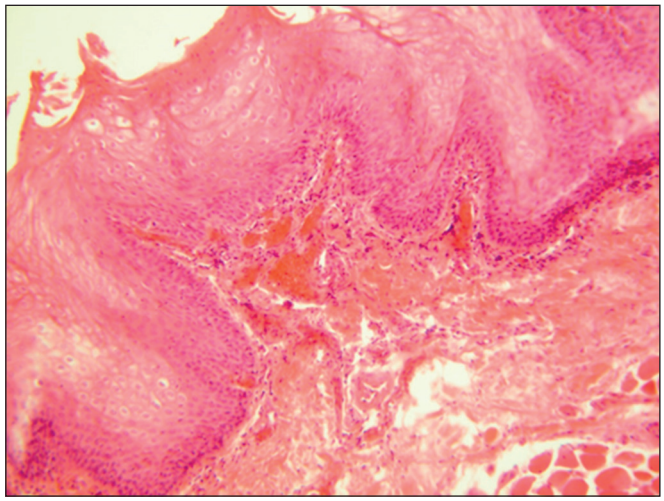

Fig. 2. Uneven thickening of the stratum corneum and hyperkeratosis of the mucous membrane of the tongue 8 weeks after the beginning of ESID modeling. Hematoxylin and eosin staining. Photomicrograph. Magn: x200.

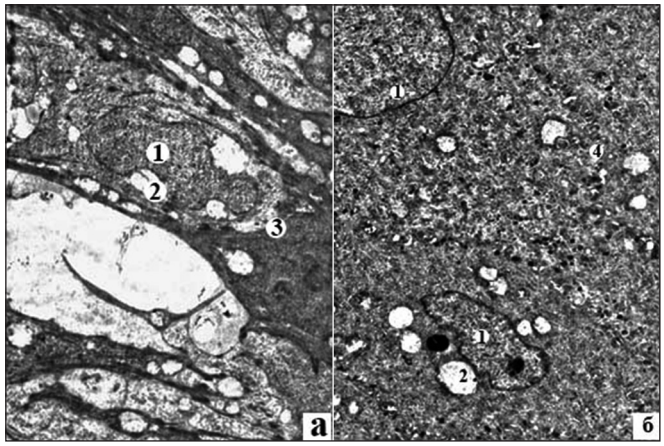

Fig. 3. Vacuolization of the cytoplasm of superficial epitheliocytes and sharp expansion of the intercellular space (a); appearance of a large number of keratohyalin granules, karyopyknosis in granular epitheliocytes (b) 8 weeks after the beginning of ESID modeling. Electronic microphotographs. Magn.: a) x 12000, b) x10000. Designations: 1 – nucleus, 2 – vacuoles, 3 – intercellular contacts, 4 – keratohyalin granules.

tively (p<0.05), with an increase of cells of differentiation stage IV and especially stage III.

Study of impression smears of MMT reveals cells of differentiation stages I-II (Table 1), which significantly affects the changes in the ICD. Its values decrease by 26.1%.

Correlation analysis of morphometric parameters show that the direct proportional relationship between various values is disturbed. At the same time, with increasing cell area, the values of NCR significantly increase (r=0.79, p<0.05).

8 weeks after the beginning of ESID modeling, the number of epitheliocytes of differentiation stages I-III continues to increase (Table 1), and the number of epitheliocytes of differentiation stages IV-VI decreases, which leads to a significant decrease in ICD and increase in NCR. Such changes in impression smears of MMT indicate active processes of epithelial desquamation with increasing duration of ESID.

The analysis of KI values revealed a number of patterns in changes of MMT in ESID. KI decreases to 58.9±3.56, compared with the control (p<0.05), which indicates a disturbance of the processes of epithelial cell differentiation and exposure of deeper layers of MMT.

8 weeks after the beginning of ESID modeling on MMT preparations, the stratum corneum is irregular, sharply thickened, hyperkeratosis is detected, which is characterized by the presence of layers of keratin masses (Fig. 2).

The surface layer of flattened epitheliocytes is thinned and there are cavities in it. The adhesion of microorganisms is revealed in a large number of observations and, mainly, it is diffuse. On superficial epitheliocytes there are mainly coccal forms of microorganisms.

According to tinctorial properties, dark and light epitheliocytes are differentiated among spinous epitheliocytes. In the nuclei of light cells, the nucleoli are not revealed. In dark cells, pyknotically deformed nuclei of irregular shape are often observed. Partially spinous epeliocytes are replaced by fine-grained, heterogeneous material. In the layer of these cells there is an intense transepithelial migration of lymphocytes.

At the ultrastructural level, superficial epitheliocytes form numerous irregular microforms. The vacuolation of the cytoplasm of superficial epitheliocytes and uneven intracellular distribution of keratohyalin are observed. Large granules are found more often, at the same time cytoplasmic accumulations of dusty keratinosomes sometimes occur. In the surface layer during the expansion of the intercellular space between individual epitheliocytes, large vacuoles are formed (Fig. 3 a). Some cells contain nuclei of chimeric shape, many nuclei are in a state of pyknosis.

In the cytoplasm of granular epitheliocytes, thin and short tonofilaments are diffusely distributed, creating the effect of medium osmophilicity. Small single granules of keratohyalin, lipid drops, vesicles of different sizes with small heterogeneous rounded inclusions are detected (Fig. 3b). Some vacuoles look optically transparent. Under electron microscopy, the shape of spinous epitheliocytes is irregular, polygonal, and sometimes flattened. 1-2 to 4-5 vacuoles are often found in their cytoplasm. They contain a fine-grained or electron-light matrix surrounded by a membrane. Spinous epitheliocytes with apoptotic bodies are dominant in some samples. Sometimes, there are single epitheliocytes with signs of complete destruction of most mitochondria.

There is a large number of pinocytic vesicles, bundles of tonofibrils and single granules of glycogen in the cytoplasm of basal cells. Disturbance of intercellular contacts with formation of optically transparent intercellular spaces in which fragments of cytoplasmic processes of the neighbouring epitheliocytes are observed is defined. The basement membrane is thickened, in some areas its integrity is violated and osmophilicity is reduced.

There is a swelling of the lamina propria of MMT. Along with this, intense lymphocyte-leukocyte infiltration is detected, the number and thickness of collagen bundles increase, and they acquire a chaotic orientation. The amount of connective tissue elements is particularly high in the paravasal space, which adversely affects the transport of nutrients to epitheliocytes.

DISCUSSION

As the duration of ESID increases to 8 weeks, the morphological changes of MMT increase and are characterized by a diverse combination of atrophic and hyperplastic processes and as a result, the stratified squamous epithelium thickens unevenly. There are pronounced dystrophic changes in epitheliocytes of the stratum corneum (dyskeratosis, parakeratosis). All areas of MMT are inflamed. The size of spinous cells increases, and their intercellular space decreases. Epithelial growth reaches large size and various shapes.

At all times of observation in the course of ESID, the process of keratinization of the epithelium of MMT undergoes changes. There are structural changes in epithelial cells: vacuolation of the cytoplasm, pyknosis, cytolysis, nuclear exposure, karyorexis, dinuclearity. Some authors [7] revealed accumulation of adipose tissue in the submucosal layer, which, in their opinion, is a specific sign of diabetes.

Cytological studies of impression smears of MMT in ESID showed increased desquamation of epithelial cells, which is confirmed by an increase in the number of epithelial cells of differentiation stages I-III and a decrease in the number of epithelial cells of differentiation stages IV-VI, which leads to a significant decrease in ICD and increased NCR. Such changes lead to an inflammatory reaction of MMT with the subsequent deterioration of the cytological picture due to maladaptation of the cellular response, aimed at decompensating the disorders that develop in ESID [8].

The processes of epithelium differentiation reflected by cytograms, were changed. In particular, there is a decrease in the prevalence of cells of stage IV by an average of 10.2% ($p<0.05$) and, to a lesser extent, of differentiation stage VI against a significant increase in the percentage of epithelial cells of stage V by an average of 18.3% ($p<0.05$). This may indicate a decrease in the regenerative function of the epithelium. In keratinized areas of MMT, the percentage of cells of differentiation stage IV was likely to decrease by an average of 2.4-fold, while the prevalence of non-nuclear scales was likely to be higher than in the control. This indicates an increase in the processes of keratinization.

The KI values had a tendency to increase, which confirms the above data of cytograms, and is also an unfavourable prognostic sign. This was especially pronounced in ESID at the stage of decompensation. Patients with ESID have a tendency to increase keratinization in all areas of MMT [9].

The 8th week of ESID course was characterized by a tendency to reduce the ICD compared with that in control animals, as indicated by other authors [5]. The tinctorial properties of epitheliocytes in ESID were changed. At the same time, the dark and light cells at staining by azure differentiate. The difference in microbial adhesion of these cells is revealed. Dark epitheliocytes are characterized by the presence of an increased number of microorganisms on the surface of plasmolemma. According to our data, tinctorial features explain the differences in the percentage of adhered microorganisms [10, 11].

It was noted that diabetic glossitis has its own morphological specificity, which differs significantly from other inflammatory processes of the oral mucosa. Experimental studies] showed that rats with hyperglycemia have a large amount of epithelial plaque in MMT. This is confirmed by the data of some authors [10, 12] who proved that patients with hyperglycemia have higher levels of pathogenic factors of the oral mucosa, among which are various microorganisms: Candida albicans, Prevotella intermedia, Bacteroides gracilis, Eikenella corodens and the like.

The cytological examination revealed a significant amount of pathological bacterial microflora on the surface of the tongue. Analyzing the literature, it can be concluded that many studies have proven the presence of pathological changes in MMT under the influence of hyperglycemia. In our study, the colonization of MMT was judged by the number of adhered bacterial cells per one epitheliocyte, ie, the adsorption reaction of microorganisms was determined. The colonization of epitheliocytes by microorganisms revealed individual fluctuations in the colonization of the epithelium of the tongue. Decreased resistance to bacteria of MMT in patients with uncontrolled hyperglycemia may be caused by impaired chemotaxis [13] and phagocytosis of neutrophils [14], which are common for diabetes.

CONCLUSIONS

Morphological changes of MMT in ESID are characterized by various combination of atrophic and hyperplastic processes, owing to that the multilayered squamous epithelium is unevenly thickened. There are pronounced dystrophic

changes in the epitheliocytes of the stratum corneum (dyskeratosis, parakeratosis) in the area of the taste buds. All areas of MMT are inflamed which indicates the development of diabetic glossitis.

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Conflict of interest:
The Authors declare no conflict of interest.

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ORIGINAL ARTICLE

DETECTION OF TORQUE TENO VIRUS ANTIGEN AND ASSOCIATED RISK FACTORS AMONG HEMODIALYSIS PATIENTS

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ABSTRACT

The aim: To determine the prevalence of TTV in patients undergoing hemodialysis and to evaluate the possible risk factors.

Materials and methods: This study was conducted in 93 patients, attending hemodialysis unit at AL-Imammain AL-Kadhimain Medical City Hospital for a period from November 2020 to March 2021. The demographic and clinical characteristics including age, sex, underling medical condition, hepatitis B and C status and laboratory tests such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline Phosphatase (ALP) and total serum bilirubin (TSB) were obtained from the record of the patients in hemodialysis unit in the hospital. Direct detection of TTV-Ag was done by enzyme linked immunosorbent assay (ELISA).

Results: TTV-Ag was detected in 38 out of 93 (40.9%) hemodialysis patients. Demographic, clinical and risk factors i.e. sex, age, history of diabetes, history of hypertension, history of blood transfusion, number of blood transfusion, the hemodialysis duration, history of surgery and liver enzymes levels did not show significant relation ($P>0.05$).

Conclusions: This study showed high prevalence of Torque Teno virus in hemodialysis patients, however, TTV did not play a role in liver injury among these patients.

KEY WORDS: Torque Teno Virus, risk factors, hemodialysis

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INTRODUCTION

The use of hemodialysis for end-stage renal disease has increased patient life span significantly. It also makes these patients more susceptible to infections, particularly those caused by blood-borne viruses, which play an important role in morbidity and mortality in hemodialysis patients [1]. Some of the most common viral infections are caused by hepatotropic or other hepatitis-associated viruses such as hepatitis C virus (HCV), hepatitis B virus (HBV), SEN Virus (SENV), and torque Teno virus (TTV) [2-7]. TTV is a non-enveloped, single stranded DNA virus, classified as a part of Anelloviridae family [8,9]. TTV is thought to be transmitted by blood transfusion, and its frequency is mainly associated with populations with a history of blood transfusion [10], but there are another ways of transmission due to it is presence in wide range of human samples, including breast milk, synovial fluid, feces, bile juices, and saliva [11]. TTV is associated with some clinical conditions. For example, TTV prevalence is ranging from 30% to 42.9% in hemodialysis patients [12,13], 20% in intravenous drug abusers [14], 46.7% in hepatocellular carcinoma patients, 40 % in cirrhotic patients [15], 75% in hemophiliacs, 46% in non A-G viral liver infections, 48%in Fulminant hepatitis patients, and 84.2 % in HIV-infected persons [13]. In addition, TTV is detected in 23.3% of healthy blood donor [5].

THE AIM

In the current study, we aimed to investigate TTV prevalence by using ELISA technique among hemodialysis patients, to evaluate the relation between TTV infection and demographical, clinical characteristics, risk factors and liver enzymes.

MATERIALS AND METHODS

SUBJECTS

This cross-sectional study included 93 patients undergoing hemodialysis in AL-Imammain AL-Kadhimain Medical City Hospital, from November 2020 to March. The ethical approval was obtained by the Institutional Review Board (IRB) at AL-Nahrain University on 20th September 2020 (No. 20200977).

SPECIMEN'S COLLECTION

Five (5) ml blood was drawn from each patient prior to starting hemodialysis in sterile gel tubes and allowed to clot at room temperature 25˚C for one hour, then centrifuged at 3000 rpm for 10 minutes. Serum samples were divided into aliquots in sterile Eppendorf tubes then stored at -20°C until being used.

IMMUNOASSAY

Ninety-three serum samples have been tested for TTV Ag presence using Human Transfusion Transmitted Virus (TTV) ELISA Kit (Abbexa, England) which is a sandwich enzyme-linked immune-sorbent assay for qualitative de-

tection of TTV Ag in serum. An antibody specific to TTV covered the wells of the microtiter plate. All the samples were diluted (1:5) with the dilution buffer, by adding 10µl of serum to 50µl sample diluents. Measurement of optical density is done at 450nm. The cut-off value is equivalent to 0.15. The test is considered positive if the absorbance of the test is the same or higher than 0.15; otherwise it is considered negative.

STATISTICAL ANALYSIS

Statistical Package for Science Services (SPSS) version-19 has been used to computerize statistical analysis. Comparison is obtained by using of Chi-square (χ^2 - test) for the categorical data, represented as count and percentage. In contrast, the differences in mean were examined by using t-test for the numerical data, presented as mean and standard deviation. The P-value same as or below 0.05 has been considered statistically significant, and below 0.01 has been considered highly significant, while, P value above 0.05 was considered non-significant.

RESULTS

In this study, 93 sera sample were screened by ELISA for TTV-Ag. Results showed that 38 (40.9%) out of 93 samples were positive to TTV-Ag, as shown in the table I. Several studies on the epidemiology of TTV showed that the infection is found worldwide. The frequency of TTV infection may differ, depending on the genomic region tested and the place where the study is undertaken (geographical location). It also depends on the group of dialysis patients [16]. In the present study, the prevalence of TTV-Ag was 40.9% in hemodialysis patients, this result is close to what's mentioned by Wahid & Saadoon (2019), who detected that TTV prevalence was 38.7% in hemodialysis patients [17].

DISCUSSION

The current study showed that there was no significant association between TTV infection and any of demographic, clinical characteristics and risk factors such as age, sex, diabetes (DM), hypertension (HT), blood transfusion, number of blood transfusions, duration of hemodialysis, history of previous surgery, liver enzymes levels and viral hepatitis, as shown in the table I. Considering age of studied population, TTV-Ag positive individuals were with mean age of (51.68±13.77) years according to table I, there was no significant difference in age between TTV positive and TTV negative in hemodialysis patients, which is in agreement with Irshad et al., (2010) [18].

In addition, when comparing the rate of TTV-prevalence between males and females, the results showed that male had a higher rate (42.6%) than female (38.5%) with no significant differences between them. This result is in accordance with Takemoto et al., (2015)including the Torque teno virus (TTV, who showed that there is no significantly difference between gender and TTV infection and also found that the majority of dialysis patients were males (55%) with a mean age of (53.8) years [16]. This variation in number between females and males may be due to the differences in pathophysiology, physiological nature, the type of work and nutrition which may lead to males being more possible than female to have kidney disease or kidney failure [15,7]. Considering the underling medical condition, 31.0% of TTV positive patients were suffering from diabetes (DM) and 40.2% of them had hypertension (HT). There was no significant statistical association between TTV positivity and diabetes mellitus or hypertension according to the table I, this results are in agreement with Akbari et al., (2018), who observed that there is no significant relation between TTV infection and hypertension [20].

Another study also in accordance with the current findings done by Gallian et al., (1999)we tested 150 attendees of two hemodialysis (HD found that the TTV prevalence in hemodialysis diabetic patients was not significantly higher than that detected in a diabetic patients without renal disease [21]. Interestingly, 61% of hemodialysis patients have a history of blood transfusions. However, there were no significant relation between the TTV infection and blood transfusion or the number of blood transfusions in hemodialysis patients which is in accordance with Irshad et al., (2010) study of comparative analysis, conducted among patients with a history of blood transfusion [18].

The use of erythropoietin to treat renal anemia resulted in a significant reduction in blood transfusions; however, infections in hemodialysis units can still occur in the absence of other parenteral risk factors [22-23]. This suggested other route of viral transmission since the virus present in healthy individual and can transmit by different mechanism. Regarding the hemodialysis duration, the current study showed no significant difference between the TTV infection and the period of hemodialysis. This is near to other study done by Akbari et al., (2018), who indicated that viral infection was not significantly associated with duration of hemodialysis [20]. Although nosocomial infection still may play an important role, but we can't be ruled out that TTV had other transmission routes such as fecal-oral route, salivary droplet, sexual transition, breastfeeding [24-25], as well as, through presence of TTV in environment and many animal reservoirs [26]. In addition, the present study showed that there was no noticeable significant association between the positivity of TTV infection and people who undergone surgery, in agreement with Khudair et al., (2019) [27]. These findings disagree with Spandole-dinu et al., (2018) who found a significant difference between the two variables, TTV and surgery. Also, it was showed that human anelloviral DNA was higher in healthy women who had at least one surgical procedure such as abortion or cesarean section [28]. This could be explained by the differences in the study population, sample size, geographical region, the percentage of virus in the normal people in the community. In this study, 46 (49.5%) of hemodialysis patients were tested negative to HBV&/or HCV and 19 (41.3%) of them had TTV infection.

Table I. Relation of TTV Ag and demographic, clinical characteristic and risk factors in studied population (n=93)

Parameter		Total No (%)	TTV Ag		P value
			Negative No (%)	Positive No (%)	
Age (mean ± S.D.)		93 (100%)	48.98±16.03	51.68±13.77	0.400NS
Sex	Male	54 (58.1%)	31 (57.4%)	23 (42.6%)	0.689NS
	Female	39 (41.9%)	24 (61.5%)	15 (38.5%)	
	Total	93 (100%)	55 (59.1%)	38 (40.9)	
DM	No	64 (68.8%)	35 (54.7%)	29 (45.3%)	0.194NS
	Yes	29 (31.2%)	20 (69.0%)	9 (31.0%)	
	Total	93 (100%)	55 (59.1%)	38 (40.9%)	
HT	No	11 (11.8%)	6 (54.5%)	5 (45.5%)	0.741NS
	Yes	82 (88.2%)	49 (59.8%)	33 (40.2%)	
	Total	93 (100%)	55 (59.1)	38 (40.9%)	
History of Blood Transfusion	No	32 (34.4%)	15 (46.9%)	17 (53.1%)	0.081NS
	Yes	61 (65.6%)	40 (65.6%)	21 (34.4%)	
	Total	93 (100%)	55 (59.1%)	38 (40.7%)	
No. of blood transfusions (time)	< 1	41 (44.1%)	21 (51.2%)	20 (48.8%)	0.348NS
	1-4	34 (36.6%)	23 (67.6%)	11 (32.4%)	
	≥4	18 (19.4%)	11 (61.1%)	7 (38.9%)	
	Total	93 (100%)	55 (59.1%)	38 (40.9%)	
History of Previous Surgery	No	49 (52.7%)	30 (61.2%)	19 (38.8%)	0.666NS
	Yes	44 (47.3%)	25 (56.8%)	19 (43.2%)	
	Total	93 (100%)	55 (59.1%)	38 (40.9%)	
Hemodialysis duration (year)	< 1	3 (3.2%)	3 (100%)	0 (0.0%)	0.129NS
	1-3	26 (27.9%)	18 (69.2%)	8 (30.8%)	
	3-5	22 (23.7%)	14 (63.6%)	8 (36.4%)	
	≥5	42 (45.2%)	20 (47.6%)	22 (52.4%)	
	Total	93 (100%)	55 (59.1%)	38 (40.9%)	
HBV &/or HCV Status	No	46 (49.5%)	27 (58.7%)	19 (41.3%)	0.986NS
	HBV	2 (2.1%)	1 (50.0%)	1 (50.0%)	
	HCV	42 (45.2%)	25 (59.5%)	17 (40.5%)	
	HBV& HCV	3 (3.2%)	2 (66.7%)	1 (33.3%)	
	Total	93 (100%)	55 (59.1%)	38 (40.9%)	

While the remaining 47 (50.5%) out of 93 of hemodialysis patients had HCV&/or HBV.

TTV was detected in 1 (50%) out of 2 of hemodialysis patients who had co-infected with HBV and 17 (40.5%) out of 42 of hemodialysis patients who had co-infected with HCV. In addition, one patient had triple infection with HBV, HCV and TTV. This is logical since these viruses share the parenteral route of transmission. However, current study showed that there was no significant association between these viruses and TTV status, as shown in the table I. In addition, there was no significant difference between the level of liver function tests between TTV-positive and TTV-negative on in hemodialysis patients, as shown in table II.

Table II. Liver enzymes level in relation to TTV Ag status in studied population

Parameters	TTV-Status		P-value
	Negative Mean ± S.D.	Positive Mean ± S.D.	
ALT mg/dl	19.24±13.06	18.05±18.72	0.719 NS
AST mg/dl	18.37±12.37	21.42±18.19	0.338 NS
TSB mg/dl	0.37±0.22	0.35±0.15	0.613 NS
ALP mg/dl	193.36±192.64	203.26±186.51	0.806 NS

This suggests that TTV presence in the liver did not cause severe damages to it. This is similar to study done by Irshad et al., (2006)a novel agent, in relation to its molecular characteristics, epidemiological features, modes of transmission, tissue tropism, pathogenesis, role in various diseases and its eradication from the body. TTV, a DNA virus, is a single stranded, non-enveloped, 3.8 kb long DNA virus with a small and covalently closed circular genome comprising 3852 bases. It was tentatively designated Circinoviridae virus. TTV genome sequence is heterogeneous and reveals the existence of six different genotypes and several subtypes. TTV has been reported to transmit not only via parenteral routes, but also via alternate routes. This virus has been detected in different non-human primates as well. At present, TTV is detected by polymerase chain reaction (PCR who stated that the hemodialysis patients with TTV infection did not necessarily have liver dysfunction [29]. Another study by Akbari et al., (2018) showed that there was no association found between a variety of epidemiological and laboratory variables including liver enzymes and TTV infection[20]. Also, this could be similar to the fact that HCV related liver damage in hemodialysis patients is mild due to immunosuppressant. Hemodialysis patients decreased secretion of Th-1 associated cytokine (INF-γ), but increased secretion of Th-2 associated cytokine (IL-10), resulting in immunological deficiency [30]. However, the pathogenicity of TTV is not clear; several experiments have been conducted to determine its target organs. Initial study suggests hepatotropic tropism and its replication in liver cells [21,32]. Torque Teno virus infection was found to be very common in people who suffered from idiopathic Fulminant hepatitis and those suffering from cryptogenic chronic liver diseases including liver cirrhosis, chronic hepatitis, and hepatocellular carcinoma [33]. In addition, researchers discovered that TTV frequency was the same in patients suffering from liver disease and in assorted cases of non-B and non-C liver diseases, and also in few healthy individuals. They settled that TTV does not cause harm to the liver [32]. Lemon et al. (2000), observed that there are harmless viruses referred to as orphan viruses and are beneficial to the body in a way that they maintain homeostasis [34]. These viruses were isolated but not yet associated with any infection, so they are considered "simple guests". Although it may be difficult to attribute the term "guest" or "endosymbiont" to viral agents, but they have a characteristic responsible for altering the normal functioning of cells [35]. It is thought that the factor that might cause the difference in infection is the immunity of an individual or viral load. The finding that TTV is most likely to be found in serum of HIV-infected abusing drugs is an indication that there is a possibility of a high viral titers due to immunosuppressant. It has also been confirmed because of it is high capability of genetic diversity; only specific TTV strains are clinically important and can cause hepatitis [36].

CONCLUSIONS

This study concluded that the prevalence of TTV Ag was high (40.9%) among hemodialysis patients. However, TTV did not seem to be associated with any of the risk factors; therefore, it may transmit by different routes rather than blood transfusion. Also, TTV have no role in increasing the level of liver enzyme or the severity of HCV infection in hemodialysis patients.

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ORIGINAL ARTICLE

MODERN DIAGNOSTIC CRITERION FOR ESTABLISHING THE REGIONAL ORIGIN OF BLOOD IN SEXUAL CRIMES

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ABSTRACT

The aim: Determination of regional blood origin in cases of sexual violence, establishing the possibility of using prostaglandin F2alpha as a marker of blood of menstrual origin.

Materials and methods: The material for the study were samples of vaginal fluid, menstrual blood and capillary blood from females, the age distribution of women was carried out according to the gynecological classification according to the age periods of women's lives depending on the functional state of their reproductive system: the first group – women at the age of 18-29, the second group – women at the age of 30-45.

Results: Among objects of biological origin, fluids, especially blood, occupy an important place. The content of PGF2α has age fluctuations: its content is higher by 6-12% in women aged 30-45 than in women 18-29 years old. PGF2α levels above 13.1 ng / mg of dry tissue are a reliable sign of blood of menstrual origin, which is very important in determining the regional origin of blood in forensic examinations for sexual violence / sexual crimes.

Conclusions: In cases of sexual violence against women, in addition to recording external harm, attention should also be paid to the examination of objects of biological origin, identification by species, sex, regional, organ or cell.

KEY WORDS: sexual abuse, laboratory diagnostics, blood, forensics, COVID-2019

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INTRODUCTION

The current and most important problem today is the spread of a dangerous infection caused by a new virus that leads to the development of respiratory diseases in humans, namely acute respiratory disease COVID-19. Given the rapid spread of COVID-2019 worldwide, the severity of the disease in 20-30% and high mortality, governments in almost all countries have taken measures to implement global quarantine and declare a global emergency [1-5]. Under quarantine conditions due to the COVID-19 pandemic, the majority of the population is forced to stay at home, significantly reducing the number of contacts. At the same time, on a domestic basis quite often there are conflicts between family members, both psychological, physical and sexual, as a result of which women often suffer. Thus, the number of appeals to law enforcement agencies, hotlines of social services and charitable organizations about cases of domestic violence against women has increased sharply, sometimes to 1,500 per day, according to information sources. Therefore, it is very important in case of suspicion of domestic violence to respond quickly for law enforcement agencies, social services, medical workers in order to provide assistance to victims, timely detection and recording of signs of psychological, physical, sexual violence, impartial investigation. Timely appointment and forensic examination, including examination of physical evidence, are required to detect the presence and record of physical and sexual violence against female victims. The most important task of forensic examination is to obtain from the minimum amount of biological material the data necessary for its comprehensive characterization. The study of micro-traces and micro-objects of biological origin, the identification of which by species, sex, regional, organ or cellular affiliation can provide significant assistance to justice in the investigation of crimes against human life and health [6, 7].

THE AIM

Determination of regional blood origin in cases of sexual violence, establishing the possibility of using prostaglandin F2alpha as a marker of blood of menstrual origin.

MATERIALS AND METHODS

The material for the study were samples of vaginal fluid, menstrual blood and capillary blood from females of reproductive age 18-45 years old, which were taken during their examination in the KNP "Center of Primary Health Care" №1 Shevchenkivskyi district of Kyiv, during 2015-2016. Sampling was performed with the informed consent of virtually healthy patients. The age distribution

of women was carried out according to the gynecological classification according to the age periods of women's lives depending on the functional state of their reproductive system: the first group – women at the age of 18-29 (n = 28), the second group – women at the age of 30-45 (n = 23). A sample of vaginal contents during menstruation on a tampon, a sample of vaginal contents in the postmenstrual period and a sample of capillary blood on gauze were removed for the study. The removed objects were dried and stored until examination. Prostaglandins in the samples were determined by the method of preparative isolation and systematic analysis of prostaglandins obtained by biosynthesis [2]. A standard solution of prostaglandin F2alpha, namely Enzaprost F ("CHINOIN" Pharmaceutical and Chemical Works Co.Ltd., Hungary) was used as a control. The evaluation of the quantitative content of PGF2α in the blood was performed directly on the chromatograms, taking into account the relationship between the size of the stain and the mass of the object, namely, a method was used that allows using software to automatically determine the the size of the stain of the standard to accurately determine its quantitative content [8]. The digital data obtained in the studies was processed statistically according to the generally accepted methods of variation statistics, comparing the values of the content of PG F2α in different liquids. Differences between liquids were considered significant under the condition P <0.001 * (* P - achieved level of significance of PGF2α).

The work was carried out in accordance with the requirements of the «Instructions on the forensic medical examination» (Order of the Ministry of Health of Ukraine No. 6 of 01/17/1995), in accordance with the requirements and norms, a typical provision on ethics of the Ministry of Health of Ukraine No. 690 of 09/23/2009, «The procedure for the removal of biological objects from the dead, whose bodies are subject to forensic examination and pathological examination, for scientific purposes» (2018).

RESULTS

In total, PGF2α content in the effluent was studied in 51 samples, along with 28 samples derived from virtually healthy women aged 18-29 and 23 samples of women aged 30-45. A similar number of samples were studied to determine the content of PGF2α in blood of menstrual origin and 32 samples with capillary blood. The total number of studied samples obtained from practically healthy women is 134.

Studies of the content of PGF2α in the vaginal fluid of almost healthy women showed that its content in the vaginal fluid in healthy women aged 18-29 is 9.25 ± 0.03 ng / mg with individual fluctuations from 2.75 ng / mg to 16 As for almost healthy women aged 30-45, the PGF2α content is 10.35 ± 0.04 ng / mg, with individual variations from 5.79 ng / mg to 16.05 ng / mg. According to the data in the table and figure, it is seen that the content of PGF2α in the vaginal fluid of almost healthy women 30-45 years old, almost 11% more than in almost healthy women 18-29 years old. Comparison of their content taking into account age showed the dependence of PGF2α content on age (p <0,001) (Table I).

Using the method of TLC, we studied the content of PGF2α in the blood of menstrual origin. Studies of the content of PGF2α in the menstrual blood of almost healthy women showed that its content in the menstrual blood of healthy women aged 18-29 is 13.62 ± 0.04 ng / mg with individual fluctuations from 5.58 ng / mg to 21, 48 ng / mg. As for almost healthy women aged 30-45, the content of PGF2α is 14.48 ± 0.02 ng / mg, with individual variations from 5.64 ng / mg to 20.95 ng / mg. According to the data in the table and figure, it is seen that the content of PGF2α in the blood of almost healthy women aged 30-45, almost 6% more than in almost healthy women 18-29 years old. Comparison of their content depending on age showed a significant significant difference (p <0,001). Thus, the content of PGF2α in the menstrual blood of almost healthy women varies depending on age (Table II).

A study of the PGF2α content in the capillary blood of almost healthy women, taking into account their age, showed that there is an effect of a woman's age on the content of PGF2α in the capillary blood. According to the data in the table and figure shows that women aged 18-29 it was 5.07 ± 0.06 ng / mg, with individual variations from 2.75 ng / mg to 7.09 ng / mg, and at the age of 30-45 is 5.44 ± 0.06 ng / mg , with individual variations from 2.54 ng / mg to 8.62 ng / mg (Table III). Thus, the content of PGF2α in vaginal fluid and capillary blood is age-dependent. (P <0.001).

The obtained indicators of PGF2α in fluids of different regional origin (vaginal contents, blood of menstrual origin, and capillary blood) of almost healthy women allowed to conduct a comparative analysis of indicators and find out that the marker of menstrual blood is PGF2α (Table IV).

To determine the diagnostic criteria, we measured the limits of fluctuations of the mean values (M ratio), using the method of two-sigma estimation - M±2σ, where M is the arithmetic mean, σ is the standard deviation.

As a result of our research, we found the dependence of PGF2α content, firstly, on the age of women, and secondly, on the regional origin of the fluid. It should be noted that the content of PGF2α in vaginal fluid, menstrual blood and capillary blood in women of reproductive age, has an age feature, its content is higher by 6-12% for women aged 30-45. Thus, in women 30-45 years old the content of PGF2α is always, in all fluids, higher than in women 18-29 years old (p <0.001): in vaginal fluid - by 11%; in menstrual blood - by 6%; in capillary blood - by 3%. PGF2α is highest in menstrual blood compared to vaginal fluid and capillary blood. Moreover, this trend is true for women of both ages: in women aged 30-45, the content of PGF2α is 2.6 times higher in menstrual blood than in capillary, and 1.4 times more than in vaginal fluid; in women aged 18-29, the content of PGF2α is 2.7 times higher in menstrual blood than in capillary, and 1.5 times higher than in vaginal fluid.

Table I. The content of PGF2α in the vaginal fluid of almost healthy women of reproductive age

Unit of measure	Vaginal fluid of almost healthy women					
	women 18-29			women 30-45		
	n	M±m	σ	n	M±m	σ
PG F2α ng / mg dry tissue	28	9,25±0,03	0,19	23	10,35±0,04	0,21
P	P<0,001*					

Note: * P– achieved level of significance of PGF2α

Table II. The content of PG F2α in the blood of menstrual origin of almost healthy women of reproductive age

Unit of measure	Menstrual blood of almost healthy women					
	women 18-29			women 30-45		
	n	M±m	Σ	n	M±m	σ
PGF2α ng / mg dry tissue	28	13,62±0,04	0,25	23	14,48±0,02	0,1
P	P<0,001*					

Note: * P– achieved level of significance of PGF2α

Table III. The amount of PGF2α in the capillary blood of almost healthy women of reproductive age

Unit of measure	Capillary blood of almost healthy women					
	women 18-29			women 30-45		
	n	M±m	σ	n	M±m	Σ
PGF2α ng / mg dry tissue	16	5,07±0,06	0,26	16	5,44±0,06	0,26
P	P<0,001*					

Note: * P– achieved level of significance of PGF2α

Table IV. The content of PGF2α in fluids of different regional origin of almost healthy women of different reproductive ages

Age group of women	PG F2α ng / mg dry tissue, M ± 2σ		
	Vaginal fluid	Menstrual blood	Capillary blood
18-29 (n = 28)	9,25±0,03 (DI 2,75-16,59)	13,62±0,04 (DI 5,58-21,48)	5,07±0,06 (DI 2,75-7,09)
30-45 (n = 23)	10,35±0,04 (DI 5,79-16,05)	14,48±0,02 (DI 5,64-20,95)	5,44±0,06 (DI 2,54-8,62)
P	P<0,001*	P<0,001*	P<0,001*

DISCUSSION

As a result of our analysis, it can be safely stated that Ukraine has come much closer to European standards in terms of preventing domestic violence and respecting women's rights, becoming the 17th state to accede to the Istanbul Convention on November 7, 2011 and supporting the main objectives of the Convention. namely: "..protection of women from all forms of violence and prevention, prosecution and eradication of violence against women and domestic violence…", and a number of legislative documents were adopted and existing changes were made [9-11]. Thus, it should be noted that the Law of Ukraine № 2229-VIII "On Prevention and Counteraction to Domestic Violence" (came into force on 01/07/2018) defines the organizational and legal framework for preventing and combating domestic violence, the main directions of state policy in the field of prevention and counteraction domestic violence, aimed at protecting the rights and interests of victims of such violence, including sexual violence. The law clearly states that sexual violence is "a form of domestic violence that includes any acts of a sexual nature committed against an adult without his or her consent or against a child regardless of his or her consent, or in the presence of a child, as well as other offenses against sexual freedom or sexual integrity of a person, including those committed against a child or in his or her presence (Article 54 of Section I of the Law) [7].

In view of the above, it should be noted that in cases of sexual violence against women, in addition to fixing external injuries, attention should also be paid to the study of microtrace and microobjects of biological origin, identification by species, sex, regional, organ or cell affiliation, which can provide significant assistance in the investigation of crimes. Among objects of biological origin, fluids, especially blood, occupy an important place as evidence of various crimes that are accompanied by

external bleeding. Traces of blood (in forensic medicine) is any amount of fresh or altered blood outside the living organism that does not have morphological characteristics [6]. At the same time, one of the issues addressed in the study of physical evidence of biological origin in forensic practice is to determine their regional origin, including the identification of menstrual blood, especially in cases of sexual violence. After studying the data of domestic and world literature, we came to the conclusion that from a forensic point of view, this problem is not fully developed, is fragmentary, known diagnostic criteria do not take into account inherent in menstrual blood components, which leads to further study of this area, so this study is of great importance for forensic examination. In search of ways to solve this problem, we paid attention primarily to biochemical studies, which are increasingly used in forensic practice, in particular the biochemical properties of menstrual blood [12-14]. It is well known that menstruation (from the Latin mensis - month) - part of the menstrual cycle of the female body during which there is a rejection of the functional layer of the endometrium (uterine mucosa), accompanied by bleeding. The main role at the beginning of menstruation is played by spasm of arterioles. It is known that vasoconstrictors, which are PG F2a, endothelium-1 and platelet-activating factor (TAF) are produced within the endometrium and are involved in the contraction of blood vessels. They also contribute to the onset of menstruation and subsequent control over it. These mediators are regulated by vasodilators such as PG E2, prostacyclin, nitric oxide, which are also produced by the endometrium. PG F2a has a pronounced vasoconstrictive effect, exacerbates arterial spasm and endometrial ischemia, causes contraction of the myometrium, which on the one hand, reduces blood flow, on the other - helps to remove the rejected endometrium. Menstrual blood does not clot and has a darker color than the blood circulating in the vessels, contains a number of enzymes. Also, menstrual blood, mixed with the contents of the vagina, contains components of the vaginal epithelium, the epithelium of the mucous membrane, as well as a large number of bacteria - cocci, bacilli, etc. But the same components are contained in the blood from the female genital tract (for example, in sexual crimes), but not of menstrual origin [15, 16]. Therefore, it is important to find a criterion that would be a reliable marker of menstrual blood. Such a marker may be the vasoconstrictor prostaglandin F2alpha (PGF2α), which is produced in the endometrium during menstruation.

Given the above, we studied the possibility of using PG F2α as a marker of menstrual blood [17, 18]. However, to verify this, it was first necessary to check whether the content of PG F2α in the menstrual blood of different age groups of women who are practically healthy. Prior to that, we determined the content of PGF2α in the blood of menstrual origin, and capillary blood in almost healthy women aged 18-45.

Thus, we obtained statistically significant indicators that indicate the possibility of establishing the menstrual origin of the blood by the quantitative content of PG F2α. In our opinion, the content of PGF2α above 13.1 ng / mg of dry tissue is a reliable sign of menstrual blood, which is of great diagnostic value in the differential diagnosis of regional origin of objects of biological origin (blood) in cases of sexual violence / sexual crimes.

CONCLUSIONS

1. Legal assistance in cases of domestic violence in Ukraine is provided at the legislative level, as evidenced by the constant development and improvement of measures to prevent and combat domestic violence. Due to the increase in cases of domestic violence, especially sexual violence, in emergencies, including quarantine due to the COVID-19 pandemic, the issue of preventing and combating domestic violence needs special attention and further solution.
2. Timely forensic examinations to identify injuries with subsequent evidence of domestic violence, especially sexual violence, help the pre-trial investigation / court party to take effective administrative, criminal or other measures against the perpetrator. In cases of sexual violence against women, in addition to recording external harm, attention should also be paid to the examination of objects of biological origin, identification by species, sex, regional, organ or cell, which can provide significant assistance to justice in the investigation of crimes. Among objects of biological origin, fluids, especially blood, occupy an important place.
3. The content of PGF2α is the highest in menstrual blood 1.5 times compared to vaginal fluid and 2.7 times compared to capillary blood. The content of PGF2α has age fluctuations: its content is higher by 6-12% in women aged 30-45 than in women 18-29 years old. PGF2α levels above 13.1 ng / mg of dry tissue are a reliable sign of blood of menstrual origin, which is very important in determining the regional origin of blood in forensic examinations for sexual violence / sexual crimes.

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ORIGINAL ARTICLE

EFFECT OF BONE MARROW ASPIRATE IN DENERVATION-INDUCED SKELETAL MUSCLE ATROPHY

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ABSTRACT

The aim: To evaluate muscle changes after sciatic nerve damage with the injection of bone marrow aspirate cells.

Materials and methods: 36 rabbits underwent sciatic nerve cross-section and neuroraphy, bone marrow aspirate cells were injected directly or 7 weeks after neuroraphy. Changes in skeletal muscle morphology (photomicrographs of histological sections were analyzed for morphometric analysis of collagen region, quantitative analysis of conducted collagen density and measurement of muscle fibers diameter) and biochemical parameters (catalase activity, superoxide dismutase and glutathione peroxidase measurements and level of TBARS was determined) at 8, 12, and 16 weeks were examined.

Results: There is atrophy of muscle fibers in denervated muscles, and it has a negative tendency between 8 and 12 weeks. Delayed bone marrow aspirate cells injection into the muscles at 7 week – delayed atrophy and formation of TBA reactive substances. But bone marrow aspirate cells injection into the muscles directly after neuroraphy increased collagen formation, and development of fibrosis in areas of atrophy.

Conclusions: Sciatic nerve injury results in atrophy of muscle tissue, which is partially delayed after delayed bone marrow aspirate cells injection at week 7. Muscle atrophy was characterized by a sharp increase in TBARS levels at 12 and 16 weeks and catalase activity at 12 weeks, and changes in biochemical parameters were partially normalized after the use of cell aspirates, to a greater extent with delayed injection.

KEY WORDS: muscles, denervation, atrophy, fibrosis, bone marrow aspirate cells

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INTRODUCTION

Innervation play a critical role in skeletal muscle function. Denervation leads to muscle atrophy, dramatically impairs quality of life and has a poor prognosis for recovery [1]. Atrophy can be associated with the weight loss of individual fibers, without reducing the total number of fibers, or muscle fibers atrophy and even necrosis with prolonged denervation and severe muscle damage [2]. In damaged muscles (atrophy, denervation, or ischemia) number of muscle fibers and their diameter decreases within 9 months, and the amount of intramuscular adipose and connective tissue increases, at this time size of the muscle itself can both to decrease or doesn't change [3]. At the same time, a significant percentage of thin muscle fibers in the period from 6 months gives hope for the emergence of new fibers, ie the manifestations of recovery [3]. Unfortunately, in long-term reinnervation, which is often unlikely after severe nerve injury, regenerative effects associated with new muscle fibers formation by satellite cells are short-lived, focal, and not significant enough to compensate for skeletal muscle atrophy.

Metabolic processes in muscles on the background of atrophy are also not unidirectional, in particular on the background of protein amount reducing in muscle revealed accumulation of peroxidation products (malonic dialdehyde, diene conjugates, protein carbonyls) and 3-fold decrease in NO-synthase activity (NOS) [4], but enzymes activity of antioxidant system on the contrary increases, or their activity is multidirectional (increase in SOD and reduction of GR, GPx) [5]. Morphometric characteristics of muscles and metabolic processes are criteria for assessing the state of denervated muscles and the dynamics of atrophy.

Large number of ways to use mesenchymal cells from bone marrow to stimulate regeneration of damaged nerves and muscle reinnervation are described in literature. These cells have a trophic effect on tissues, activate nerve fibers regeneration, secrete growth factors, stimulate synthesis of extracellular matrix proteins, in particular collagen, fibronectin. Injection of these cells is considered as a potential way of denervated muscles trophic support and reinnervation. [6,7].

Injection of bone marrow mesenchymal stem cells in m.gastrocnemius has already been studied and even some encouraging results have been obtained [8], however, little is known about delayed muscle changes that occur in denervated muscles and how muscle tissue responds to cell injection.

THE AIM

To evaluate muscle changes after sciatic nerve damage with the injection of bone marrow aspirate cells.

MATERIALS AND METHODS

EXPERIMENTAL GROUPS

Experimental studies were performed on rabbits weighing 3-4 kg. Animals were divided into 5 groups of 3 animals per group (for each experimental period):

1) Control group - intact animals;
2) Group of shame-operated animals – only surgical approach to sciatic nerve and wound suturing;
3) Group 1 – surgical approach, neurotomy and nerve suture at the hip level, wound suturing;
4) Group 2 – same as Group 1, and bone marrow aspirate harvest from femur, with its injection into m.gastrocnemius, wound suturing;
5) Group 3 - same as Group 1, and at the beginning of muscle reinnervation (7 weeks) - bone marrow aspirate harvest from femur, and its injection into m.gastrocnemius.

SCIATIC NERVE INJURY

Two end-to-end nerve sutures by EHICON PROLENE® 7/0 (Johnson'n'Johnson Init) using microsurgical techniques was applied. In order to worsen conditions of reinnervation, nerve suture is applied through the entire thickness of sciatic nerve, with significant tension, and without adaptation of nerve stumps. Skin was sutured with COROLENE® 2/0 (Peters SURGICAL). Operating field was treated three times with Sterillium® Classic Pur disinfectant (BODE Chemie GmbH, Germany).

BONE MARROW ASPIRATE

Animals of group 2 after performing a nerve suture was injected with purified bone marrow aspirate in m.gastrocnemius. Animals of group 3 purified aspirate was injected 7 weeks after nerve suturing.

0.2 ml of 4% ACD-A solution was collected in a 5 ml syringe. A 1.2 mm diameter needle was used to puncture the skin in the projection of greater trochanter of femoral bone. Drilling of the greater trochanter outer cortical layer was performed, and immersion of needle to the inner cortical layer. Using a 0.9 mm thick conductor, needle is cleaned of bone debris. Syringe was attached and 2 ml of bone marrow aspirate was harvested. Needle was removed, puncture site was treated with Sterillium® classic pur disinfectant (BODE Chemie GmbH, Germany). Bone marrow aspirate was purified of spongy bone particles using a Tulip® EmulsifierTM subcutaneous fat aspirate filter. A 0.6 mm thick injection needle is connected to a syringe with a purified bone marrow aspirate. Purified bone marrow aspirate was inserted into m.gastrocnemius.

SKELETAL MUSCLE HISTOLOGY

Skeletal muscle (m.gastrocnemius) was fixed in a 10% solution of neutral formalin. After fixation, samples were embedded in paraffin via isopropanol-paraffin method and 8μm microsections were made on a Thermo Microm HM 360 microtome (Thermo Scientific, USA). Dewaxed sections were stained for 30 min at 25° red Sirius (0.5 g Direct Red 80 (Magnacol Ltd, UK) in 500 ml of saturated picric acid solution) [9], dehydrated, enclosed in balm (Merck, Germany). Photomicrographs were obtained on an Olympus BX51 microscope. Photomicrographs of histological sections were analyzed using ImageJ software (Wayne Rasband, USA). Microphotographs (lens magnification ×10) were selected for morphometric analysis of collagen region. Quantitative analysis of conducted collagen density is presented as a percentage (%).

To measure muscle fibers diameter, 10-15 microscopic fields were randomly selected for each sample to examine at least 200 fibers. Diameter was measured using Carl Zeiss AxioVision SE64 Rel.4.9.1 software as a linear projection between short ends of a single fiber.

BIOCHEMICAL RESEARCH

Homogenates were obtained from muscle samples using an Glas-Col electric homogenizer (USA) (100 mg tissue mass, 1 ml chilled 0.05 M phosphate buffer, 0.1 mM EDTA, pH 7.6). Homogenates were centrifuged at 10,000g for 20 min to obtain supernatants. Protein levels were measured by Lowry O.H. method [10]. Indicators were determined by spectrophotometric methods using a spectrophotometer μQuant, Bio-Tek, (USA). Catalase activity was determined by Aebi H. method [11], superoxide dismutase was determined by Mirsa method [12]. Glutathione peroxidase was measured by decrease in NADPH levels in conjugated glutathione reductase reaction [13]. Level of TBARS was determined by method described in the article published by Uchiyama M. [Uchiyama M.].

STATISTICAL RESEARCH

Distribution normality of data samples was carried out according to Kolmogorov-Smirnov criterion. Intergroup differences were assessed using one-parameter variance analysis of ANOVA variations with Bonferroni correction. Difference between the groups was considered significant at $P < 0.05$. Correlation analysis was performed according to Spearman's criterion. Statistical studies were conducted using OriginPro software ver. 8 (OriginLab Corporation, USA) and StatPlus ver. 7.3.0. (AnalystSoft Inc. USA). Data are presented as mean and mean error (Mean ± SEM).

BIOETHICS

All manipulations with animals were carried out in accordance with European Convention for Protection of Vertebrate Animals (Strasbourg, March 18, 1986) and recommendations of Bioethics Commission of the SI "Institute of Traumatology and Orthopedics of National Academy of Medical Sciences of Ukraine".

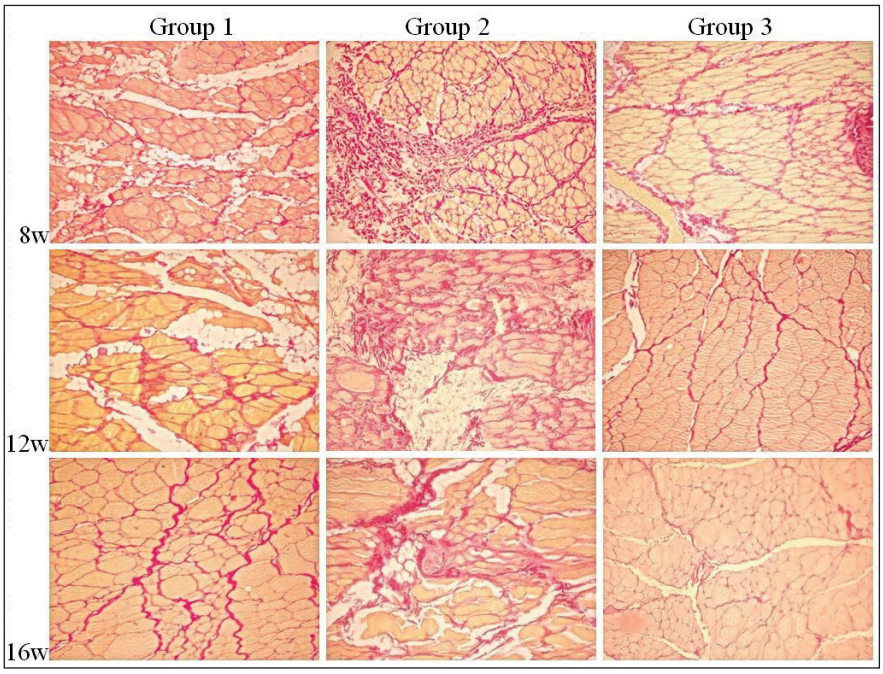

Fig. 1. Photomicrograph of m.gastrocnemius after picrosirius red staining with muscle fibers (yellow) and collagen (red).

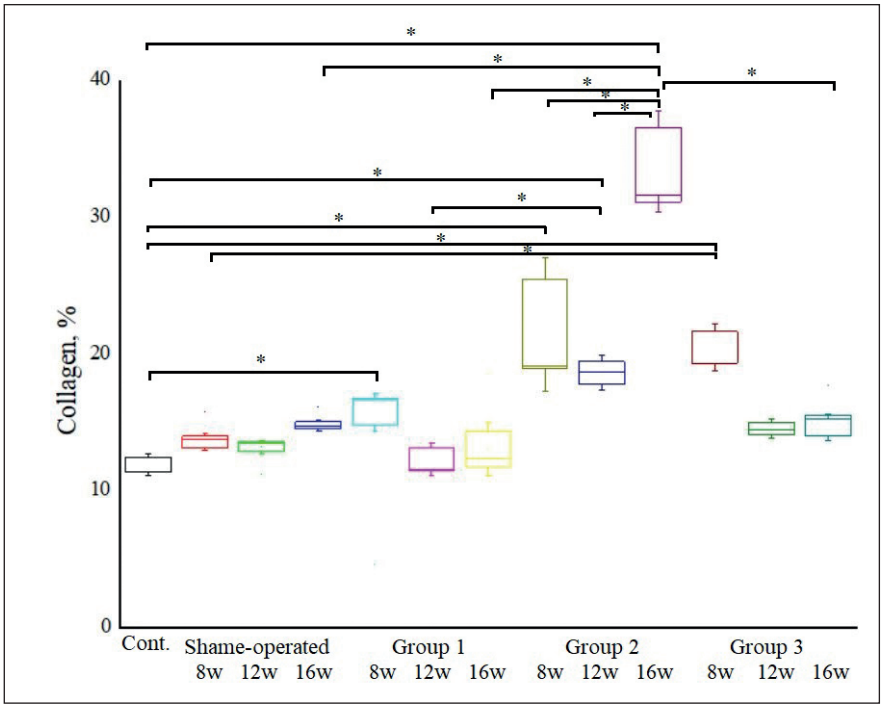

Fig. 2. Graph of collagen quantitative measurement in m.gastrocnemius histological micropreparations after staining with picrosirius red. * P <0.05

RESULTS

MUSCLE FIBROSIS

In skeletal muscles of group 1 at 8 weeks revealed a higher density of positively stained regions with picrosirius red compared to control (difference 4.38 ± 1.63%, P <0.05), which is the result of an increase in the amount of connective tissue (Fig. 1). In group 2, density of Sirius Red-positive regions was significantly higher relative to control group and group of pseudooperated animals by 8 (difference 10.2 ± 2.75%, P <0.05), 12 (difference 6.71 ± 1.98%, P < 0.05) and 16 (difference 22.1 ± 1.97%, P <0.05) weeks (Fig. 2). There was a statistical difference relative to group 1, namely an increase in positively colored regions by 12 (difference 6.33 ± 1.78%, P <0.05) and 16 (difference 21.0 ± 2.21%, P <0.05) weeks. These data indicate that injection of bone marrow aspirate after neuroraphy causes cellular reactions of connective tissue with collagen synthesis, stimulates collagen genesis. In group 3, increased collagen density was detected at 8 weeks relative to control (difference 8.56 ± 2.15%, P <0.05). At 12 and 16 weeks, no difference between control group and group of pseudooperated animals was detected; collagen density was higher relative to group 1 at 8 (difference 2.24 ± 0.96%, P <0.05) and 12 (difference 5.61 ± 1.64%, P <0.05) weeks. Indicator was significantly lower than in group 2 at 16 weeks (difference 17.5 ± 2.94%,

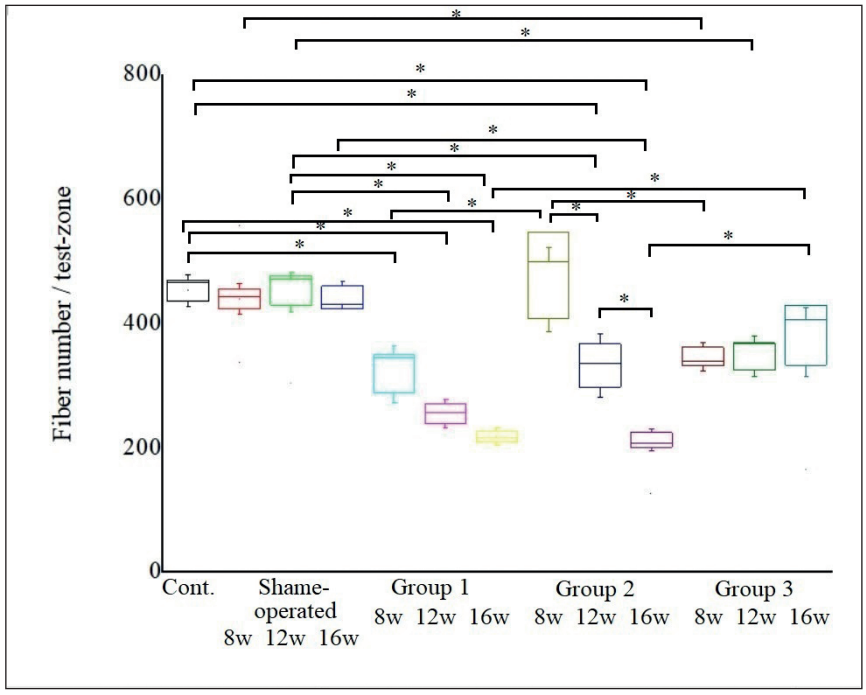

Fig. 3. Muscle fibers number in m.gastrocnemius, at the background of denervation. * P <0.05

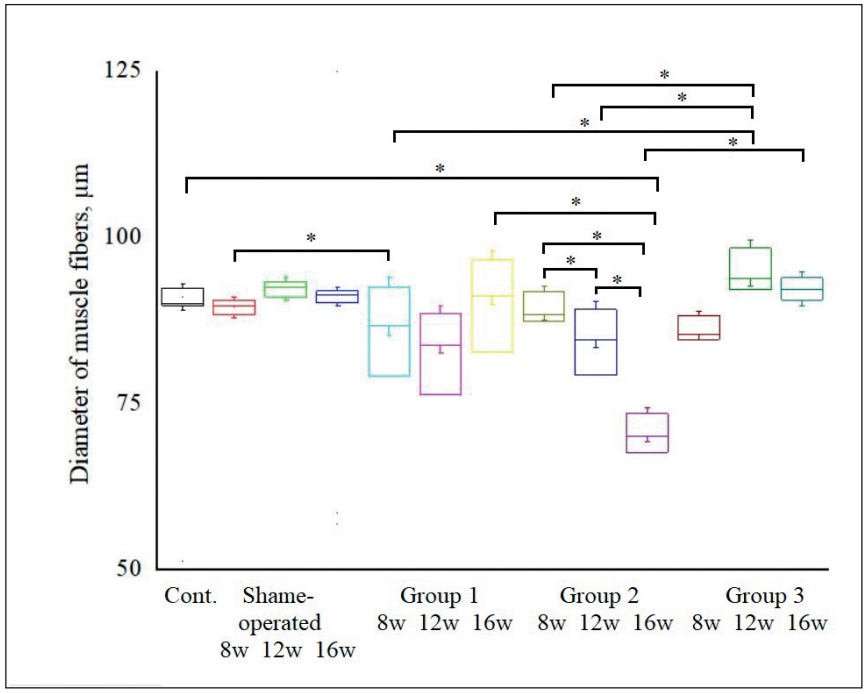

Fig. 4. Muscle fibers diameter in m.gastrocnemius, at the background of denervation. *P<0,05

P <0.05). Difference between groups 1, 2 and 3 indicates that injection of bone marrow aspirate cells after neurorapy stimulates more collagen formation than after injection of aspirate cells at 7 weeks after neuroraphy.

MUSCLE ATROPHY

Analysis of muscle fibers number in m.gastrocnemius shows a significant decrease in group 1 and group 2 (Fig. 3). Dynamics of change in groups 1 and 2 was similar, but in group 2 difference between the terms of 8 and 12 weeks (difference 27.5%; P <0.05), 12 and 16 weeks (difference 27.7%; P <0.05) was reliable. At 8 weeks, muscle fibers number in group 2 was significantly higher than in group 1 (difference 27.5%; P <0.05), at 12 and 16 weeks the difference in change was within the statistical error. This may indicate that injection of bone marrow aspirate cells immediately after injury partially prevents atrophy, but in subsequent stages of change progress.

In group 3, muscle fibers number was statistically lower compared to group of pseudooperated animals. Rate between groups 1 and 3 did not differ at 8-12 weeks, but statistically significant difference was found at 16 weeks of the experiment (P <0.05). Compared with group 2, figure

Table I. Results of biochemical studies of m.gastrocnemius, at the background of denervation

Group	TBARS nmol×mg-1	CAT μmol×min-1×mg-1	GPx nmol×min-1×mg-1	GR nmol×min-1×mg-1	SOD U×min-1×mg-1
8 weeks					
Shame	0.62±0.16	5.14±0.43	2.15±0.15	2.15±0.24	4.16±0.40
Group 1	1.11±0.17	3.88±0.31	3.88±0.37	3.17±0.35	4.95±0.54
Group 2	1.51±0.34	5.66±0.39*	4.03±0.72	3.40±0.46	5.18±0.46
Group 3	1.58±0.51	4.06±0.38^	3.60±0.27	4.19±0.30	4.66±0.35
12 weeks					
Shame	0.75±0.07	3.42±0.02	3.39±0.56	4.59±0.26	4.77±0.52
Group 1	4.33±0.78#**	5.17±0.29#	4.04±0.39	3.22±0.34	5.42±0.16
Group 2	0.97±0.29*	2.47±0.17*	2.15±0.21*#	2.56±0.62	4.10±0.29
Group 3	2.04±0.78*	2.98±0.20*	2.86±0.14	3.55±0.15	4.58±0.18
16 weeks					
Shame	0.66±0.13	2.16±0.37#	1.94±0.14	2.34±0.14	4.67±0.47
Group 1	5.89±1.94#**	3.62±0.41	3.3±0.50	3.85±0.43	5.37±1.02
Group 2	3.05±0.12*,**	4.37±0.17@**	3.05±0.37	3.46±0.23	4.01±0.22
Group 3	0.43±0.23*	2.29±0.32^#	1.96±0.24*#	2.10±0.28*#	5.22±0.31

* P <0.05 to group 1; # P <0.05 before 8 weeks; ** P <0.05 to the group of pseudooperated; ^ P <0.05 to group 2; @ P <0.05 to 12 weeks

was significantly lower at 8 weeks, no difference at 12 weeks, and muscle fibers number was significantly higher than in groups 1 and 2 at 16 weeks. This indicates that injection of bone marrow aspirate cells at 7 weeks after injury reduces dynamics of muscle fibers atrophy in the following terms.

Negative correlation was found between muscle fibers number and level of fibrosis in m.gastrocnemius. In group 1, a high negative dependence was found at 12 (r = -0.75; p = 0.001) and 16 (r = -0.84; p = 0.002) weeks; in group 2 high dependence on 8 (r = -0.73; p = 0.05), weak and medium on 12 (r = -0.26; p = 0.34) and 16 (r = -0.54 ; p = 0.03) weeks; in group 3 weak and medium correlation strength by 8 (r = -0.36; p = 0.18), 12 (r = -0.55; p = 0.03) and 16 (r = -0.33); p = 0.22). These results suggest an association of muscle fiber malnutrition with collagenogenesis in m.gastrocnemius, especially in group 1.

Difference in m.gastrocnemius morphometry was not limited to atrophic changes in individual muscle fibers. Additionally, analysis of muscle fibers diameter and fibers distribution by diameter (Fig. 4 and 5) was made. At the background of denervation, two manifestations of changes in muscle fibers were detected simultaneously: appearance of atypically thin, atrophic fibers (less than 40 microns in diameter) and hypertrophy of individual fibers (more than 160 microns in diameter). As can be seen from the diagrams (Fig. 5), in group 1 <5% of fine fibers were found in all terms of the experiment, and hypertrophied ≥6%. In the group of 2 ~ 5% of fine atrophic fibers were detected at 16 weeks, and hypertrophied fibers - 2.3%, 7.5% and 1.9% according to the terms of experiment. In group 3, relative number of atypically thin muscle fibers was <2%, and hypertrophied were recorded at 12 and 16 weeks (10.7% and <2%). That is, dynamics are similar. Analysis of muscle fiber distribution patterns indicates that in group 3, malnutrition develops at a slower rate than in groups 1 and 2, and hypertrophy is likely to be both a compensatory response to denervation and to injection of bone marrow aspirate cells. It should be noted that appearance of hypertrophied fibers was reflected in the morphometry of studied muscle samples, so the conclusions about action of aspirate cells were formed based on the assessment of several indicators (number, diameter and distribution of muscle fibers).

RESPONSE OF MUSCLE ANTIOXIDANT SYSTEM

Analysis of changes in thiobarbituric acid reactive substances (TBARS) was selected as an indicator of metabolic changes in muscle during injury. As can be seen from table I, level of TBARS increased relative to control at all times of the experiment. Marked trend of TBARS accumulation at 8 weeks increased to a statistically significant difference at 12 and 16 weeks (difference of 3.9 and 5.3 times; P <0.05). In group 2, level of TBARS differed from pseudooperated animals at 16 weeks (difference 4.5 times; P <0.05) and was significantly lower than group 1 (difference 48.1%; P <0.05). In group 3, the level of TBARS was significantly lower than in group 1 at 12 and 16 weeks and did not differ from pseudo-operated animals. These data indicate that lipid peroxidation products accumulate during muscle atrophy, which is an indicator of cell damage, and they decrease after injection of bone marrow aspirate cells, to a greater extent in group 3.

In this study, we found a decrease in catalase activity (CAT) in pseudooperated animals between 8 and 16 weeks. This muscle response has been evaluated as the response of tissues to surgical approach to sciatic nerve. In group 1, activation of CAT at 12 weeks and then decreased. In group 2, rate varied similarly to the group of pseudooperated

animals, but at 16 weeks a significant increase was found, which is probably a reaction in response to atrophy. In group 3, the dynamics is similar to those of pseudooperated animals, and the rate is statistically significantly lower than in group 2 at 8 and 16 weeks.

Glutathione peroxidase (GPx) activity did not differ significantly between groups. In group 2, difference compared to group 1 was observed at 12 weeks, and in group 3 at 16 weeks. In both groups, these values were probably lower than original. Glutathione reductase (GR) activity decreased only in group 3 at 16 weeks, as a manifestation of metabolic reactions restoration. Analysis of changes in superoxide dismutase (SOD) activity did not reveal an intergroup difference. Based on the analysis of described indicators, it was concluded that in group 3 metabolic changes are less striking compared to group 2 and especially group 1, both in the level of TBARS formation and response of the antioxidant system to surgery and denervation.

DISCUSSION

To date, some morphological and molecular mechanisms of skeletal muscle atrophy have been discovered [15]. Main morphological manifestation of muscle fiber atrophy is a decrease in its thickness, which in our study was estimated by fibers diameter. If skeletal muscle is affected at an early time of denervation and innervation is restored, stabilization of muscle morphology and even partial restoration of muscle fiber diameter can be achieved, as shown after muscle electrical stimulation [16]. However, amount of thin fibers can remain for a long time and this is a morphological manifestation of atrophy in denervated muscles.

Results show that both the percentage of fibrotic changes and atrophic muscle fibers are lower after injection of bone marrow aspirate cells 7 weeks after denervation, whereas early injection stimulate collagenogenesis. However, data on muscle fibers number at 8 weeks in groups 2 and 3 may indicate a delay in muscle atrophy in early stages of cell injection. Obviously, third observation period (16 weeks) is crucial and ultimately based on the comparison of muscle morphology, morphometry results and biochemical data, delayed route of administration can be considered as potentially favorable. We are not the first to find a similar relationship between the level of fibrosis and injection of bone marrow cells. Liu X, et al described a significant increase in fibrosis and muscle inflammation in contusive muscle injury [6]. Mesenchymal cell transplantation has caused infiltration of leukocytes and macrophages, increased levels of inflammatory cytokines and chemokines, matrix metalloproteinases and oxidative stress in muscles, ie processes associated with inflammatory muscle damage [6]. Under other conditions, injection of cells with fibrin did not have such consequences [17]. Obviously, opposite results in different works depend on the experimental model, for example, nerve transection and suturing or autografting [18]. In our own research, we obtained similar results. Injection of aspirate cells immediately after nerve injury was characterized by increased fibrosis, while injection of cells 7 weeks after denervation had no such effect. However, it should be borne in mind that the reference point in experiments is the period after nerve injury, while the time difference between cells injection in groups 2 and 3 is 7 weeks. We do not rule out that fibrosis may increase in the future, so we consider the error of this assessment, but in general, bone marrow aspirate cells injection exacerbated fibrosis.

Dynamics of muscle atrophy can be assessed not only by morphological methods, although changes in structure are the most convincing indicator. We found that the level of TBARS, as a product of lipid peroxidation, increases at all times. After bone marrow aspirate cells injection, their level decreased, which can be assessed as a manifestation of their utilization and inhibition of formation. In response to the level of TBARS, catalase activity increased, as the antioxidant system is activated in response to oxidative stress. At the same time, protective mechanisms of this system were more pronounced in group 3. These results should be taken into account when using bone marrow aspirate cells in regenerative medicine and traumatology.

CONCLUSIONS

Sciatic nerve injury results in atrophy of muscle tissue, which is partially delayed after delayed injection of bone marrow aspirate cells. Direct injection of aspirate cells caused fibrotic changes in the areas of atrophy, more than after late injection and without it. Muscle atrophy was characterized by a sharp increase in TBARS levels at 12 and 16 weeks and catalase activity at 12 weeks, and changes in biochemical parameters were partially normalized after the use of cell aspirates, to a greater extent with delayed injection.

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ORIGINAL ARTICLE

EVALUATION OF NLRP3 INFLAMMASOME PROTEIN EXPRESSION IN ULCERATIVE COLITIS

DOI: 10.36740/WLek202203113

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ABSTRACT

The aim: This study aimed to evaluate the NLRP3 immunoreactivity in ulcerative colitis in various clinical presentations.

Materials and methods: The retrospective study involved 80 formalin fixed paraffin embedded tissue blocks. These were divided into 50 samples of ulcerative colitis and 30 with normal colonoscopy findings. NLRP3 protein expression patterns were evaluated in various cellular components in tissue sections using immunohistochemistry method.

Results: NLRP3 inflammasome was significantly expressed with higher percentage among ulcerative colitis tissue sections compared with normal tissue sections. Intense staining was observed in Paneth cells at the base of crypts, inflammatory cells infiltrated between glands and near the base of crypts. Variable intensities of NLRP3 staining were observed in surface epithelial cells and glandular epithelium. Statically higher percentage of expression was found among active disease and patients with extra-intestinal complications.

Conclusions: The NLRP3 protein expression pattern was upregulated among various cellular compartments among ulcerative colitis and correlated with disease activity.

KEY WORDS: NLRP3 protein, immunohistochemistry,ulcerative colitis

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INTRODUCTION

The innate immune system activation is involved in immunological response mechanisms of inflammatory cells against invading pathogens. However, the Inflammasome protein complex composed from the nucleotide-binding domain and leucine-rich repeat (LRR)-containing (NLR) family and the pyrin and HIN domain (PYHIN) family [1]. NLRs are a family of intracellular innate immune recognition molecules [2]. These receptors found to play essential roles through interaction with microbes and environmental stimuli in mucosal immunity and inflammation [3]. NLRP3 is the best characterized member belongs to this family, its fusion with apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC), and caspase-1 to form large complex protein named as 'inflammasome' resulting in production of inflammatory cytokines IL-1β and IL-18 [4]. Several studies highlighted the pathogenic role of NLRP3 in experimental intestinal inflammation [5-6]. However, NLRP3 deficient mice experienced less sever pathological changes compared to wild type mice, indicating the essential role for IL-1B and IL-18 production could exacerbate DSS mice model [7]. Interestingly, mice deficient model of NLRP3 and ASC displayed higher rate of bacterial colonization, increased tissue pathology and sever weight loss, indicating early activation of NLRP3 and its role in prevention of bacterial colonization on intestinal epithelium as an essential innate immune defense mechanism as well as tissue repair after injury [8].

THE AIM

This study aimed to evaluate the NLRP3 immunoreactivity in ulcerative colitis in various clinical presentations.

MATERIALS AND METHODS

STUDY DESIGN AND SETTING

This case-control study involved 50 ulcerative colitis patients and 30 subjects with negative endoscopic findings. All subjects were recruited from three hospitals in Baghdad: The Gastroenterology and Hepatology Teaching Hospital, Medical city and Al-Emamain Al-Kadhemain medical city as well as private hospitals in the period March 2018- June, 2019. Diagnosis of cases was done according of the guideline of diagnosis and treatment of inflammatory bowel disease [9] in terms of clinical, endoscopic, radiographic and histopathological evaluation. All patients were either newly or previously diagnosed. Demographic data were collected through direct interview with the patient, and by seeking his/her hospital record as well as previous medical reports in addition to the score for UC.

NLRP3 IMMUNOHISTOCHEMISTRY STAINING

All tissue biopsies were processed as formalin fixed paraffin embedded tissue. They were cut into 5 micrometers on positive charge slide (Fisher brand). After removal of wax and sufficient rehydration step, antigen retrieval made by

Table I. Descriptive analysis of demographic and clinical parameters of study groups

		UC (n=50)	HC (n=30)	P value
Age (year)	Mean±SE	34.00±1.80	37.11±1.24	0.154^{NS}
Sex type	Female	26 (52.00%)	15 (50%)	0.778^{NS}
	Male	24 (48.00%)	15 (50%)	

NS: None statistically significant (p>0.05)

Table II. NLRP3 immunoreactivity pattern among study groups

Parameter		Ulcerative colitis (n=50)		Healthy control (n=30)		P value
NLRP3 %		**39.31±9.32**		**14.10±6.97**		**<0.001****
Intensity score	Negative	3	6.00%	3	10.00%	<0.001**
	Weak	9	18.00%	20	66.67%	
	Moderate	27	54.00%	5	16.67%	
	High	11	22.00%	2	6.67%	
Expression score	0	2	4.00%	5	16.67%	<0.001**
	1	6	12.00%	21	70.00%	
	2	29	58.00%	4	13.33%	
	3	9	18.00%	0	0.00%	
	4	4	8.00%	0	0.00%	
Combined score	Low expression (<6)	22	44.00%	26	86.67%	<0.001**
	High expression (7-12)	28	56.00%	4	13.33%	

**: High statistically significant (p<0.001)

autoclave for 3 minutes using citrate buffer pH: 6.0. Endogenous peroxidase was blocked, and protein block was added on slide, Cryopyrin antibody (orb182473) purchased from Biorbyte® diluted as 6 pg/ml of antibody diluents (orb90427) and added on slide (50 micoliter), and stained by Super Sensitive IHC Detection System Kit (orb219874) Biorbyte®, Counterstained by Myers hematoxylin (Dako). Both intensity score was classified as 0=negative, 1=weak, 2=moderate and 3 - strong. Staining score was classified as 0 (0%), 1 (1%–25%), 2 (26%–50%), 3 (51%–75%), and 4 (76%–100%). Then both scores were multiplied and patients were divided into two groups: 0-6= low expression group and 7-12: high expression group.

STATISTICAL ANALYSIS

Categorical data were formulated as count and percentage. Chi-square test was used to describe the association of these data. Alternatively, Fisher exact test was used if there is 25% of cells less than expected count. Numerical data were described as mean, and standard deviation. Independent sample t-test used for comparison between two groups. The lower level of accepted statistically significant difference is bellow or equal to 0.05, all statistical analysis was done using Graph Pad software Prism 7 ® Mac OSX version.

RESULTS

The results in table I showed age of patients and control, the mean age in ulcerative colitis patients was 34 years, while in controls - 37.11 years old. However, they were none statically different (p=0.154). There were more representatives of females in the study: 26 (52%) in ulcerative colitis group, and 15 (50%) among controls. The results showed none statistically significant difference in the frequency of sex types among study groups (table I).

NLRP3 INFLAMMASOME IS UPREGULATED IN ULCERATIVE COLITIS MUCOSA

The results in table II describes the NLRP3 protein expression among study groups, in terms of relative percentage, intensity score, expression score and combined score. It was found that NLRP3 over-expressed in ulcerative colitis (39.31±9.32), while, in controls were low (14.1±6.97) p<0.001. It was noted that intensity score was almost moderate to high in ulcerative colitis, while being weak and negative in controls (p<0.001). In addition to that, higher immunoreactivity scores were prevalent in ulcerative colitis in contract to controls the lower scores were reported. The combined score (combined intensity and expression score) was high among 28 (65%) in ulcerative colitis, while it was inversely reported (lower expression) in controls 4/30 (13.33%). Furthermore, in ulcerative colitis patients with left sided lesions (49.4%) there was higher percentage of expression than in those with extensive colitis (38.9%) p=0.038.

NLRP3 protein expression localized polyclonal rabbit anti-Cryopyrin (orb182473) diluted as 6 pg/ml, visu-

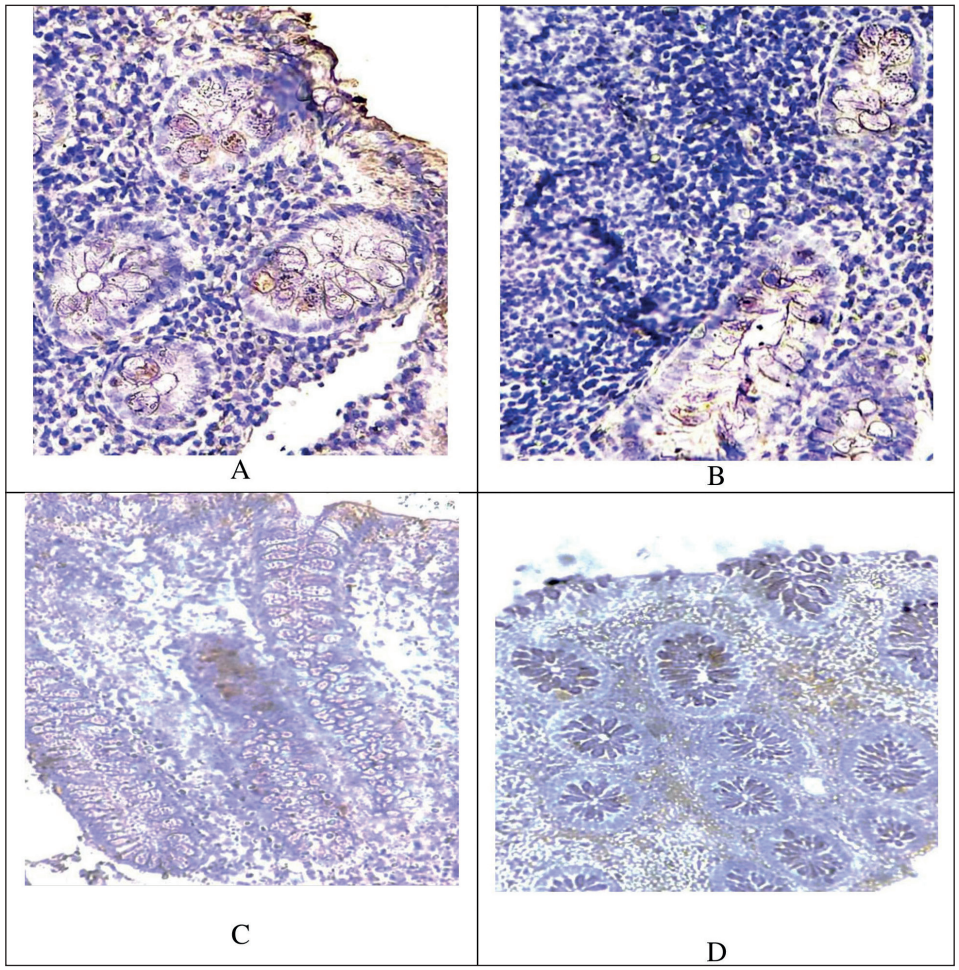

Fig. 1. NLRP3 protein expression localized polyclonal rabbit anti-Cryopyrin (orb182473)

alized by Super Sensitive peroxidase Detection System Kit (orb219874) A-C, Showing cytoplasmic staining of glandular epithelium and dark cytoplasmic and nuclear staining of infiltrated inflammatory cells D. Diffuse cytoplasmic staining between glands, original magnification (400X) (fig 1).

DISCUSSION

Its widely accepted that inflammasome activation is a prominent feature in inflammatory bowel disease [10]. In this study, NLRP3 protein was expressed in the majority of ulcerative colitis and controls suggesting an active role of inflammasome protein in the pathogenesis of disease. In argument with our results, several human studies highlighted the association of certain genetic polymorphism (s) in NALP3 and CARD8with UC such as: rs35829419 (Q705K) and rs10754558 as a risk factor for developments of UC leading to elevation of IL-18 and T-helper 1 inflammation in colonic mucosa of inflamed area [11-14]. Pathogenic role of IL-1b have been reported in inflammatory bowel disease suggesting its possible therapeutic target [15]. Furthermore, study by Liu, et al., in 2017 suggested an abnormal activation in NLRP3 protein that plays an important pathogenic role in chronic colitis IL-10 mice and humans [5]. It was found that NLRP3 protein higher expression is found in active disease and patients with extra-intestinal complications. This finding was originally reported in the current study, suggesting a predictive role of worse outcome in patients [5]. It`s argued by Ranson, et al., who in 2018 also reported upregulation of NLRP3 expression in active form of IBD16. Further details observed via exploring role of IL-18 and IL-1b in Nlrp3$^{-/-}$mice is protected in the acute DSS colitis model [17]. The current study highlighted the importance of NLRP3 targeting as a therapy for IBD patients. Study by Bauer et al., suggested involvement of inhibiting NF-κB activation and decreasing mitochondrial reactive oxygen species. This supports our recommendation that future targeting of inflammasome will help to reduce inflammation and its development into sever and poor outcome of disease.

CONCLUSIONS

The NLRP3 protein expression pattern was upregulated among various cellular compartments among ulcerative colitis and correlated with disease activity.

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ORIGINAL ARTICLE

POSSIBILITIES OF METABOLIC AND FUNCTIONAL DISORDERS CORRECTION IN OSTEOARTHRITIS WITH COMPLEX COMORBIDITY

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ABSTRACT

The aim: To assess the impact of complex metabolic therapy of primary osteoarthritis and type 2 diabetes mellitus under conditions of comorbidity on the course and progression of these pathologies. Patients with comorbidities of primary osteoarthritis and diabetes mellitus are a special group of patients because the importance of these comorbidities is the additional difficulty in diagnosing and conducting adequate therapy, given the close etiopathogenetic links of these conditions, which leads to poor quality of life, increased costs of diagnosis and treatment, increasing the frequency and duration of hospital stay.

Materials and methods: We examined 67 patients with primary osteoarthritis in comorbidity with diabetes mellitus. Patients were comparable by clinical, gender criteria, the severity of primary osteoarthritis, and treatment received and were divided into two groups: 1st group (n=32) - patients received treatment for OA and diabetes mellitus in accordance with international recommendations; 2nd group (n=35) - patients received treatment as in group 1 + drug alpha-lipoic acid. Determination of the level of the studied parameters was performed before and after treatment.

Results: The analysis of the obtained results revealed statistically significant positive dynamics after treatment for symptoms of primary osteoarthritis in both study groups of patients ($p<0.05$), but the therapeutic effect in the 2nd group was more significant ($p<0.05$). There was a statistically significant positive dynamics on the scale of VAS at rest and movement ($p<0,05$), WOMAC index for pain, stiffness, and functional insufficiency ($p<0,05$), and Leken index in the 2nd group after treatment compared with the 1st ($p<0.05$).

Conclusions: The obtained results indicate a statistically significant positive effect of alpha-lipoic acid on the course and progression of primary osteoarthritis under conditions of comorbidity with diabetes mellitus.

KEY WORDS: primary osteoarthritis, diabetes mellitus, exocrine pancreatic insufficiency, family medicine

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INTRODUCTION

Osteoarthritis (OA) is one of the most common diseases of the joints, which is accompanied by high comorbidity, which greatly complicates the drug therapy of this disease. Diseases of the pancreas with a high frequency are detected as a comorbid pathology in primary OA [1-3]. Most diseases of the pancreas are considered diseases affecting both the secretory and incretory parts of the pancreas, leading to the development of chronic pancreatitis (CP) and diabetes mellitus (DM) [3-5]. The problem of diabetes is becoming increasingly important, as this disease is one of the world's most common chronic diseases. Type 2 diabetes (DM2) is now seen as a social problem. This is due to the fact that there is an increase in the number of people suffering from diabetes and an increased risk of developing various complications [5-7]. Exocrine pancreatic insufficiency (EPI) often develops on the background of DM2, which worsens the course of comorbid pathologies, as well as complicates the choice of treatment tactics [3, 5, 7]. Patients with comorbidities of primary OA and diabetes mellitus are a special group of patients because the importance of these comorbidities is the additional difficulty in diagnosing and conducting adequate therapy, given the close etiopathogenetic links of these conditions, which leads to poor quality of life, increased costs of diagnosis and treatment, increasing the frequency and duration of hospital stay [5-7].

THE AIM

The aim of the study was to assess the impact of complex metabolic therapy of primary OA and DM2 under conditions of comorbidity on the course and progression of these pathologies.

MATERIALS AND METHODS

We examined 67 patients with primary OA in comorbidity with diabetes mellitus, who were on outpatient treatment at the Ternopil Center for Primary Health Care during 2019-2020. The average age of patients was (58.56±7.97) years (from 28 to 79 years); there were 34 women (50.75%) and 33 men (49.25%). The control group consisted of 30 healthy people. Exclusion criteria were cancer, acute and exacerbation of chronic pathologies of vital organs, type 1 diabetes, active gastric and duodenal ulcers, viral hepatitis

Table I. Dynamics of symptoms of primary OA in the study groups before and after treatment

Symptom of OA	Comparison group				
	Control (n=30)	1st group (n=32)		2nd group (n=35)	
		Before treatment	After treatment	Before treatment	After treatment
VAS index, calm, mm	1,11±0,12	35,54±3,76 p_{1-2}<0,05	29,16±1,54 p_{2-3}<0,05	34,41±2,88 p_{1-4}<0,05	21,54±1,28 p_{4-5}<0,05
VAS index, movement, mm	2,12±0,43	48,65±3,48 p_{1-2}<0,05	39,71±1,88 p_{2-3}<0,05	49,17±3,69 p_{1-4}<0,05	31,65±1,75 p_{4-5}<0,05
WOMAC index, pain, points	0,79±0,09	16,85±1,97 p_{1-2}<0,05	13,26±1,47 p_{2-3}<0,05	17,01±1,71 p_{1-4}<0,05	10,59±1,22 p_{4-5}<0,05
WOMAC index, stiffness, points	0,12±0,02	5,58±0,89 p_{1-2}<0,05	4,18±0,12 p_{2-3}<0,05	5,69±0,83 p_{1-4}<0,05	3,89±0,15 p_{4-5}<0,05
WOMAC index, func. insufficiency, points	1,15±0,03	43,77±3,43 p_{1-2}<0,05	37,95±2,03 p_{2-3}<0,05	43,81±3,77 p_{1-4}<0,05	32,14±2,08 p_{4-5}<0,05
WOMAC index, total, points	2,38±0,05	72,89±5,23 p_{1-2}<0,05	65,77±2,19 p_{2-3}<0,05	73,53±5,02 p_{1-4}<0,05	61,22±3,07 p_{4-5}<0,05
Leken index, points	0,21±0,04	6,76±0,97 p_{1-2}<0,05	5,54±0,54 p_{2-3}<0,05	6,51±0,88 p_{1-4}<0,05	4,63±0,52 p_{4-5}<0,05

Notes:
1. p_{1-2} , p_{1-4} – statistically significant difference between groups in relation to the control group;
2. p_{2-3}, p_{4-5} – statistically significant difference in relation to their group before treatment.

and liver cirrhosis, Crohn's disease, nonspecific ulcerative colitis, cystic fibrosis.

The materials of the clinical study were considered at the meeting of the commission of bioethics of I. Horbachevsky Ternopil National Medical University Minute № 60 from 01.09.2020 The work was carried out in accordance with the Code of Ethics of the Declaration of Helsinki. All patients signed an information agreement to participate in the study.

The diagnosis of OA was established on the basis of diagnostic criteria of the International Association for the Study of OA (OARSI (2019)), the American Association of Rheumatologists (ACR (2020), and the European Association of Rheumatologists (European League Against Rheumatism, EULAR, 2017). Examination of the joints included examination, palpation, and objective assessment of pain at rest and during VAS movements. OA symptoms were also assessed by the WOMAC index (Western Ontario and McMaster University) and the Leken index. Radiological stages of OA were evaluated according to the classification of J.H. Kellgren and J.S. Lawrence.

The diagnosis of diabetes mellitus 2 was verified by the Order of the Ministry of Health of Ukraine from № 1118 from 21.12.2012 "On approval and implementation of medical and technological documents for standardization of medical care for type 2 diabetes". Fecal α-elastase was determined by an enzyme-linked immunosorbent assay. Also, to determine the presence and depth of reduction of exocrine function of the pancreas and concomitant enterocolitis was evaluated coprogram on a 5-point scale. To diagnose incretory insufficiency of the pancreas used to determine the level of fasting blood glucose, glycosylated hemoglobin (HbA1c) (using a kit for rapid determination of HbA1c by ion-exchange chromatography), and the HOMA-IR index, calculated by the formula:

HOMA = (fasting glucose (mmol/l) × fasting insulin (μIU/l))/22.5.

The assessment of the state of the pancreas was performed according to the parameters of ultrasound examination of the pancreas, which was summarized to determine the severity of the process according to the criteria of the Marseille-Cambridge classification of CP in points.

Patients were comparable by clinical, gender criteria, the severity of primary OA, and treatment received and were divided into two groups:

1st group (n=32) - patients received treatment for OA and diabetes mellitus in accordance with international recommendations;

2nd group (n=35) - patients received treatment as in group 1 + drug alpha-lipoic acid by intravenous administration of the drug at a dose of 20 ml per day, corresponding to 600 mg of alpha-lipoic acid 1 time per day for 2 weeks. After this course, patients orally took the drug alpha-lipoic acid 2 capsules (600 mg) 1 time per day for 4 weeks. Determination of the level of the studied parameters was performed before and after treatment.

The correspondence of the data distribution of the clinical study to the law of normal distribution was checked by the Kolmogorov-Smirnov test. The arithmetic mean and standard error (M±m) were used to describe the data in the normal distribution. Because the data obtained from the clinical study had deviations from the normal distribution of the variation series, we used nonparametric statistical methods to compare the groups - Mann-Whitney U-test (for independent groups)

Table II. Dynamics of indicators of the functional state of the software in the study groups before and after treatment

Indicator functional state of the pancreas	Comparison group				
	Control (n=30)	1st group (n=32)		2nd group (n=35)	
		Before treatment	After treatment	Before treatment	After treatment
α-elastase, μg/g	251,63±5,28	132,77±4,65 p_{1-2}<0,05	135,36±4,54 p_{2-3}>0,05	129,21±4,97 p_{1-4}<0,05	144,81±3,58 p_{4-5}<0,05
Blood glucose, mmol/l	4,14±0,12	7,61±0,51 p_{1-2}<0,05	7,53±0,44 p_{2-3}>0,05	7,87±0,49 p_{1-4}<0,05	6,49±0,35 p_{4-5}<0,05
HbA1c, %	4,57±0,12	7,25±0,87 p_{1-2}<0,05	7,06±0,47 p_{2-3}>0,05	7,11±0,31 p_{1-4}<0,05	6,59±0,21 p_{4-5}<0,05
HOMA index	1,47±0,11	3,54±0,19 p_{1-2}<0,05	3,48±0,12 p_{2-3}>0,05	3,37±0,11 p_{1-4}<0,05	3,11±0,12 p_{4-5}<0,05
Coprogram, points	0,12±0,03	5,89±0,69 p_{1-2}<0,05	5,15±0,33 p_{2-3}>0,05	5,68±0,38 p_{1-4}<0,05	4,73±0,29 p_{4-5}<0,05
Ultrasound indicator of pancreas structure, points	1,06±0,02	5,67±0,42 p_{1-2}<0,05	5,23±0,36 p_{2-3}>0,05	5,47±0,59 p_{1-4}<0,05	4,72±0,39 p_{4-5}<0,05

Notes:
1. p_{1-2}, p_{1-4} – statistically significant difference between groups in relation to the control group;
2. p_{2-3}, p_{4-5} – statistically significant difference in relation to their group before treatment.

and Wilcoxon test (for dependent groups). We used the software and mathematical complex for the personal computer "Microsoft Exel 2016" (Microsoft) and computer programs for statistical analysis and data processing "STATISTICA® 8.0".

RESULTS

The analysis of the obtained results revealed statistically significant positive dynamics after treatment for symptoms of primary OA in both study groups of patients (p<0.05), but the therapeutic effect in the 2nd group was more significant (p<0.05). There was a statistically significant positive dynamics on the scale of VAS at rest and movement (p<0,05), WOMAC index for pain, stiffness, and functional insufficiency (p<0,05), and Leken index in the 2nd group after treatment compared with the 1st (p<0.05) (Table I).

According to the indicators of the functional state of the pancreas in the 1st group, no statistically significant improvement was found for any indicator, but there was a positive trend (p> 0.05) (Table II). In the 2nd group after treatment, there was a statistically significant increase in the level of fecal α-elastase (p<0,05), there was also a statistically significant decrease in blood glucose (p<0,05) and a statistically significant decrease in the level of glycated hemoglobin and HOMA index (p<0.05), a similar statistically significant trend was observed for the scores of the coprogram and ultrasound criteria of the pancreas structure (p<0.05) (Table II).

DISCUSSION

An important result of our study is that the use of alpha-lipoic acid in patients with comorbidity of primary OA and DM2 contributes to the positive dynamics of symptoms of both diseases. The most studied is the effect of exogenous alpha-lipoic acid in DM [7]. According to the results of many studies, alpha-lipoic acid when taken orally or intravenously has a positive effect on various conditions in which oxidative and inflammatory processes are involved [8, 9]. Against the background of diabetes, oxidation leads to the development of hyperglycemia, which, in turn, causes even more pronounced oxidative processes, which in turn causes the development of a vicious pathophysiological cycle. Alpha-lipoic acid, by inhibiting oxidation, is able to break this cycle, and this makes it a promising tool in the treatment of prediabetes and diabetes [10, 11]. Understanding the structure of alpha-lipoic acid and its use as an exogenous drug makes it possible to consider alpha-lipoic acid in the treatment of diabetes and diabetic peripheral neuropathy [12]. However, we have not found studies aimed at studying the pathogenetic mechanisms of the influence of alpha-lipoic acid on the course of primary OA under conditions of comorbidity with DM2, which makes our work relevant.

CONCLUSIONS

The use of alpha-lipoic acid in the complex treatment of patients with comorbidity of primary OA and DM2 has a positive effect on the course of both comorbid pathologies (p<0.05). In the group of additional appointment of alpha-lipoic acid was found a statistically significant decrease in the index of VAS at rest and in movement (p<0,05), statistically significantly decreased WOMAC index in the group with additional appointment of alpha-lipoic acid after treatment compared with the group protocol treat-

ment was found (p<0.05). There was also a statistically significant decrease in the Leken index in the group with the additional prescription of alpha-lipoic acid compared with the group in which only protocol treatment was used (p<0,05). The obtained results indicate a statistically significant positive effect of alpha-lipoic acid on the course and progression of primary OA under conditions of comorbidity with diabetes mellitus (p<0.05). There was also a statistically significant positive effect of alpha-lipoic acid on the indicators of exocrine and endocrine functions of the pancreas (p<0.05), which indicates the feasibility of using alpha-lipoic acid in the treatment of patients with primary OA and diabetes mellitus under comorbidity conditions.

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ORIGINAL ARTICLE

EFFECT OF IMUNOFAN INFLUENCE ON THE STRUCTURE OF THE TESTES, HORMONAL AND CYTOKINE PROFILE OF IMMATURE EXPERIMENTAL ANIMALS

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ABSTRACT

The aim: It was the establishing the features of changes in the structure of the testes of experimental animals, as well as immunological, hormonal and cytokine parameters of blood plasma during stimulation**.**

Materials and methods: The study was carried out on 60 white male immature rats. Imunofan was used at a dosage of 50 μg. The organs were weighed, the relative mass was calculated, and the linear dimensions were determined. The morphometriv parameters of the epitheliospermatogenic layer were measured. The number of supporting cells and interstitial endocrinocytes was counted, as well as the volume of cell nuclei. The level of reproductive hormones in the plasma and the concentration of cytokines were determined.

Results: The results obtained indicate the development of readaptation processes in the testes after the use of the Imunofan against the background of environmental immunosuppression. The ability of the drug to stimulate the production of cytokines and hormones normalizes the function of immunocompetent cells, which is manifested in the stabilization of the immune homeostasis of the testes.

Conclusions: In response to the immunostimulating effect of Imunofan, a pronounced reaction is observed on the part of the testes of immature animals, which is due to the sensitivity of morphogenetic processes in the organ to external influences and the formation of mechanisms of their regulation, characteristic of this period of ontogenesis.

KEY WORDS: testis, immunostimulation, imunofan, rats, reproductive hormones, cytokines

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INTRODUCTION

Based on comprehensive studies from around the world, WHO has concluded that children's health is significantly affected by environmental phenomena caused by climate change, the presence of persistent organic pollutants, ozone degradation and declining natural flora and plankton diversity. Among the significant consequences for the health of the child is a decrease in immunity [1]. In recent years, there has been an increase in the number of diseases associated with impaired functioning of the immune system, caused by a sharp deterioration in the ecological state of the environment [2, 3]. At the same time, a special position among the causes of this problem is occupied by immunodeficiency states, the occurrence of which is associated with the increasing influence of unfavorable anthropogenic factors, a significant increase in immune-dependent pathological conditions and allergies [4, 5]. It is known that any effect on the body changes its homeostasis, which leads to a complex reaction, primarily from the regulatory systems - nervous, endocrine and immune, which naturally causes morphofunctional rearrangements in all organs that are not directly involved in the processes of modulating the response to environmental changes, including testes [6]. It is known that the critical periods of ontogenesis include long periods of childhood and puberty, which are characterized by increased sensitivity and vulnerability of the organs of the reproductive system to exo- and endogenous influences. However, today, against the background of the abundance of scientific information regarding the clinical and pathogenetic features of the course of certain diseases of the male reproductive system, there are practically no results of studying the problem of the structure of the gonads in the state of immunostimulation, the need for which is justified by the above problems, especially in childhood and puberty. In this regard, the aim of this study was to establish the features of changes in the structure of the testes of experimental animals, as well as immunological and hormonal parameters of blood plasma during stimulation.

THE AIM

It was the establishing the features of changes in the structure of the testes of experimental animals, as well as immunological, hormonal and cytokine parameters of blood plasma during stimulation**.**

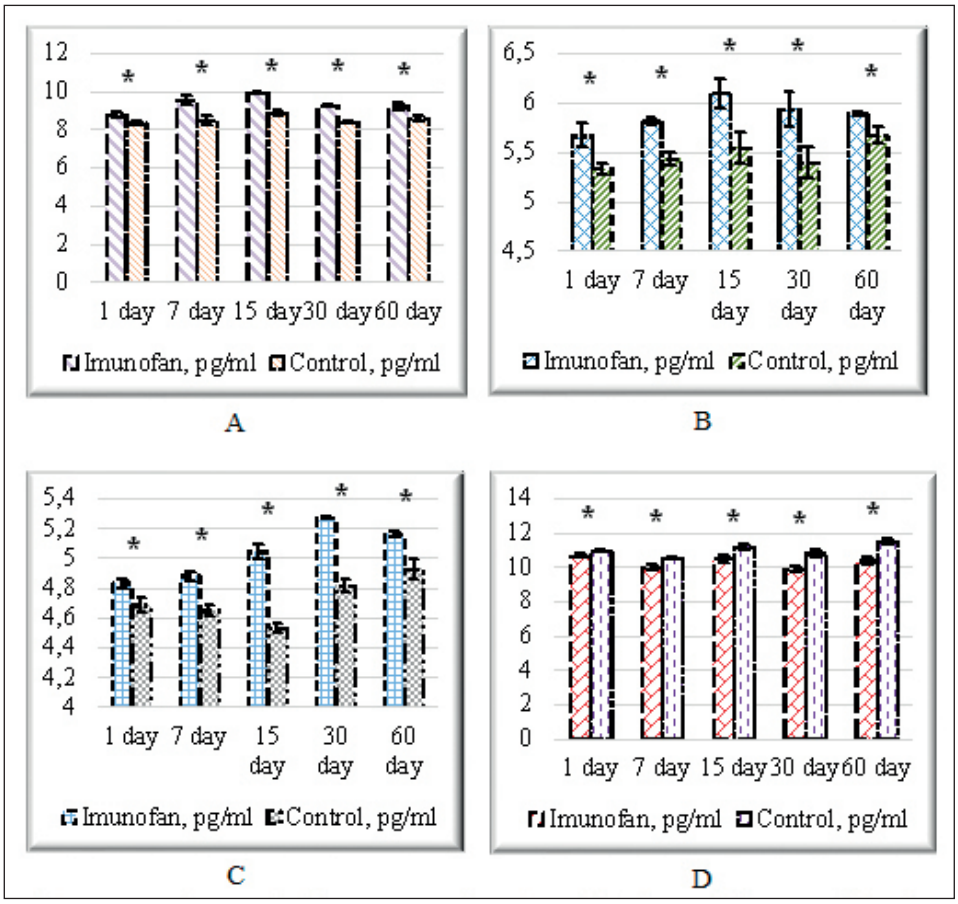

Fig. 1. Changes in the concentration of IL-1β (A), IL-2 (B), IL-6 (C) and TNF-α (D) in the blood plasma of immature rats after application of Imunofan and in the control. * - significant difference from the control data ($p < 0.05$).

MATERIALS AND METHODS

The study was carried out on 60 white outbred male immature rats. When working with animals, we were guided by the current ethical standards [7]. The study protocol was approved by the ethical committee of the Saint Luka Lugansk State Medical University (Protocol No. 1 dated 04/10/2019). To create a model of an immunostimulated state, we used Imunofan, a representative of the IV generation of thymic hormone derivatives. The drug was administered on days 1, 3, 5, 7, 9 of the experiment at a dosage of 50 μg. Rats receiving 0.9% sodium chloride solution in equivalent volumes according to the same scheme served as control. The animals were taken out from the experiment 1, 7, 15, 30 and 60 days after the cessation of drug administration. The organs were weighed, the relative mass was calculated, and the linear dimensions were determined: length, width, and thickness. The volumetric index was calculated using the formula for the volume of an ellipsoid of revolution:

$$V = \frac{\pi ABC}{6},$$

where A is length, B is width and C is thickness.

After standard histological examination, sections of the testes were stained with hematoxylin-eosin and photographed using an automated morphometric complex. At the light-optical level, the larger and smaller diameters, the area of the convoluted seminiferous tubule, the height and area of the epitheliospermatogenic layer were measured. The number of supporting cells and interstitial endocrinocytes was counted per unit area (1725 μm^2), as well as the volume of cell nuclei as an indicator of their functional activity. The spermatogenesis index (I) was calculated using the formula:

$$I = \frac{\Sigma A}{n},$$

where A is the number of rows of spermatogenic cells at different stages of development found in each tubule; n is the number of studied tubules.

The level of hormones in the plasma of peripheral blood, as well as the concentration of mediators of intercellular interaction IL-1β, IL-2, IL-6 and TNFα were determined by enzyme immunoassay. An enzymatic conjugate "Rat Elisa Kit", Elabscience (USA), with antibodies to follitropin, luteinizing hormone and testosterone was added to the studied blood sample, followed by calibration of the solution.

The data obtained were processed using a licensed statistical program StatSoft Statistica 12. The Mann-Whitney U-test was used to assess the statistical significance of the differences. Differences were recognized as statistically significant when the level of reliability (p) was more than 95.0% ($p < 0.05$), in other cases the differences were recognized as statistically insignificant ($p > 0.05$).

RESULTS

After the use of Imunofan, the dynamics of the levels of intercellular interaction mediators in the blood plasma of immature rats was found, indicating the development

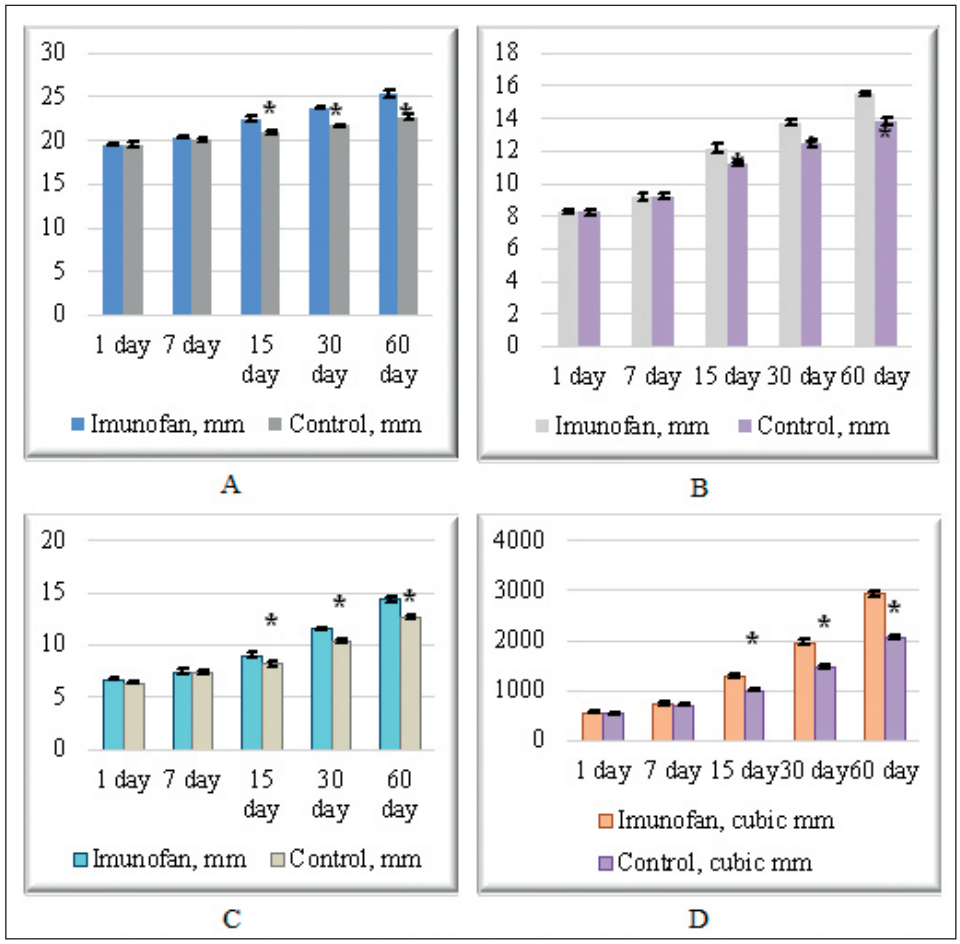

Fig. 2. Changes in the length (A), width (B), thickness (C) and volume (D) of the testes of immature rats after the use of Imunofan and in the control. * - significant difference from the control data ($p < 0.05$).

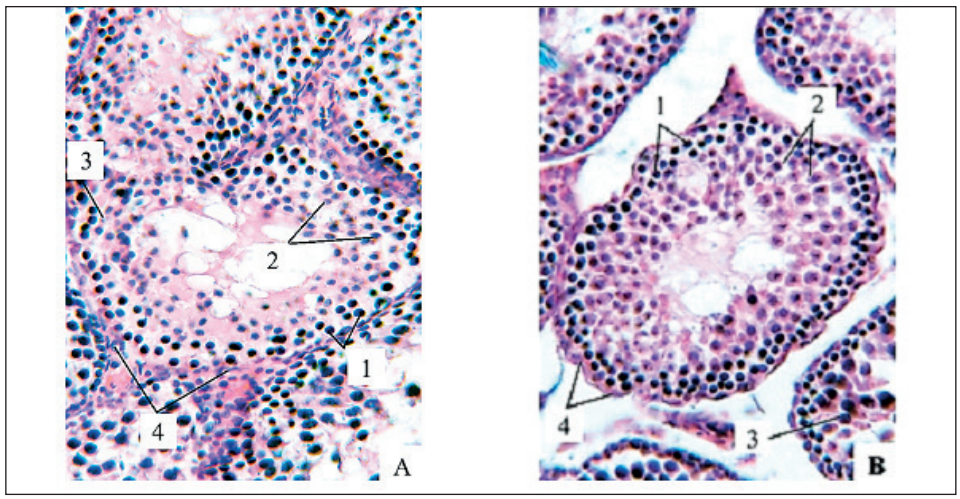

Fig. 3. Fragment of the testis of immature animals on the 30th day of observation: A - in the control; B - after using Imunofan. 1 - spermatogonia, 2 - spermatocytes, 3 - sustentocyte, 4 - tubule wall. Hematoxylin-eosin staining. Magnification: Approximation: Zoom 18.5; lens: PlanCN 40x / 0.65 ∞ / 0.17 / FN22.

of the state of immunostimulation throughout the entire observation period (Figure 1).

The general plan of the structure of the testes of immature animals after the administration of the drug retained typical morphological features: the gonads were covered with a thin connective tissue capsule, the convoluted seminiferous tubules, predominantly of a rounded shape, were densely packed in the lobules of the organ, on the histological section many did not have a lumen and were lined with rows of cells of the epitheliospermatogenic layer without presence of mature sperms.

At the same time, after the administration of Imunofan, changes in the absolute and relative weight of the testes of immature animals were revealed. Thus, a statistically significant increase in these indicators was observed on 15, 30 and 60 days by 9.38%, 10.13%, 11.00% and 5.94%, 6.07%, 6.83%, respectively. The linear-volumetric parameters of the gonads changed in the same way (Figure 2).

Administration of imunofan also caused changes in the micromorphometric parameters of the testes. Thus, the larger and smaller diameters of the convoluted seminiferous tubules increased statistically significantly on days 15,

30 and 60 of observation, the positive dynamics was 7.92%, 13.44%, 14.69% and 8.05%, 10.06%, 12.66% respectively.

The immunostimulating effect was also reflected in the sizes of the tubule and epitheliospermatogenic layer areas. The height of the germinal epithelium was statistically significantly higher than the control values in the same observation period by 10.68%, 11.75% and 16.07%, respectively. The spermatogenesis index, which reflects the functional activity of the testes, increased under conditions of immunostimulation. Thus, the indices of the control groups of immature animals after 7, 15, 30 and 60 days were statistically significantly lower than those of the rats receiving Imunofan, by 4.02%, 7.58%, 10.40% and 15.91%, respectively (Fig. 3).

When determining the number of sustentocytes and endocrinocytes, an increase was noted during immunostimulation in both indicators on the 15th, 30th and 60th days of observation in comparison with the data of the control groups of animals by 7.00%, 13.81%, 14.63% and 8.02%, 11.41%, 14.34% respectively. The volume of the nuclei of Sertoli and Leydig cells changed in a similar way. The positive dynamics was 5.19%, 7.33%, 10.27%, 14.51% and 4.68%, 9.70%, 12.00%, 14.29% at 7, 15, 30 and 60 days of immunostimulation.

The study of the parameters of the gonads of immature animals did not reveal statistically significant differences between the data of the experimental and control groups at the early stages of observation (1 and 7 days).

The immunostimulating effect, simulated by the introduction of Imunofan, led to moderately pronounced shifts in the hormonal parameters of the blood plasma of immature animals. Thus, an increase in testosterone concentration was found on the 15th, 30th and 60th days of observation after the end of the drug administration by 5.42%, 7.92% and 11.22%, respectively.

At the same time, there was a slight decrease in the level of luteinizing hormone by 4.85% (7 days), 7.31% (15 days), 7.63% (30 days) and 9.05% (60 days). On 15, 30 and 60 days after the cessation of the administration of Imunofan, a moderate decrease in the concentration of follitropin in the blood plasma was found by 7.43%, 8.75% and 9.77%, respectively.

DISCUSSION

It has been proven that immunosuppressive effects change the morphogenesis of the testes, which is accompanied by their dysfunction in the form of impaired spermatogenesis up to azoospermia [9, 10]. The results obtained indicate the development of readaptation processes in the testes after the use of the immunomodulator against the background of environmental immunosuppression, which is reflected in the prevalence of the morphometric parameters of the studied organ over the data of the control groups of animals. So, J. Gold and V. Vardhani in studies on mice proved an increase in the level of testicular DNA after the use of an immunostimulant [11]. This can be explained by the properties of imunofan to increase the resistance of the genetic material of cells to unfavorable exogenous influences. The ability of the drug to stimulate the production of cytokines normalizes the function of immunocompetent cells, which is manifested in the stabilization of the immune homeostasis of the testes. At the same time, the long-acting phase of Imunofan is accompanied by an intense antioxidant and detoxifying effect, which prevents damage to actively dividing gonadal cells. The development of such changes, apparently, is associated with the normalization of metabolic processes in the body of animals, including restoration of the balance of redox reactions, optimization of anabolic and catabolic processes, and adaptation of regulatory systems to disorganizing exogenous influences [12]. Optimization of the parameters of the antioxidant system and cell lipid peroxidation, as well as the membrane stabilizing effect resulting from prolonged use of Imunofan, stimulated the activation of proliferative and synthetic processes, which was manifested by an increase in the index of spermatogenesis, as well as linear-volumetric and micromorphometric parameters of the testes. Research conducted by L.M. Yaremenko et al. proved that the systemic use of Imunofan led to the normalization of the expression of neuronal cytoskeleton proteins and an increase in the amount of mRNA encoding them [13]. A similar effect can take place among germ cells of the reproductive system, mediating an increase in the stability of mitotic and intracellular transport processes. At the same time, the dynamics of the hormonal parameters of blood plasma indicates the active involvement of systemic endocrine mechanisms in the regulation of testes morphogenesis during immunostimulation at a later follow-up period. Changes in the concentration of the above regulators were reflected both in the intensity of the processes of differentiation of germ cells and in the activity of sustentocytes and interstitial endocrinocytes. The effect of the drug on the levels of cytokines in blood plasma is associated with its effect on the morphogenesis of the organs of the reproductive system, which is determined by the property of imunofan to change the formation and concentration in the cytoplasm of immunocompetent cells of secondary messengers: cAMP, inositol-1,4,5-triphosphate, diacylglycerol, calcium ions and nitric oxide (II) with subsequent signal transmission to various inductors. Imunofan is able to stimulate the synthesis of group E prostaglandins, the action of which is probably realized through cGMP. Also, cytokines acting on sustentocytes regulate the formation of integral proteins in their plasmolemma that control endocytosis processes using clathrin-mediated mechanisms, which optimizes the activity of the blood-testis barrier.

CONCLUSIONS

1. In response to the immunostimulating effect of Imunofan, there is a pronounced reaction of the testes of immature animals, which is due to the sensitivity of morphogenetic processes in the organ to external influences and the formation of mechanisms of their regulation, characteristic of this period of ontogenesis.

2. The most intense immunostimulating, membrane stabilizing and detoxifying effect of Imunofan was observed on the 15th, 30th and 60th days of observation, which was confirmed by the statistically significant dynamics of morphometric parameters of the organ.
3. Micromorphometric data of the testes supplement the results obtained at the macroscopic level at the later stages of observation after the use of Imunofan and indicate the full participation of all structural components of the organ in its response to immunostimulation.
4. The established dynamics of the hormonal parameters of the blood plasma of animals suggests the development of a systemic endocrine reaction in response to the use of imunofan, which indicates a close integration of the immune and humoral mechanisms of regulation of the morphogenesis of testes at immature age.
5. The data obtained allow us to conclude about the positive effect of immunostimulation on morphogenetic processes in the testes in childhood, which must be considered when carrying out the immunotropic therapeutic and prophylactic measures in practical pediatric health care.

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ORIGINAL ARTICLE

FUNCTIONING OF NO-CYCLE IN THE ORAL FLUID IN CHILDREN OF PRIMARY SCHOOL AGE WITH TYPE 1 DIABETES MELLITUS IN THE TREATMENT OF CHRONIC CATARRHAL GINGIVITIS

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ABSTRACT

The aim: To determine the activity of NO-synthase and arginase in oral fluid in children with type 1 diabetes mellitus and to evaluate the efficacy of the treatment scheme we elaborated in the treatment of chronic catarrhal gingivitis.

Materials and methods: 82 children were examined, they were divided into groups by presence of gingivitis and diabetes mellitus. NO-synthase (NOS) activity was determined in oral fluid by the difference in nitrite concentration before and after incubation. The arginase activity was determined in oral fluid by the difference in the concentration of L-ornithine before and after incubation.

Results: Use our treatment scheme in children with chronic catarrhal gingivitis and type 1 diabetes mellitus lead to a change in the polarization of oral macrophages towards the predominance of M2 polarization in 1 month. The polarization of macrophages changed to the predominance of M1 polarization activity in 1 year.

Conclusions: We have elaborated a scheme for the treatment of chronic catarrhal gingivitis in children with type 1 diabetes mellitus. It normalizes the polarization of oral macrophages caused by exposure to chronic catarrhal gingivitis as a local pathogenetic factor.

KEY WORDS: type 1 diabetes mellitus, macrophages, gingivitis, iNO-synthase, arginase

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INTRODUCTION

Diabetes mellitus is one of the most common metabolic diseases. According to the International Diabetes Federation (IDF), the number of people with diabetes will exceed 500 million by 2030. Local changes in periodontal tissues in persons with diabetes mellitus are characterized by increased production of reactive oxygen species, and pro-inflammatory cytokines, which are based on the accumulation of glycation products and their active interaction with receptors [1, 2]. An increase in the level of pro-inflammatory cytokines leads to the development of oxidative stress and stimulation of further inflammation in periodontal tissues. One of the central roles in the development of inflammation is played by macrophages. They have either pro-inflammatory M1 or anti-inflammatory M2 phenotype. It depends on the microenvironment [3-5].

There is a possibility of transformation of the M1 phenotype into M2 by changing the spectrum of stimulating cytokines. One of the differences between M2 and M1 phenotypes is that they metabolize L-arginine differently. M2 macrophages transform L-arginine into L-ornithine and polyamines, while M1 macrophages produce nitric oxide and L-citrulline. It is proved that the determination of the expression of the inducible isoform of the nitric oxide synthase enzyme (iNOS) makes it possible to assess the polarization of macrophages towards "classically" activated M1 phenotype with the significant formation of nitric oxide and other proinflammatory cytokines and mediators. It is believed that the activity of iNOS characterizes the features of polarization and differentiation of macrophages into different types, namely, M1 or M2 [6-8].

A better understanding of the etiological factors and pathogenetic mechanisms of periodontal diseases in diabetes mellitus is one of medical and healthcare priorities [9-11], which will enable to elaborate more effective approaches for the prevention and treatment of oral diseases.

THE AIM

The aim of this study is to determine the activity of NO-synthase and arginase in oral fluid in children of primary school age with type 1 diabetes mellitus and to evaluate the efficacy of the treatment scheme we elaborated in the treatment of chronic catarrhal gingivitis.

MATERIALS AND METHODS

We examined 82 children (from six to twelve years old), including 56 children with type 1 diabetes mellitus and 26 children without somatic diseases.

According to our dental examination, all the patients were divided into the following subgroups:

Group 1 - 13 children with clinically healthy periodontium and no comorbidities, this group was a control one;

Group 2 - 13 children of children without comorbidities and with chronic catarrhal gingivitis (CCG);

Group 3 - 26 children with type 1 diabetes mellitus without signs of periodontitis;

Group 4 - 30 children with type 1 diabetes mellitus who had chronic catarrhal gingivitis.

We studied the PMA index (papillary–marginal–alveolar index) modified by Parma (1960). General NO-synthase (gNOS) activity was determined in oral fluid by the difference in nitrite concentration before and after incubation. Activities of constitutive (cNOS) and inducible (iNOS) isoforms of NOS were determined with the usage of selective iNOS inhibitor (Aminoguanidine hydrochloride) The arginase activity was determined in oral fluid by the difference in the concentration of L-ornithine before and after incubation in phosphate buffer solution containing L-arginine [12].

The findings obtained were statistically processed using Microsoft Office Excel 2016 software pack. The distribution was checked by the Shapiro-Wilk test. The arithmetic mean (M), the representativeness error of the mean (m), and the significance level of the differences in the mean values (p) were calculated. The t-test for paired samples was used to compare values. The difference was considered statistically significant at $p<0.05$.

We taught all 82 children how to perform oral hygiene routine properly. We recommended using soft toothbrushes, toothpaste, and mouth rinse with extracts of medicinal plants "BIOMED SENSITIVE" (STS Holding Group LTD, Bulgaria) for all the participants [13]. We treated chronic catarrhal gingivitis in children of group 2 following the protocols in the specialty "Pediatric Dentistry" approved by the Ministry of Health of Ukraine.

The management of the children with chronic catarrhal gingivitis and type 1 diabetes mellitus (group 4) combined therapeutic and hygienic measures. Following the examination and diagnosis confirmation, the children received professional oral hygiene and educated how to keep oral hygiene properly. The one-month course included our recommendation to use the toothpaste mentioned above and the mouth rinse with grape extract. The children were also prescribed to take the antioxidant "Askorutin" (LLC Agrofarm, Ukraine) 1 tablet twice a day for 1 month [14]. To normalize oral mocrobiota, we recommended the patients to rinse the mouth with an antiseptic solution "Sangiva" (JSC Pharmaceutical firm "Darnitsa", Ukraine) twice a day for 5 days and to take the probiotic "BioGaia Prodentis" (BioGaia AB, Sweden) 1 tablet once a day for 10 days. The evaluation of treatment results was carried out in immediate follow up period, a month since the completion of the full course of treatment for chronic catarrhal gingivitis, and in the remote follow-up period, in 3, 6, and 12 months.

The scientific materials meet the rules of humane treatment of patients in accordance with the requirements of the Tokyo Declaration of the World Medical Association, international recommendations of the Helsinki Declaration of Human Rights, the Council of Europe Convention on Human Rights and Biomedicine, Laws of Ukraine, orders of the Ministry of Health of Ukraine.

RESULTS

Using the standard treatment complex in non-diabetic children with chronic catarrhal gingivitis leads to a decrease in iNOS activity and an increase in arginase activity in oral fluid one month after treatment, according to our results (Table I).

The concentration of nitrites does not change statistically significantly. It evidences an increase in reparative processes and a decrease in pro-inflammatory processes. It is also confirmed clinically, namely, the PMA index is 20.52% before treatment, and it decreases to 12.22% a month after treatment (Fig. 1).

The activity of iNOS and the concentration of nitrites in the oral fluid of the above-mentioned group of children increases compared to pre-treatment in 3 months after using the standard treatment complex. The activity of arginases does not change statistically significantly. The activity of iNOS and nitrite concentration increase, arginase activity decreases after 6 months. There is a change in the biochemical parameters of oral fluid in the group of non-diabetic children with chronic catarrhal gingivitis 1 year after the start of the standard treatment complex. Thus, the activity of iNOS decreases, and the activity of arginases increases compared to pre-treatment data, the concentration of nitrites stays unchanged. Thus, there is a decrease in the pro-inflammatory component and an increase in reparative function. These data coincide with the data of the clinical research, namely, the PMA index before treatment was 20.52%, and a year later it decreased to 13.35%. The use of the above-mentioned personal hygiene products (a soft toothbrush, toothpaste and rinse aid with grape extract) in children with healthy gums and type 1 diabetes leads to increased arginase activity and nitrite concentration in oral fluid in 1 month. It evidences the strengthening of reparation processes (Table II). The activity of iNOS is not statistically significant.

The iNOS activity and nitrite concentration increase, and arginase activity decreases compared to pre-treatment values in 3 months. The iNOS activity, arginase, and nitrite concentration increased in 6 months. The iNOS activity and nitrite levels in the oral fluid increased compared to pre-treatment data in 1 year after using the soft toothbrush, toothpaste, and rinse with grape extract, which was recommended by us. And the activity of arginases, respectively, did not change statistically significantly compared with pre-treatment activity.

We noted a statistically significant decrease in iNOS activity and a statistically significant increase in arginase activity and nitrite concentration in oral fluid in children with type 1 diabetes mellitus and chronic catarrhal gingivitis in 1 month after using of treatment complex elaborated by us. (Tab. III). This fact evidences an increase in reparative

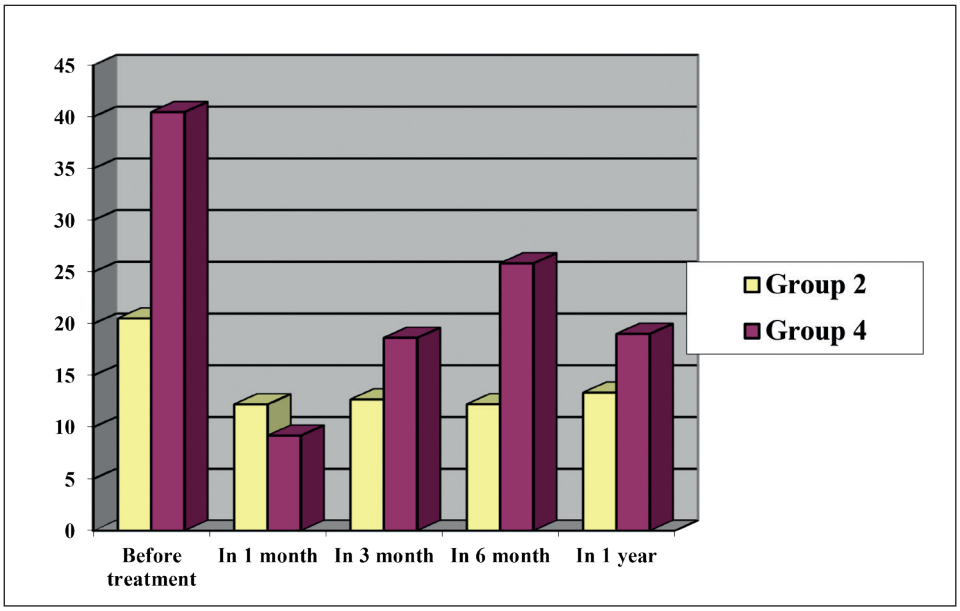

Fig. 1. The PMA index in Parma modification (1960) in non-diabetic children with chronic catarrhal gingivitis (group 2) and children with chronic catarrhal gingivitis and type 1 diabetes mellitus (group 4).

Table I. The influence of standard treatment complex on the biochemical parameters of oral fluid in non-diabetic children with chronic catarrhal gingivitis n = 13

Term of sampling of oral fluid	Biochemical parameters of the oral fluid				
	gNOS, μmol/min. per g of protein	cNOS, μmol/min. per g of protein	iNOS activity, μmol / min per g of protein	Nitrite concentration, nmol / l	Arginase activity, μmol / min per g of protein
Before treatment	0.55±0.02	0.09±0.001	0.46±0.02	2.50±0.20	0.67±0.02
After 1 month	0.34±0.01*	0.09±0.002	0.30±0.01*	2.01±0.10	0.92±0.02*
In 3 months	0.66±0.08	0.09±0.002	0.57±0.07	3.84±0.37*	0.57±0.05
In 6 months	1.43±0.13*	0.11±0.008*	1.22±0.13*	6.30±0.80*	0.34±0.04*
After 1 year	0.40±0.01*	0.09±0.002	0.29±0.01*	2.27±0.10	1.09±0.02*

Note: * - the difference is statistically significant when compared with the indicators before treatment ($p<0.05$).

Table II. The influence of preventive measures on the biochemical parameters of oral fluid in children with healthy gums and type 1 diabetes mellitus, (M ± m). n = 26

Term of sampling of oral fluid	Biochemical parameters of the oral fluid				
	gNOS, μmol/min. per g of protein	cNOS, μmol/min. per g of protein	iNOS activity, μmol / min per g of protein	Nitrite concentration, nmol / l	Arginase activity, μmol / min per g of protein
Before treatment	0.87±0.07	0.11±0.001	0.76±0.07	1.93±0.15	0.42±0.03
After 1 month	0.65±0.04*	0.09±0.001*	0.64±0.04	3.24±0.23*	0.78±0.04*
In 3 months	1.75±0.05*	0.17±0.002*	1.72±0.05*	10.98±0.22*	0.24±0.01*
In 6 months	1.18±0.08	0.14±0.006*	0.93±0.09	9.04±0.53*	0.63±0.04*
After 1 year	1.37±0.08*	0.13±0.005*	1.20±0.07*	8.15±0.57*	0.43±0.03

Note: * - the difference is statistically significant when compared with the indicators before treatment ($p<0.05$).

processes and reduction of pro-inflammatory processes. Clinical manifestations are consistent with laboratory data. The PMA index was 40.47% (moderate degree of inflammation) before treatment in this group of children, and it decreased more than 4 times to 9.2% (mild degree of inflammation) in 1 month (Fig. 1).

We noted the increase in the activity of iNOS and concentration of nitrites, and also decrease in activity of arginases in 3 months and 6 months after using the treatment complex developed by us in this group of children. The iNOS activity did not change statistically significantly, arginase activity and nitrite concentration increased statistically significantly compared to pre-treatment values in 1 year. We can conclude that the treatment complex elaborated by us, enhances the repair process (it is evidenced by the increase in arginase activity) even in 1 year. But the activity of iNOS and the concentration of nitrites stayed stable and did not change after a year. We believe that it is a consequence of the presence of type 1 diabetes mellitus in these children and, consequently, systemic inflammation in the body as a whole.

Table III. The influence of the treatment complex elaborated by us on the biochemical parameters of oral fluid in children with type 1 diabetes mellitus and chronic catarrhal gingivitis, (M ± m). n = 30n = 30

Term of sampling of oral fluid	Biochemical parameters of the oral fluid				
	gNOS, µmol/min. per g of protein	cNOS, µmol/min. per g of protein	iNOS activity, µmol / min per g of protein	Nitrite concentration, nmol / l	Arginase activity, µmol / min per g of protein
Before treatment	0.75±0.04	0.13±0.004	0.62±0.04	1.34±0.14	0.72±0.03
After 1 month	0.45±0.01*	0.09±0.002*	0.36±0.01*	4.80±0.24*	0.88±0.02*
In 3 months	1.78±0.02*	0.18±0.002*	1.62±0.02*	14.04±0.13*	0.21±0.01*
In 6 months	2.05±0.04*	0.13±0.005	1.92±0.05*	12.62±0.23*	0.22±0.01*
After 1 year	0.72±0.02*	0.13±0.004	0.61±0.02	7.97±0.41*	0.51±0.02*

Note: * - the difference is statistically significant when compared with the indicators before treatment ($p<0.05$).

DISCUSSION

The most indicative terms for the assessment of treatment schemes and prevention measures are 1 month and 1 year according to our research. A decrease in the iNOS activity and an increase in arginase activity and the absence of changes in nitrite concentrations in oral fluid indicates a predominance of anti-inflammatory polarization in non-diabetic children with chronic catarrhal gingivitis in 1 month and in 1 year after using the standard treatment scheme. This fact, together with the clinical condition of the gums, indicates sufficient effectiveness of the standard method of treatment of chronic catarrhal gingivitis.

The preventive complex in children with healthy gums and type 1 diabetes leads to an increase in arginase activity after 1 month, which indicates an increase in reparative processes. However, there are no changes in the activity of arginases, and the activity of iNOS even increases in 1 year. Thus, despite the absence of inflammation, proinflammatory polarization of oral macrophages predominates. Such changes may be associated with the change of the normal oral microbiota to the so-called "diabetic" [15]. It is characterized by the presence of specific pathogenic strains. The increase in nitrite concentration can be considered not as a damaging factor, but as an adaptive response aimed at suppressing bacterial pathogens [16]. It is evidenced by the intact periodontium.

Using our own treatment complex in children with chronic catarrhal gingivitis and type 1 diabetes mellitus leads to a change in the polarization of oral macrophages towards the predominance of M2 polarization in 1 month. It is evidenced by increased arginase activity and a statistically significant decrease in iNOS activity. The polarization of macrophages changes to the predominance of M1 polarization in 1 year. It is evidenced by the decrease in arginase activity, and no changes in the iNOS activity. Thus, taking into account the state of the periodontium in this group of children, we can conclude that changes in biochemical parameters of oral fluid in 1 month are associated with the elimination of chronic catarrhal gingivitis, and changes in biochemical parameters in 1 year are due to systemic pathogenetic factor, namely, type 1 diabetes mellitus. Increasing nitrite concentrations may also be an adaptive response of oral macrophages to diabetes-induced changes in the composition of the oral microflora [17, 18].

CONCLUSIONS

Our treatment complex of chronic catarrhal gingivitis in children with type 1 diabetes mellitus normalizes the polarization of oral macrophages caused by the influence of chronic catarrhal gingivitis as a local pathogenetic factor.

Standard treatment of chronic catarrhal gingivitis in non-diabetic children leads to the predominance of anti-inflammatory polarization of oral macrophages.

The complex of preventive measures for children with type 1 diabetes mellitus and healthy gums preserve the pro-inflammatory polarization of macrophages in the long term but prevent damage to periodontal tissues.

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ORIGINAL ARTICLE

FUNCTION ROLE OFIL-6-174 GENE POLYMORPHISMS IN ASSOCIATION WITH IL-6 LEVELS IN TYPE 2 DIABETES MELLITUS

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ABSTRACT

The aim: To distinguish the function role of IL-6 protein levels as well as IL-6 (-174) gene polymorphism in diabetes mellitus patients.

Materials and methods: in present case-control study160 (cases 86, control 74) volunteers were enrolled in this study 2.5 ml were added to EDTA tube for molecular investigation IL6 (-174), and other 2.5 ml use for measurement of fasted glucose by spectrophotometry and insulin levels as well as IL-6 level by ELISA.

Results: Regarding IL-6-174 (*rs*1800795), results revealed significant difference at genotype CC (p- value 0.05 with odds ratio 3.49) and allele frequency C (p-value 0.02 with odds 2.17), also at protein level the IL-6 showed significant difference between IL-6 levels and T2DM especial in insulin resistance (IR) group (p-value 0.03) compared with control one (Cont).

Conclusions: There is potent relation between IL-6 levels with T2DM and IR groups. There is potent relationship between CC variation of IL-6 (-174) and risk of each T2DM and IR condition.

KEY WORDS: type 2 diabetes mellitus, interlukine-6, IL-6 (-174), insulin resistance

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INTRODUCTION

Diabetes mellitus one of the most important diseases worldwide, characterized by increased blood sugar [1]. According to the International Diabetes Federation, in 2020 approximately 463 million patients were registered in world with diabetes mellitus [2]; in report of the WHO (2018) around 1.4 million,of Iraqis, have diabetes [3]. There are many factors that may be considered as risks for developing of T2DM including obesity, hypertension, lifestyle, age, nutrition regimen and physical activity, as well as genetic factor [4, 5]. Accumulated evidences reported on strong correlation between immune factors and hyperglycemia [6, 7]. Particularly, IL-6 at both protein and genetic levels showed significant relation with development of T2DM and special insulin resistance [8]. Elevation of IL-6 level involves in the pathogenicity of T2DM, effecting the insulin signaling pathway, leading to prevent the glucose uptake as well as insulin resistance [9]. On the other hand, IL-6-174 gene polymorphism (rs1800795) effects the rate of IL-6 gene expression and production of IL-6 [10, 11]. The mutant allele of IL-6-174 gene polymorphism is higher in patient with T2DM rather than in control [12].

THE AIM

To distinguish the function role of IL-6 protein levels as well as IL-6 (-174) gene polymorphism in diabetes mellitus patients.

MATERIALS AND METHODS

Present case-control study was conducted in 160 Iraqi participants (30 – 70 years): 86 T2DM patients diagnosed by consultant internists and 74 healthy volunteers in ALEmamain AL-Kadhemain Medical City at Baghdad, during the period December 2020 - March 2021. Patients were divided according to HOMO-IR into following groups: Insulin resistance (24 participants, 9 male and 15 female) and Insulin sensitive (62 participants, 22 male and 40 female). Five ml of venous blood were collected from each subjects, 2.5 ml were added to EDTA tube for measurement (SNP) for IL-6 (-174). Sera were isolated from the remaining 2.5 mL of the blood then stored until performing the biochemical and immunological assay (ELISA). Allele specific PCR was used to determine the gene polymorphism of IL-6 -174 using specific primers design online, as shown in table I.

Biochemical assay (Human, Germany) was used to measure FBS, while ELISA assays (Sunlong, Chinese) were used to measure levels of each of fasted insulin and IL-6 levels.

STATISTICAL ANALYSIS

This case control report was analyzed statistically with SPSS version 24.0 and Microsoft Excel 2010.Using (ANOVA) for Way Analysis of Variance to test the discrepancy between the groups tested. The Pearson test was used to determine the degree of association between the variables under consideration.

RESULTS

Level of Fasted blood sugar has shown the significant difference between each of IS (135.58 ± 49.82, P value ≤0.001) and IR (192.31 ± 64.31, P value ≤0.001) groups compared to cont. (90.40 ± 19.74) group. Also we detected significant difference between IS (P value ≤0.001) compared to IR group, as shown in table II. Data related to the level of insulin, showed non-significant and significant difference in IS (3.87 ± 2.67, P value 0.31) and IR (8.58 ± 2.38, P value ≤0.001) groups respectively compared to cont. (4.32 ± 2.64) group, while there was significant difference between IS (P value ≤0.001) compared IR groups, as shown in table III. Results related to the level of HOMO-IR, showed non-significant and significant difference in IS (1.13 ± 0.68, P value 0.12) and IR (3.90 ± 1.39, P value ≤0.001) groups respectively compared to cont. (0.92 ± 0.58) group, while there was a significant difference between IS (P value ≤0.001) compared to IR group, as shown in table IV.

Level of IL-6 showed non-significant and significant difference in IS (20.25 ± 0.87, P value 0.39) and IR (25.17 ± 1.87, P value 0.03) groups respectively compared to cont. (21.34 ± 0.83) group, with significant difference between IR (P value 0.01) and IS groups, as shown table V.

VARIATION OF IL-6 (RS 1800795)

Gel electrophoresis of ARMS-PCR production was done to determine the variations in the studied SNPs that had three genotypes in IL-6: heterozygous genotype (GC), homozygous genotype (CC) and Wild genotype (GG), shown in figure 1.

ASSOCIATION OF (RS1800795) WITH STUDY GROUP

Interleukin-6 SNP rs1800795 which had G>C Allele variations in control group made 33 (45%) Wild genotype (GG), 24 (32%) of GC and 17 (23%) of CC variation, with DM2 patient variation equaling 27 (31%) of GG, 41 (48%) of GC and 18 (21%) of CC. We used Logistic regression test for association of variation with incidence of DM2, which revealed insignificant association with each of variation (p value > 0.05). Furthermore (G and C) allelic distribution did not show significant differences (p value > 0.05) between control group and DM2 patients as shown in table VI.

ASSOCIATION OF (RS1800795) WITH CONTROL AND IR DM2

The studied of SNP rs1800795 of IL-6 had G>C Allele. The heterozygous genotype frequency (GC) was found in 10 (42%) DM2 patients and control group 24 (32%), whereas wild genotype (GG) was present in (21%) 5 patients with DM2 and 33 (45%) among healthy control, while homozygous genotype frequency (AA) was in 9 (38%) of DM2 patients and 17 (23%) of healthy control group. As shown by the outcomes, there was a positively significant difference (P value 0.05, odds 3.49) between DM2 patients for genotype (CC) compared to GG control group and allele frequencies showed statistically significant difference between DM2 patient and healthy control group (P value = 0.02, odds 2.17), as shown in Table VII.

Table I. The sequencing of primer

Primer	Sequence (5'-3')	Product size
Wild type Forward	CCCTAGTTGTGTCTTGGG	281bp
Mutant Forward	CCCTAGTTGTGTCTTGGC	
Common Reverse	GCACTTACTTGTGGAGAAGG	

Table II. the levels of FBS and insulin as well as HOMO-IR in studied groups

FBS		P value (sg≤0.05)	
Groups	M±SD	Sensitive	Resistance
Control	90.40 ± 19.74	0.00	0.00
Sensitive	135.58 ± 49.82		0.00
Resistance	192.31 ± 64.31		

Table III. the levels of insulin in studied groups

INSULIN		P value (sg≤0.05)	
Groups	M±SD	Sensitive	Resistance
Control	4.32 ± 2.64	0.31	0.00
Sensitive	3.87 ± 2.67		0.00
Resistance	8.58 ± 2.38		

Table IV. the levels of HOMO-IR in studied groups

HOMO IR		P value (sg≤0.05)	
Groups	M±SD	Sensitive	Resistance
Control	0.92 ± 0.58	0.12	0.00
Sensitive	1.13 ± 0.68		0.00
Resistance	3.90 ± 1.39		

Table V. value of IL-6 in studied groups

IL-6		P value (sg≤0.05)	
Groups	M±SD	Sensitive	Resistance
Control	21.34 ± 0.83	0.39	0.03
Sensitive	20.25 ± 0.87		0.01
Resistance	25.17 ± 1.87		

DISCUSSION

There are many clinical studies referring presence of strong correlation between development of T2DM with many proinflammatory factor such as (IL-6, IL-1β and TNF-α) [13-15]. IL-6 is major responsible for the insulin resistance by effecting the internal signal of cells to uptake the glucose [9]. The result of our study showed that levels of fasted blood sugar in IR patients is higher than level in IS patients with significant value and both of them are significantly different, compared to control group. Elevation of FBS may be resulted by increasing of IL-6

Table VI. Distribution of Genotypes and Alleles of IL-6 gene polymorphism (Rs1800795) in study groups

		Rs1800795 Genotype Frequency (%)			
IL-6 Variations	Control (74)	DM2 (86)	P value	Odds ratio	C.I (95%)
GG	33 (45%)	27 (31%)	0.38	1	
GC	24 (32%)	41 (48%)	0.41	1.35	0.662-2.769
CC	17 (23%)	18 (21%)	0.17	1.76	0.781-3.967
		Allele frequency (%)			
IL-6 alleles	Control (148)	DM2 (172)	P value	Odds ratio	C.I (95%)
G	90 (61%)	95 (55%)	0.31	1.26	0.81-1.97
C	58 (39%)	77 (45%)			

Table VII. Distribution of Genotypes and Alleles of IL-6 gene polymorphism (Rs1800795) between control and Insulin resistance

		Rs1800795 Genotype Frequency (%)			
IL-6 Variations	Control (74)	IR DM2 (24)	P value	Odds ratio	C.I (95%)
GG	33 (45%)	5 (21%)	0.12	1	
GC	24 (32%)	10 (42%)	0.09	2.75	0.832-9.088
CC	17 (23%)	9 (38%)	0.05	3.49	1.011-12.074
		Allele frequency (%)			
IL-6 alleles	Control (148)	IR DM2 (48)	P value	Odds ratio	C.I (95%)
G	90 (61%)	20 (42%)	0.02	2.17	1.12-4.21
C	58 (39%)	28 (58%)			

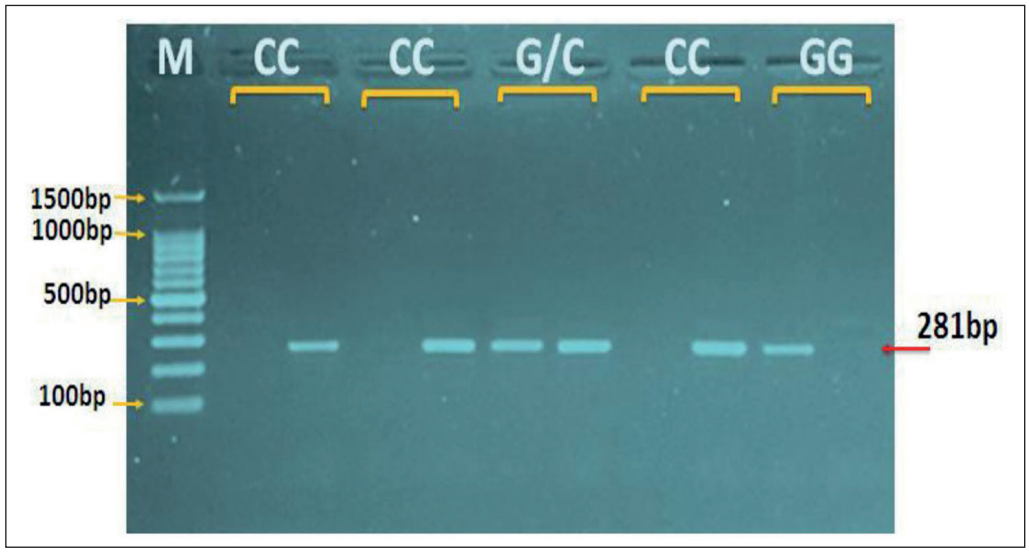

Fig. 1. Agarose gel electrophoresis image showed the ARMS-PCR product analysis of rs1800795 (C/G) gene polymorphism. Where M: marker (1500-100bp). The presence of A or G allele was observed at 281bp product size. The (GG) wild type homozygote was present in G allele only, the (CC) mutant type homozygote showed in C allele only, whereas the (G/C) heterozygote showed in both G and C allele.

level that we detected in our study. The result of our study agrees with another clinical study, conducted by Park et al; (2015) that showed significant increasing of FBS in IS and IR groups [16]. Fryk et al; (2021) also detected it in patients with insulin resistance compared with control group with normal glucose level [17]. The results of our studying of related insulin levels showed non-significant and significant differences in each of IS and IR patients respectively compared with control group. There was also a significant difference between IS patients compared to IR groups. Accumulated studies conducted by many research teams support the result of our study, regarding the level of insulin in each of studied group [18-20]. Present study revealed the elevation of HOMO-IR in IR group compared to IS group, as well as its increased level in IR compared to control group. This significant elevation in IR comes from increasing of glucose and insulin levels due to defect in insulin signaling pathway. This fact is proved by accumulated studies, supported by results of our study [21-23]. In present study the Level of IL-6 showed non-significant and significant difference in IS and IR groups respectively compared to control group, with parallel significant difference between IR and IS groups. This increasing of IL-6 level in association with increasing of FBS may refer to its effects on blood sugar metabolism leading to IR condition due to affected of internal signaling responsible for cell

glucose uptake. Furthermore, non-elevation of IL-6 levels in IS group in association with non-elevated of FBS may agree the results mentioned above. This result consent with the findings of Bashir et al; (2020) who stated a statistical difference between the levels of IL-6 in each of IS patients and IR patients [23].

Also, Ayelign et al; (2021) have reported the association between IL-6 level as pro-inflammatory cytokine and T2DM; they mentioned that chronic elevation of IL-6 at low-grade leads to rising of glucose levels, then to predisposition of T2DM [24]. Regarding genetic investigation of IL-6-174 (*rs*1800795) SNP reveled non-significant association of each variation with control group and T2DM. However, detected association between IR compared control at CC variation (mutant allele) means the patient-carrier of CC genotype has high risk for development of IR condition, compared to patient carrier GG genotype. Furthermore, the allelic C -carriers have risk higher by 2.04 times to development of IR condition than those carrying allelic G. These results agree with Todendi et al; (2015) study that showed non-associated IL-6 gene variation and level of IL-6 [8]. While Frota, et al; (2021) reported potent effect of polymorphism IL-6-174 on this gene expression [25]. IL-6 SNP (*rs1800795)* is associated with diseases such as T2DM and insulin resistance [26, 27]. Whereas other study conducted by Tabassum et al; (2012) showed not associated IL-6-174 SNP (*rs1800795),* obese and T2DM [28]. A study was conducted in London in 571 patients. 76 % of them were patient-carriers of CC genotype with metabolic syndrome, compared to patients who had GG genotype 56%. The C allele has association with metabolic syndrome compared to patient without this syndrome [29]. According to a study performed by Qi et al; (2006) IL-6-174 G/C polymorphism is not associated with the risk of T2DM development [30]. Other study by Dhamodharan et al; (2015) showed that IL-6-174 GC and CC variation conferred protection against of T2DM, and also reported the C allele of CC and GC variation has significant protection against T2DM [31].

CONCLUSIONS

There is potent relation between IL-6 levels with abnormality of blood sugar metabolism in T2DM, this effect was highly obvious with protein levels in IR groups of IL-6 (-174) variations. A potent relation between CC variation of IL-6 (-174) and risk of each of T2DM and IR condition in T2DM, is supported by the risk of C allele with incidence in both of T2DM and IR condition of T2DM.

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ORIGINAL ARTICLE

MORPHOMETRIC ANALYSIS OF TOPOGRAPHIC VARIABILITY OF THE LEFT AND RIGHT MANDIBULAR CANALS IN CASE OF LOSS OF THE MASTICATORY TEETH

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ABSTRACT

The aim: To study the topographic variability of the left and right mandibular canals in case of bone atrophy caused by the loss of the masticatory teeth.

Materials and methods: 136 digital scans were selected for morphometric analysis, 68 for each side taken with the Vatech PaX-i 3D Green extra-oral radiography system. The readout of absolute morphometric values, laying the left and right MC was performed in the projection of 3.7, 3.6, 4.6, 4.7 teeth using standardized Ez3D-I software.

Results: The alveolar part is characterized by distance to the alveolar ridge, and primarily exposed to pronounced atrophic processes of bone tissue. Distance to the lingual ridge directly proportionally indicates the morphological transposition vector of the mandibular canals for the distance to the buccal ridge, by the same length to its reduction. Morphometric analysis on a short toothless segment determines the variability of laying the mandibular canals but it is characterized by constant regular values of the ridge of the mandibular base.

Conclusions: Dentition defects, moving towards the missing teeth, lead to a decrease in the biophysical stimulus on bone tissue, causing pronounced morphological changes with the loss of significant volume and restructuring of its trabecular layer, which synchronously affects the topographic variability of the left and right MC.

KEY WORDS: mandible, computed tomography, mandibular canal, bone atrophy

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INTRODUCTION

Early tooth loss in the distal parts of the lower jaw is the most common manifestation of dental pathology, which in turn is complicated by segmental deformity of the antagonizing dentition and «disuse atrophy» of bone tissue, in general. This pathophysiological mechanism of the defect circle triggers a whole cascade of irreversible processes in the restructuring of the architectonics of bone tissue with all its components, including the mandibular canal (MC). After all, an indicator of the normal functioning of the maxillary system, first of all, is the integrity of the dentition, which through the masticatory muscles, teeth and periodontium, in particular, transmit proper pressure to bone tissue in the form of a mechanical stimulus, to which a biological reaction occurs from the bone tissue, thereby ensuring the corresponding processes of metabolic transformations. And such a non-stop process of reconstruction is usually called remodelling, with the participation of basic multicellular units of bone structure, that is, areas of bone tissue in which bone restructuring occurs. The number of such points directly depends on the stimulus spreading to its receptors, by regulating the concentration of ions and change of protein levels, as well as factors that stimulate the inhibition of mineralization. Under the opposite conditions, the absence of indirect «constant pressure» forces bone tissue to the status of the relative metabolic rest, and, accordingly, to its devastation. Reduction of the quantity of osteoblast and osteocyte forming cells that maintain the level of ionic concentration in the bone interstitial fluid thereby reduces osteonic structure and its volume. Our study proves this interpretation by comparing segments in projection 3.6, 3.7 teeth of the left side and 4.6, 4.7 teeth of the right side [1]. Defects of the dentition, moving towards the missing teeth, lead to a decrease in the mechanical stimulus on bone tissue, causing pronounced morphological changes with the loss of significant volume and restructuring of its trabecular layer, which synchronously affects the topographic variability of the left and right mandibular canals.

THE AIM

To study the topographic variability of the right and left mandibular canals in case of bone atrophy, due to the loss of the masticatory teeth (lower molars), in individuals aged 25-75 years.

MATERIALS AND METHODS

High-quality images were obtained during a thorough analysis of 243 computed tomography cone-digital scans taken by the Vatech PaX-I 3D Green extra-oral radiography

system with a scan size range of 16 x 9 cm, which minimize the possibility of artifacts caused by patient movement, a focal spot of 0.5 mm (EC60336) on a 14-bit gray scale with a size of 0.2/0.3 voxels and due to the short scanning time. For statistical analysis, we selected 136 digital scans, 68 for the left and right sides, in individuals without existing somatic pathology, which would have an indirect effect on the metabolic transformations of bone tissue, which provide the best opportunities for scientific research and have proper information content to achieve the aim of this study.

The analysis was performed using HEWLETT-SNCPUM1 computer equipment with 16.0 GB RAM, 10 Pro Software for Workstations, 2019: 00391-70000-00000-AA425 after that, using the method of «statistical selection» we separated four age groups (Table I), namely: the first study group (I) - 25-45 years, the second study group (II) – 46-60 years, the third study group (III) 61-75, the fourth control group (IV) – 25-75 years, people with preserved dentition.

We obtained the results of absolute morphometric values (Fig. 1) of laying the mandibular canal (MC), using standardized Ez3D-I software ver.5.1.9.0 in the projection of 3.7, 3.6, 4.6, 4.7 teeth relative to: the distance from the ridge of the alveolar part (AR) to the MC – ar-mc (Fig. 1-A); distance from the ridge of the mandibular base (RMB) to the MC – rmb-mc (Fig. 1-B); distance from the ridge of the buccal surface (BR) to the MC – br-mc (Fig. 1-C); distance from the ridge of the lingual surface (LR) to the MC – lr-mc (Fig. 1-D).

To study the topographic variability of the right and left mandibular canals, we used a variational analysis of statistical data with the determination of average morphometric values for each distance, which characterize such data and the standard error of average values, as well as estimates of the reliability of average values and the probability of an error-free forecast between comparison groups.

The study was conducted in compliance with the main provisions of the GCP (1996), the Council of Europe Convention on human rights and biomedicine (dated 04.04.1997), and the World Medical Association Declaration of Helsinki on ethical principles for conducting scientific medical research involving human subjects. Following the order of the Ministry of Health of Ukraine No. 110 dated 14.02.2012, the informed voluntary patients' consents were obtained to conduct diagnostics, the relevant medical documentation was drawn up and certified by patients' signatures. The provisions of the Law of Ukraine of 01.06.2010 No. 2297-VI «On personal data protection» with amendments and additions by the laws of Ukraine dated 23.02.2012 No. 4452-VI, dated 20.11.2012 No. 5491-VI regulating legal relations concerning protection and processing of personal data, and aimed at protecting fundamental human and civil rights and freedoms.

RESULTS

The result of the processing of statistical data showed absolute figures indicating the size of phenomena and their quantitative characteristics. Although they have a certain cognitive significance, their use is limited. To determine the level of the phenomenon and comparison of the indicator in dynamics we calculated relative values (indicators, coefficients), which are the result of the ratio of statistical values to each other and give an idea of the topographic variability of the left (Fig. 2, Fig. 3) and right mandibular (Fig. 4, Fig. 5) canals in case of bone atrophy under conditions of loss of the masticatory teeth.

What attracts the researcher's attention is that significant morphometric changes are observed in the distance ar-mc, that goes from the AR to the MC in all the study groups and differs significantly from the control group, which is represented by preserved dentition rows.

In the projection of 3.6 tooth with an average number of M=12.72±0.64 in the study groups I-III (S) and M=17.15±0.58 in the comparison control group IV (K), where p<0,001, with a decrease in the values of S – M=11.49±0.59, and in K – M=15.36 ±0.49, where p<0,001 in the projection of 3.7 tooth.

Topographic variability of the right MC is characterized by a slight asymmetry, however, maintains the tendency of regularity and it is S – M=12.9±0.57 and K – M=17.48±0.58, where p<0,001 in the projection of 4.6 tooth with the reflection of atrophic processes of bone tissue, which certainly affect the topography of the canal, and it is confirmed by the values S – M=11.63±0.55 and K – M=15.39±0.52, where p<0,001 in the projection of 4.7 tooth.

Another pattern defines the distance lr-mc, which runs from the lingual ridge of the body of the mandible to its channel with constant morphometric values:

S – M=3.46±0.18 in the area of the 3.6 tooth; S – M=3.11±0.18 in the area of the missing 3.7 tooth, and a slight asynchrony of the right canal and is:

Table I. Grouping of data in the study of qualitatively homogeneous aggregates – quantitative indicators that determine the topographical and anatomical features of the mandibular canal in case of bone atrophy caused by the loss of the masticatory teeth

Group	Age (full years)	Number of observations	Lower jaw	
			A - right side	B - left side
I (study group)	25-45	28	14	14
II (study group)	46-60	40	20	20
III (study group)	61-75	34	17	17
IV (control group)	25-75	34	17	17
Total observations	25-75	136	68	68

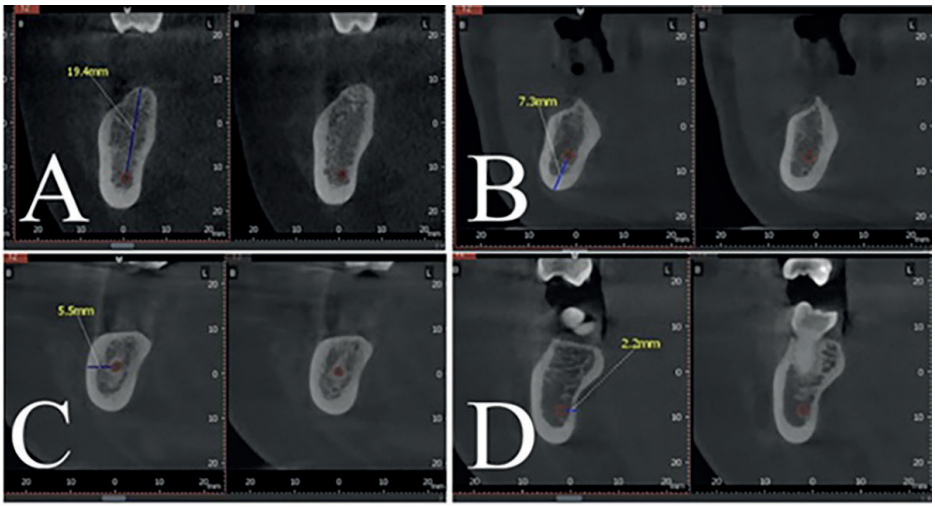
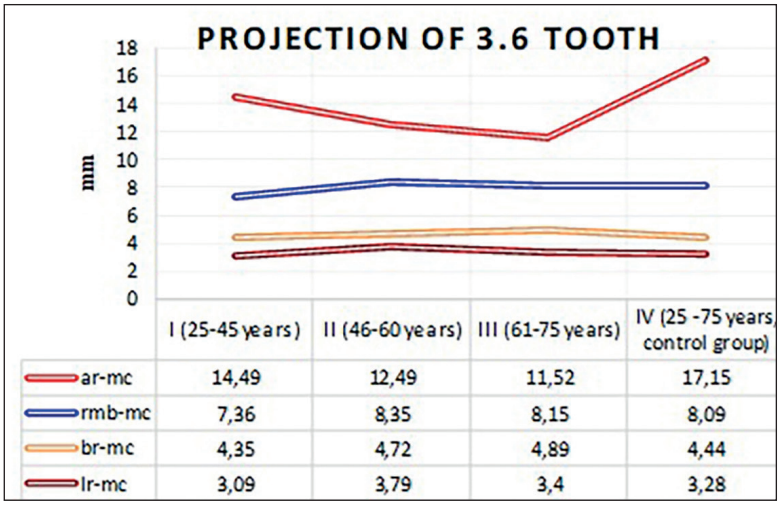
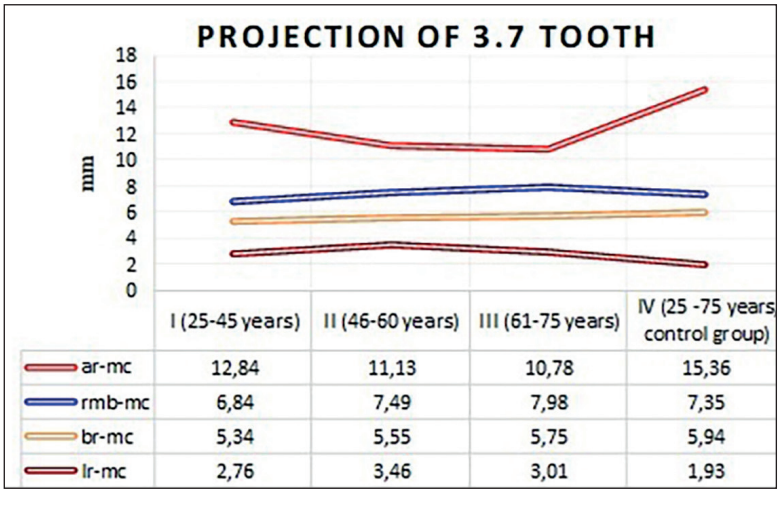


Fig. 1. 3D reconstruction model of the structural topography of the mandibular canal, sections in the sagittal plane:
A) patient of group I, terminal dentition defect, 25-45 years old;
B) patient of group II, terminal dentition defect, 46-60 years old;
C) patient of group III, terminal dentition defect, 61-75 years old;
D) patient of group IV, with a preserved dentition, 25-75 years old.



	I (25-45 years)	II (46-60 years)	III (61-75 years)	IV (25 -75 years, control group)
ar-mc	14,49	12,49	11,52	17,15
rmb-mc	7,36	8,35	8,15	8,09
br-mc	4,35	4,72	4,89	4,44
lr-mc	3,09	3,79	3,4	3,28

Fig. 2. Morphometric analysis (mm) of topographic and anatomical variability of the left MC in case of bone atrophy caused by the loss of the masticatory teeth in people aged 25-75 years, n=68: ar-mc – distance from the ridge of the alveolar part to the MC; rmb-mc – distance from the ridge of the base of the lower jaw to the MC; br-mc – distance from the ridge of the buccal surface to the MC; lr-mc – distance from the ridge of the lingual surface to the MC.



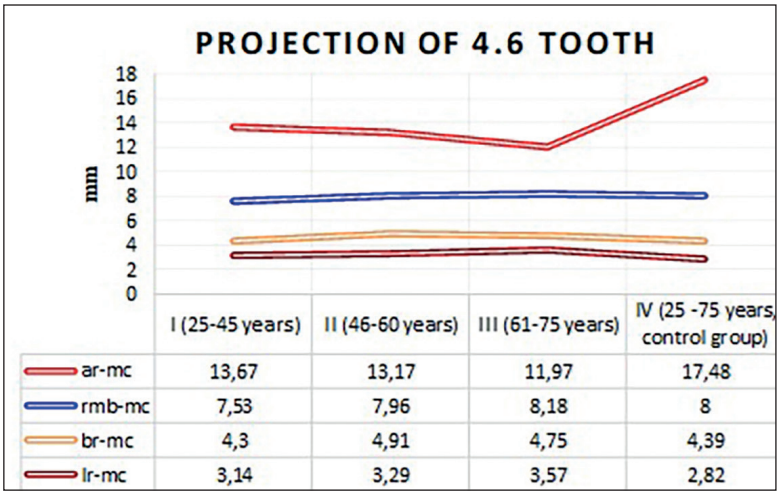
	I (25-45 years)	II (46-60 years)	III (61-75 years)	IV (25 -75 years, control group)
ar-mc	12,84	11,13	10,78	15,36
rmb-mc	6,84	7,49	7,98	7,35
br-mc	5,34	5,55	5,75	5,94
lr-mc	2,76	3,46	3,01	1,93

Fig. 3. Morphometric analysis (mm) of topographic and anatomical variability of the left MC in case of bone atrophy caused by the loss of the masticatory teeth in people aged 25-75 years, n=68: ar-mc – distance from the ridge of the alveolar part to the MC; rmb-mc – distance from the ridge of the base of the lower jaw to the MC; br-mc – distance from the ridge of the buccal surface to the MC; lr-mc – distance from the ridge of the lingual surface to the MC.

S – M=3.34±0.18 in the area of the 4.6 tooth; S – M=2.88±0.18 in the area of the missing 4.7 tooth.

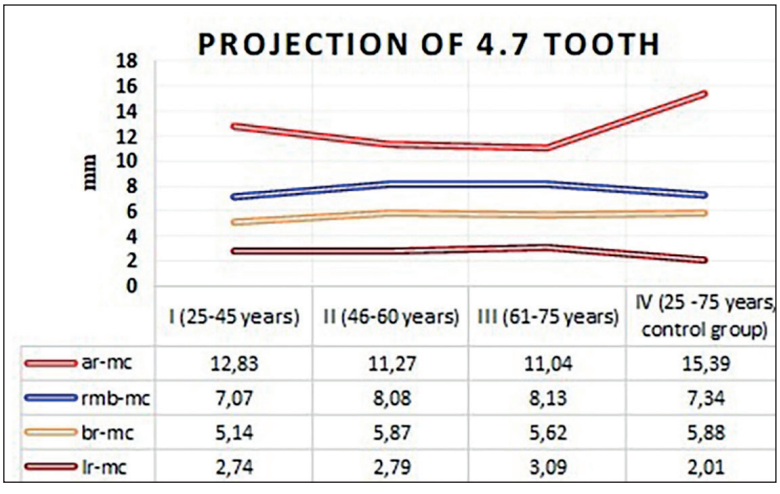
At the same time, the change in distance lr-mc directly proportionally causes the morphological transposition vector for the distance br-mc, which runs from the buccal ridge of the body of the mandible to its channel, for the same length in the direction of its decrease and is characterized by a progressive-uniform curvature of graphic images (see Fig. 2-5).

Detailed morphometric analysis of the topographic features of the left MC and right MC in case of bone atrophy on its short toothless segment determines the variability of its laying, however, it is characterized by constant values by the average number of observations on the distance



	I (25-45 years)	II (46-60 years)	III (61-75 years)	IV (25 -75 years, control group)
ar-mc	13,67	13,17	11,97	17,48
rmb-mc	7,53	7,96	8,18	8
br-mc	4,3	4,91	4,75	4,39
lr-mc	3,14	3,29	3,57	2,82

Fig. 5. Morphometric analysis (mm) of topographic and anatomical variability of the right MC in case of bone atrophy caused by the loss of the masticatory teeth in people aged 25-75 years, n=68: ar-mc – distance from the ridge of the alveolar part to the MC; rmb-mc – distance from the ridge of the base of the lower jaw to the MC; br-mc – distance from the ridge of the buccal surface to the MC; lr-mc – distance from the ridge of the lingual surface to the MC.



	I (25-45 years)	II (46-60 years)	III (61-75 years)	IV (25 -75 years, control group)
ar-mc	12,83	11,27	11,04	15,39
rmb-mc	7,07	8,08	8,13	7,34
br-mc	5,14	5,87	5,62	5,88
lr-mc	2,74	2,79	3,09	2,01

Fig. 4. Morphometric analysis (mm) of topographic and anatomical variability of the right MC in case of bone atrophy caused by the loss of the masticatory teeth in people aged 25-75 years, n=68: ar-mc – distance from the ridge of the alveolar part to the MC; rmb-mc – distance from the ridge of the base of the lower jaw to the MC; br-mc – distance from the ridge of the buccal surface to the MC; lr-mc – distance from the ridge of the lingual surface to the MC.

rmb-mc, which runs from the lower ridge of the base of the mandibular body to the MC.

The average experimental value of rmb-mc is 8.0±0.22 with the same number in the control group – K – M=8.09±0.43 in the area of the 3.6 tooth; S – M=7.47±0.21 and K – M=7.35±0.48 in the area of the missing 3.7 tooth, also represented by a slight asynchrony of the right canal and is:

S – M=7.91±0.21 and K – M=8.0±0.39 in the area of the 4.6 tooth; S – M=7.82±0.22 and K – M=7.34±0.54 in the area of the missing 4.7 tooth.

DISCUSSION

The most significant task and challenge of our time is the rehabilitation of patients with bone atrophy complicated by the topographical and anatomical features of the mandibular canal. Destruction of the trabecular layer, as a consequence of atrophic processes of bone tissue [2, 3], due

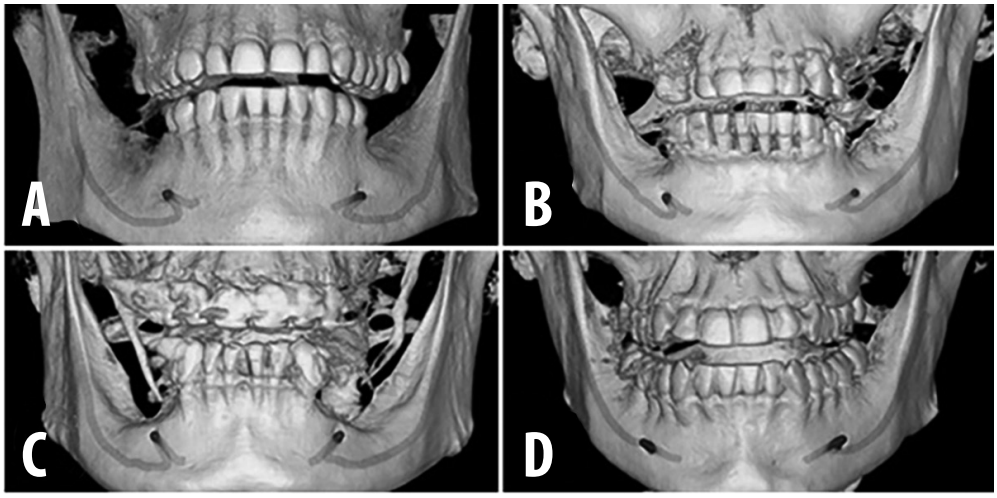


Fig. 6. 3D reconstruction model of the structural topography of the mandibular canal:
A) patient of group I, terminal dentition defect;
B) patient of group II, terminal dentition defect;
C) patient of group III, terminal dentition defect;
D) patient of group IV, with a preserved dentition.

to the loss of the masticatory teeth, leads to topographic variability of the laying of the left MC and right MC, which is presented in the results of the study, but also, accordingly, of the neurovascular bundle, which is already characterized by its morphological variability [4, 5].

In such studies, modern scientists choose digital methods of Computed Tomography as a priority according to ergonomics among similar ones, speed in processing and analysis, and reproduction, that is, visualization (Fig. 6) in the form of 3D reconstruction models [6, 7].

A thorough study with detailed morphometric values ensures a predictable clinical outcome, as the main goal of such studies. However, understanding certain patterns of topographic features described above makes it easier to choose methods of rehabilitation [8] or directed regeneration of bone tissue, before acquiring its normal physiological properties.

But the desire «researcher-patient-clinician» can not always be agreed upon when drawing up a plan for adequate treatment, due to a sequence of objective and subjective circumstances. Although available, promising scientific justifications provide an opportunity to choose effective tools or replace the proposed with similar ones according to the same principle of expediency [9, 10], we face the problem of bone atrophy, which «determines» the topographic variability of the mandibular canal and is the starting point in the rehabilitation [11], which corresponds to the aim and objectives of this work.

Such a detailed morphometric analysis of the topographic variability of the right and left mandibular canals in case of bone atrophy on the example of terminal dentition defects is a new platform for expanding further scientific research and a reliable reference point in the development of a three-dimensional model for template application in practical dentistry, in particular, maxillofacial surgery.

CONCLUSIONS

1. Morphological changes in bone tissue caused by a decrease in the biophysical stimulus through defects in the dentition with their postponement, proportionally affects the volume and restructuring of its trabecular layer and synchronous topographic variability of the left and right mandibular canal.
2. Distance from the ridge of the alveolar part to the mandibular canal of vertical morphometric values of the alveolar part of the lower jaw is characterized by the primary influence of atrophic processes.
3. The change in distance from the ridge of the lingual surface to the mandibular canal direct proportionally causes the vector of morphological transposition of the mandibular canal for the distance BR for the same length in the direction of its decrease.
4. On the toothless segment, with the loss of the masticatory group of teeth, the natural variability of laying the mandibular canal is determined and characterized by constant values based on the average number of observations on a distance of RMB, which runs from the lower ridge of the mandibular body to the mandibular canal.

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ORIGINAL ARTICLE

PREVALENCE OF SENSORY DYSFUNCTIONS IN ADULT UKRAINIAN POPULATION WITH LABORATORY CONFIRMED COVID-19

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ABSTRACT

The aim: To analyse the structure of sensory impairments, associated with COVID-19. To identify terms of recovery periods depending on severity of disease, age and gender of the patients.

Materials and methods: Within two weeks, 2225 patients with confirmed COVID-19 completed a questionnaire, created by Google Forms. General complaints, peculiarities of sensory impairments and recovery time were specified. After exclusion criteria application, data of 2108 patients were analyzed by R Statistics Package, Student's t-test, Wilcoxon rank-sum test, Fisher's exact test, Spearman's rank test.

Results: Among patients enrolled (973 males and 1135 females, mean age 28.6±0.18) the most frequent were olfactory (91.32%) and gustatory (66.03%) dysfunctions. Olfactory manifestations were usually accompanied by gustatory disorders (73.72%). Average duration of olfactory dysfunction was 15.46±0.45 days, gustatory - 11.3±0.33, hearing - 4.3±0.16, and visual - 6.53±0.23 days. It was found a correlation between duration of olfactory and gustatory impairments ($r=0.65$; $p < 0.001$), hearing and visual disorders ($r=0.49$; $p < 0.05$).

Conclusions: Olfactory and gustatory disorders are prevalent symptoms in Ukrainian population. 7.87% of respondents who had impairment of all four sensory functions had the longest recovery time. Duration of sensory impairments did not depend on age, type of treatment and severity of disease, which rises the question about the neurogenic pathway of virus.

KEY WORDS: early detection of disease, COVID-19, sensory disorders

Wiad Lek. 2022;75(3):670-677

INTRODUCTION

Pandemic infection of COVID-19 or SARS-Cov-2, recently announced by WHO, causes mild and moderate symptoms in majority of cases. As of February 24, 2021, a total of 97,464,094 confirmed cases were registered worldwide, including 2,112,689 deaths [1]. First case of this disease was confirmed in Ukraine on 3 March 2020. Since then 1,191,812 laboratory cases of COVID-19 have been confirmed, including 21,861 deaths of people infected with this disease. SARS-Cov-2 put enormous pressure on every country and now it requires implementation of preventive measures. Early diagnosis of coronavirus-19 disease is the most important factor of improving its outcomes and therefore, an important public health strategy. Being transmitted mainly through aerosols and droplets, COVID-19 primarily affects respiratory system and accompanied by such symptoms as tiredness, fever, dry cough, anorexia. Rhinorrhea, conjunctivitis, sore throat, headache, a rash on a skin are less common symptoms. It has been reported [2] that early stages of this disease are usually accompanied by various olfactory dysfunctions, such as anosmia (loss of smell) and ageusia (loss of taste). Besides, patients reported having temporary hearing loss after they had been hospitalized with COVID-19 [3]. Cases of ocular manifestations are not so common but attract an attention of scientists. It was shown that COVID-19 positive patients had ocular abnormalities [4]. Variety of sensory disorders in patients with COVID-19 requires deep studying. However, since the outbreak of pandemic, an attention has been paid to loss of smell and in many cases leaves out other sensory impairments, which determine the quality of people's life. Recognizing all kinds of sensory impairments in patients infected by SARS-Cov-2 is highly important for understanding of underlying mechanisms playing role in the spread of infection and for the developing preventive strategies.

The majority of studies concerning sensory impairments, based on self-reported symptoms or retrospective questionnaires [5-6], related to separate sensory disturbances.

Until now, a comprehensive epidemiological study concerning different types of sensory disorders among patients with confirmed COVID-19 has not been conducted.

Table I. Demographic and epidemiological characteristics of the respondents

Variable	Gender		Total n (%)
	female n (%)	male n (%)	
Average age, years	27.7±0.26 (53.84)	29.7±0.26 (46.16)	28.6±0.18 (100)
Self-reported health status			
excellent	153 (7.26)	136 (6.45)	289 (13.71)
good	879 (41.70)	779 (36.95)	1658 (78.65)
bad	98 (4.65)	68 (2.75)	156 (7.40)
very bad	5 (0.24)	-	5 (0.24)
Severity of COVID-19			
mild	629 (29.84)	529 (25.09)	1158 (54.93)
moderate	458 (21.73)	404 (19.17)	862 (40.89)
severe	44 (2.09)	34 (1.61)	78 (3.70)
critical	4 (0.19)	6 (0.28)	10 (0.47)
Type of treatment			
outpatient	1095 (51.94)	917 (43.50)	2012 (95.45)
inpatient	40 (1.90)	56 (2.66)	96 (4.55)

Table II. Number of self-reported olfactory manifestations in groups of patients with different severity of disease

Severity of COVID-19	Duration of GD, days				
	none*	1-4	5-8	9-14	>15
mild	87	148	367	248	308
moderate	88	89	249	196	240
severe	18	8	19	19	14
critical	4	1	1	1	3
Grand total, n (%)	197 (9.35)	246 (11.67)	636 (30.17)	464 (22.01)	565 (26.80)

* 4 patients reported slight OD during several hours were added in this group

THE AIM

The present article aimed to conduct an epidemiological study, characterizing the prevalence of sensory impairments (gustatory, olfactory, hearing, visual) in patients with laboratory-confirmed COVID-19 infection, analyse their prevalence, features and interactions.

MATERIALS AND METHODS

Within two weeks, 2225 patients completed a questionnaire created by Google Forms. Patients answered 25 questions including six general questions (age, gender, self-reported general health state, severity of COVID-19, presence/absence of laboratory-confirmed case of disease, outpatient/inpatient treatment), 18 questions about peculiarities of sensory dysfunctions (duration, features of impairments, associated symptoms, recovery time, presence of sensory disfunctions in anamneses), and one general clinical question about symptoms associated with SARS-Cov-2. Severity of COVID-19 was classified following the WHO recommendations [7] and included four phases (mild, moderate, severe, critical). The mean recovery time of all sensory dysfunctions was assessed according to four periods: 1-4 days, 5-8 days, 9-14 days, and more than 15 days. This gradation is widely used in the modern literature [8] and based on the fact that viral load significantly reduced after the beginning of disease [9].

Exclusion criteria were: patients under 18 years old, those who did not have confirmed COVID-19 infection by laboratory test, incomplete responses.

R Statistics Package was used to perform statistical analyses. Normally distributed data were reported as mean (deviation). Difference between means was calculated using Student's t-test. Non-normally distributed data were reported as median (interquartile range) and compared using Wilcoxon rank-sum test. Categorical data were presented as n (percentage) and compared using Fisher's exact test. Correlation analysis was performed using Spearman's rank test. A level of $p < 0.05$ was used to determine statistical significances.

RESULTS

A total of 2225 patients completed the questionnaire. After applying of exclusion criteria, 2108 participations remained. Average age of the patients was 28.6±0.18 years old (range 18-77). There were 1135 females and 973 males. The majority of the group reported having mild (n=1158;54.93%) or moderate (n=862 (40.89%) COVID-19

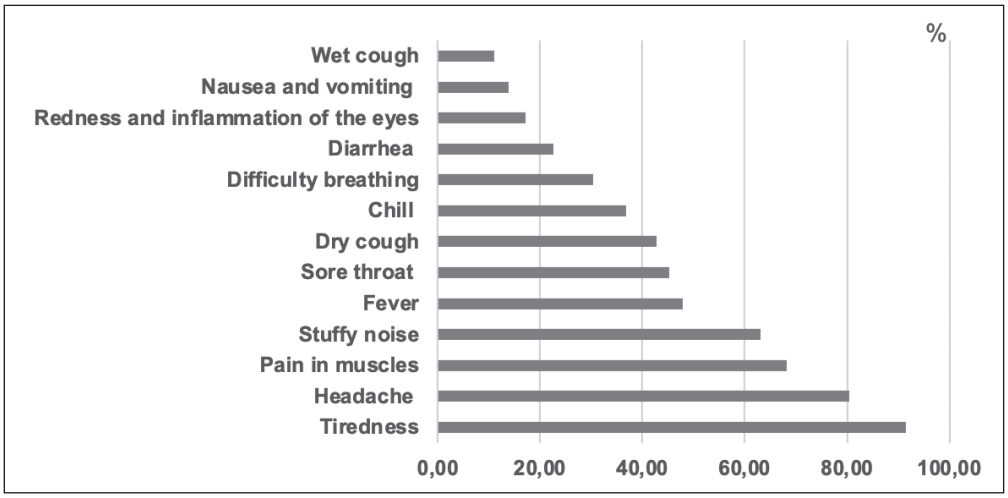

Fig. 1. General symptoms associated with COVID-19

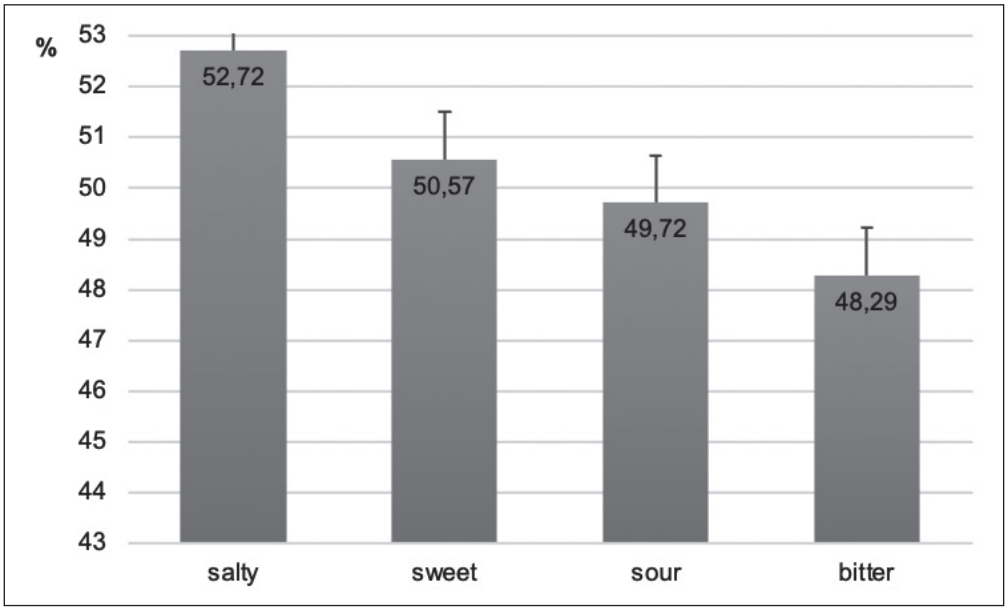

Fig. 2. Taste-quality specific changes

progression, while 78 patients (3.70%) had severe form of disease and 10 (0.47%) – critical one. Self-reported assessment of health status revealed that 289 or 13.71% of respondents had excellent health status before the disease, 1658 (78.65%) – good, 156 (7.40%) – bad and 5 (0.24%) – very bad. 96 people (4.55%) were hospitalized, 2012 (95.45%) had outpatient treatment at home. Demographic characteristics, self-reported health status and severity of disease of the respondents are shown in the Table I.

CLINICAL OUTCOMES

The distribution of self-reported symptoms reported by the patients as associated with COVID-19 is shown in the Fig.1. The most common ones were tiredness, headache, pain in muscles, stuffy noise, fever etc. There were no statistically significant differences in symptoms reported by males and females.

Less common self-reported outcomes (6.9%) included hemoptysis, dizziness, painful lymph in the neck, apathy, tachycardia, stomatitis, runny nose, constipation, low-grade fever (10 days – one month), skin rashes, faintness, pain in kidneys, mental depression, irritable bowel syndrome, pain in joints, dry nose, insomnia, hypertension within 14 days of COVID-19.

PREVALENCE AND FEATURES OF OLFACTORY DYSFUNCTIONS (OD)

A total of 1819 (86.29%) patients reported OD related to COVID-19, 96 people (4.55%) reported partial OD without anosmia and 193 (9.16%) patients did not have olfactory manifestations. Most of the patients with olfactory manifestations had mild or moderate mode of the disease (Table II).

One patient reported hyperosmia (heightened sense of smell). The majority of the respondents had OD within 15.46±0.45 days (median value was 10) and a long recovery time which lasted from two weeks to 240 days. Another reported OD were functional anosmia such as permanent feeling of smell of vinegar, festerment or other unpleasant odours, reduced sense of strong or unpleasant odours (chlorine). Among the participants was a woman who had

Table III. Duration of gustatory dysfunctions in patients with different severity of COVID-19

Severity of COVID-19	Duration of GD, days				
	none*	1-4	5-8	9-14	>15
mild	299	196	302	205	156
moderate	214	126	234	145	143
severe	28	5	19	14	12
critical	3	1	2	2	2
Grand total, n (%)	544 (25.81)	328 (15.56)	557 (26.42)	366 (17.36)	313 (14.85)

* 66 patients reported short-time GD during several hours were added in this group

Table IV. Frequency and duration of self-reported sensory impairments

Type of sensory impairment	Frequency of self-reported dysfunctions, n (%)			Average duration, days	Maximal duration, days
	significant	partial	total		
OD	1819 (46.87)	96 (4.55)	1915 (91.32)	15.46±0.45	240
GD	293 (15.6)	1099 (58.77)	1392 (66.03)	11.3±0.33	180
HD	280 (13.28)	302 (14.33)	582 (27.61)	4.3±0.16	60
VD	285 (13.52)	233 (11.05)	518 (24.57)	6.53±0.23	150

COVID-19 twice. She reported having had hyposmia after she had been infected in February. Her olfaction had not recovered yet when she was infected second time in November.

At the moment of completing questionnaire, only 100 (4.74%) patients reported anosmia while the majority of the group had partial (n=693; 32.87%) or full recovery (n=1131; 53.65%) of the ability to feel and distinguish odours. Most of the study population did not have any complaints on olfactory function before the infection (n=1982; 94.02%), 95 people (4.51%) reported nonsignificant dysfunctions and 31 (1.47%) went to doctor before being infected.

The was no correlation between duration of OD and age. Difference in average duration of OD was not statistically significant between groups of in- and outpatients and between groups with different severity of COVID-19 (p>0.05).

PECULIARITIES OF GUSTATORY DYSFUNCTIONS (GD)

There were 478 patients without gustatory dysfunctions (25.56%), whereas the majority of the participants had partial (n=1099; 58.77%) or complete (n=293; 15.67%) loss of ability to recognize taste stimuli (Table III). Average duration of GD in the study population was 11.3±0.33 days (ranged from several hours to 180 days), median value was 7 days.

Besides, participants were asked to report changes in specific taste-qualities (salty, sweet, sour, bitter). 519 (24.62%) patients reported impairments of all four taste qualities. Bitter and sour taste loss reported less frequently comparing to salty. Results are shown the Fig. 2.

Partial GD were accompanied by taste distortion. For instance, several patients reported that "all the food was bitter", other ones complained on absence of shades of taste: "when tasting a lemon, I felt only a little acid, and there was no taste of citrus" or "there was no difference between cookies and chocolate - they were just sweet". Inability to feel pleasure in eating manifested itself as following: "At the moment I cannot stand the taste of fresh cucumbers. They acquired for me a metallic-caustic-rotten taste, from which vomiting sensations arise. After eating cucumbers for several hours, nothing else interrupts this taste (and it continues to stand in the mouth). At the same time the taste of other products fades, and in some places completely disappears" or "The onion seemed to be rotting, the taste of coffee changed, the meat smelled unpleasantly". Patients also described the taste of food as "spoiled", "like a grass", "toilet paper", "plastic", "rubber".

97.11% of patients did not have any taste dysfunctions before infection, 45 people (2.13%) reported partial GD and 0.76% had to consult with doctor due to significant gustatory disorders before COVID-19.

By the end of follow-up, the majority of the patients reported partial (n=297;14.09%) or full recovery (n=1315; 62.38%) of gustatory function, whereas it had not returned to normal in 48 patients (2.28%). Duration and prevalence of GD depended neither age nor gender.

FEATURES OF HEARING DISORDERS

Hearing disorders were not so as common as OD and GD. 1526 patients (72.39%) did not have hearing disturbances associated with COVID-19, 302 people (14.33%) reported partial dysfunctions and 280 (13.28%) confirmed significant problems with auditory analyzer, affecting life quality. It is necessary to underline that the majority of the participants (n=1982; 94.02%) did not experienced HD before COVID-19. 69 people (3.27%) had nonsignificant complaints and 57 (2.70%) went to otolaryngologist before infection. Presence of auditory disorders in the anamneses (57 patients) can be a reason of hearing impairment during infection. We found that the difference between those who

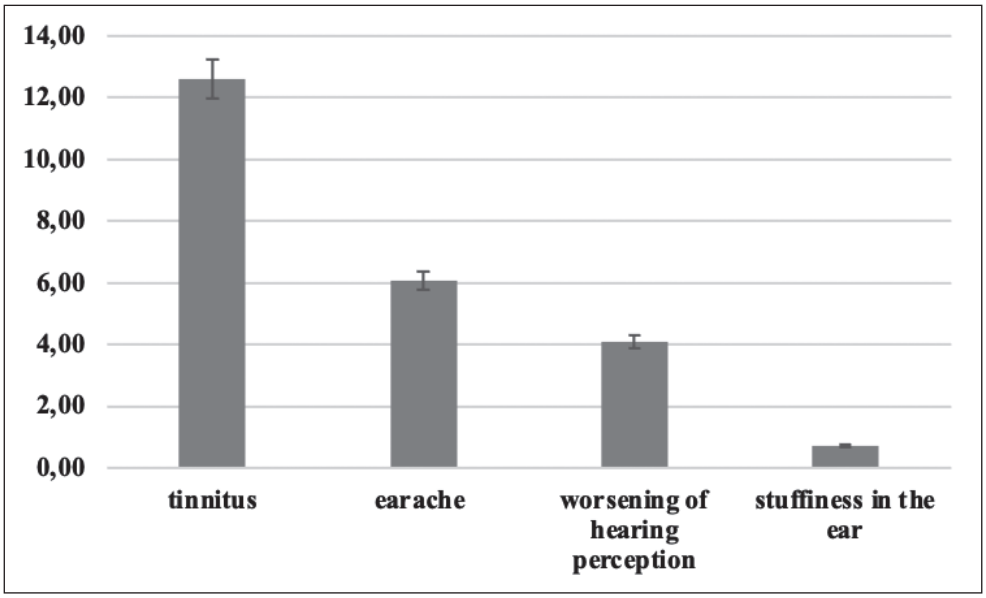

Fig. 3. The most common types of otologic dysfunctions reported by the patients with confirmed COVID-19

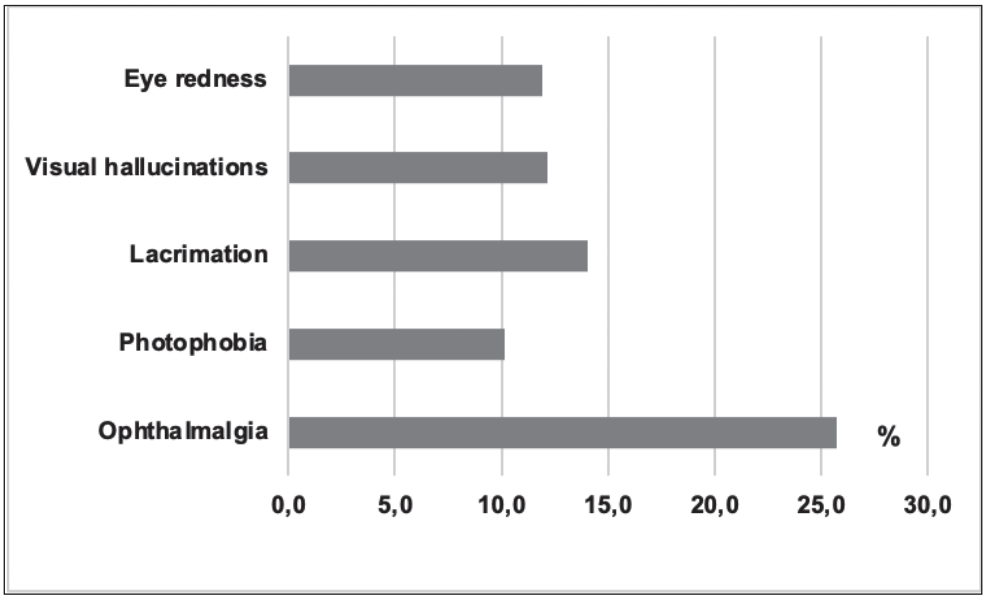

Fig. 4. Ocular manifestations reported by the patients

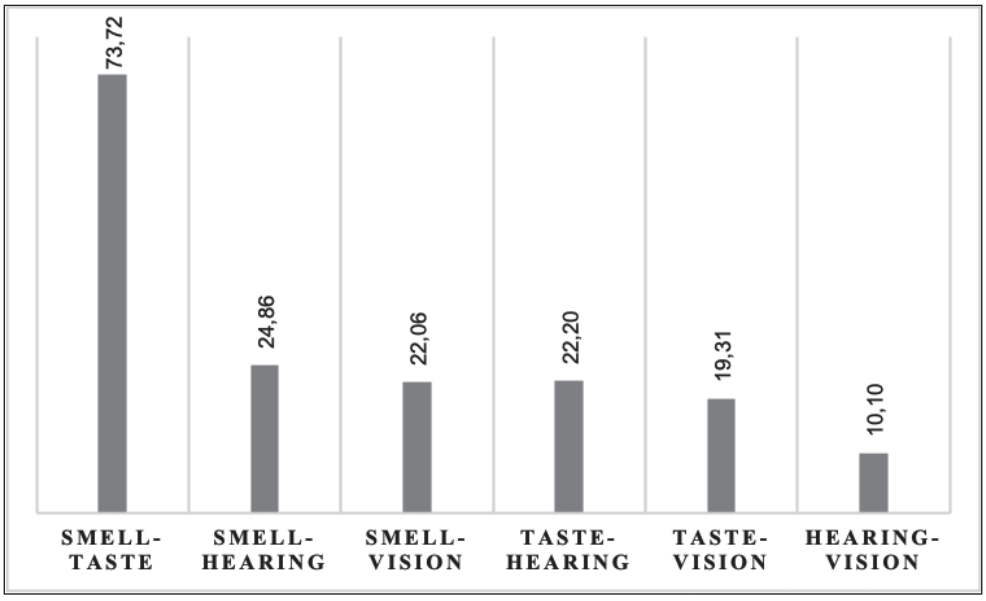

Fig. 5. Frequency of pair combination of sensory impairments regardless to other dysfunctions

reported otologic dysfunctions, associated with COVID-19 (n=27; 47.4%) and those who denied having them (n=30; 52.6%) was statistically nonsignificant. Average duration of hearing impairment associated with SARS-Cov-2 was 4.3±0.16 (ranged from 1 to 60 days). The most common dysfunctions reported by the patients (Fig. 3) were tinnitus (n=266; 12.62%) and earache (n=128; 6.07%), whereas worsening of hearing perception was reported by 86 participants (4.08%) and stuffiness in the ear – by 15 people (0.71%).

Stuffiness in an ear was compared to feelings, occurred in the mountains due to the changes of atmospheric pressure. Another patient described it as "being in an aquarium". Less common complaints reported by the participants were clack sounds in the ear, appearance of permanent irritative noise, temporary decreasing of hearing, accompanied by headache. One patient reported increasing of hearing sensitivity. HD remained in 38 patients (1.80%), 44 people (2.09%) reported partial recovery of hearing function.

CHARACTERISTICS OF OCULAR COMPLICATIONS IN STUDY POPULATION

A total of 518 of 2108 patients had ocular manifestations. Among them were partial (n=233; 11.05%) and significant (n=285; 13.52%) complications, associated with COVID-19. 1590 participants (75.43%) did not have any problems with visual analyzer. Presence of ocular complications did not depend on severity of disease and gender of the patients. Average duration of visual dysfunctions was 6.53±0.23 days (ranged from 1 to 150 days). The most common complaints reported by the patients are shown in the Fig. 4.

The main ophthalmic complaints included (in decreased order): ophthalmalgia (n=542; 25.71%), lacrimation (n=296; 14.04%), visual hallucinations, such as flickers, dots, flashes in the field of vision (n=257; 12.19%), eye redness (n=251; 11.91%), photophobia (n=214; 10.15%). Conjunctivitis was reported by 74 people (3.51%). Apart from this, patients complained on periodical reduction of visual clarity, blurred vision (for 4 weeks maximum), hypersensitivity of eyes, rapid visual deterioration.

Before having COVID-19 449 people (21.3%) had complaints on vision and went to ophthalmologist (the highest number of sensory dysfunctions before infection reported by the respondents). It is necessary to underline, that majority of patients who had in anamneses ophthalmological problems (n=293; 65.3%) did not have any ocular manifestations during COVID-19.

ASSOCIATIONS BETWEEN DIFFERENT SENSORY DISORDERS AND OTHER CLINICAL OUTCOMES

Sensory impairments reported by the respondents were different in frequency and duration (Table IV). Difference in average duration of sensory impairments was significant between all types of sensory impairments ($p<0.05$).

Besides, it was found a correlation between duration of OD and GD ($r=0.65$; $p<0.001$) and HD-VD ($r=0.49$; $p<0.05$). Probably, it can be explained by the presence of combined sensory dysfunctions (Fig. 5). In the majority of the respondents OD were accompanied by GD (n=1554; 73.72%).

Combination smell-taste-hearing dysfunction was reported by 455 people (21.58%), smell-taste-vision – by 396 respondents (18.79%), smell-hearing-vision – by 189 patients (8.97%), taste-hearing-vision – by 170 (8.06%).

Only 166 people (7.87%) had partial or significant impairment of all four sensory functions. This unique group included 95 females and 71 males who experienced different modes of COVID-19, reported all possible health states before the disease (from excellent to very bad). Most of them denied having sensory dysfunctions before infection (90.96% had no previous history of OD, 95.78% - GD, 87.34% - HD and 52.41% - VD). Interesting, that this group included people with the maximal recovery time of sensory functions (see table IV) except hearing (maximal recovery time in this group was 30 days).

DISCUSSION

Our results confirm and substantially extends previous studies concerning sensory impairments associated with COVID-19. The exact pathophysiological mechanisms of sensory impairments caused by SARS-CoV-2 remain unclear. However, according to the most popular hypothesis, coronavirus uses an olfactory pathway as a gate to the central nervous system.

Generally, OD can be either quantitative, when strength alteration takes place or qualitative involving the changing of odors. First group is classified into hyposmia (reduced sense of smell), functional anosmia (when a person can detect only occasional odors), and anosmia (total loss of the ability to detect one or more smells) [10].

The prevalence of self-reported OD and GD obtained in our study stay within data published earlier. The prevalence of OD and GD were analyzed among 8438 patients in a systematic review conducted by Agyeman A et al. [11]. They concluded that the reported prevalence of OD ranged from 3.2 to 98.3%, and the pooled prevalence was 41.0%, whereas gustatory dysfunctions were reported or objectively confirmed in the range from 5.6% to 62.7% of patients with COVID-19 and the pooled prevalence was 38.2%. Our findings about average duration of sensory impairments partially confirm results of other scientists. For example, Klopfenstein et al. [12] studied 114 patients with confirmed COVID-19 and found that the mean duration of anosmia was 8.9 days.

Our results support an idea [13] that this viral infection and inflammatory response may lead to disruption of saliva composition, normal taste transduction or the continuous renewal of taste buds. For instance, SARS-CoV-2 may occupy the binding sites of sialic acid on the taste buds. Reduction of sialic acid lead to increasing of gustatory threshold [14].

As it was mentioned above, we did not find any correlations between OD/GD and age or gender of the patients.

It corresponds to the latest research in adult population, which shows that sex is not associated with smell and taste disturbances [15]. At the same time, Giacomelli A. and Pezzati L. conducted as a verbal interview with 69 patients, hospitalized patients with COVID-19, and showed that olfactory dysfunctions were more prevalent in younger people and women [16]. Approximately the same conclusion was made another group of scientists studying prevalence of chemosensory dysfunctions in infected patients. Von Bartheld CS et al. conducted a systematic review and meta-analysis. The authors found that smell or taste dysfunction or both decreased with older age, male gender, and disease severity [17]. Their study also revealed significant ethnic difference: Caucasians had a three times higher prevalence of chemosensory dysfunctions (54.8%) than Asians (17.7%). Probably, uncontrolled factors (genetic, individual immune resistance etc.) play the main role in chemosensory dysfunctions. Testing of this hypothesis requires additional studies and usage of factorial analysis. While the underlying mechanisms of chemosensory dysfunction is still under discussion, an international research team of Harvard Medical School made a progress in their explaining [18]. It was suggested that such mechanisms are determined not by the direct infecting the neurons, but by the affection the vascular cells in the nervous system. Losing of taste and smell differs from those, caused by flu or cold. For instance, COVID-19 patients don't have a stuffy or runny nose and can breathe freely. Ageusia is usually accompanied by the significant reducing of sensitivity and inability to distinguish bitter or sweet. Combination of taste and smell impairments in 73.72% of respondents (Fig. 5), confirms that these senses are closely linked to each other, having many overlapping brain areas. Inflammatory damage of frontobasal region may alter both senses.

We did not find any comprehensive epidemiological studies devoted to self-reported hearing dysfunctions among patients infected by SARS-Cov-2 in scientific literature. Data concerning hearing dysfunctions associated with COVID-19 are scarce and usually are limited by case studies [19]. In a systematic review, published by Maharaj S. et al in 2020 [20] 62 articles were analyzed. The authors found only 5 case reports and 2 case series describing 28 patients with hearing loss, associated with COVID-19. According to results, obtained by Munro K et al., 13.2% of patients admitted to Manchester University Hospital reported changes in hearing and/or tinnitus, associated with coronavirus [21], which is comparable with our results. It is well known that sudden sensorineural hearing loss (SSNHL) is associated with viral infections (measles, mumps, meningitis). Auditory pathway, including cochlear nerve, cochlea, perilymphatic tissue can be affected due to ascending infection from the nasopharynx or damaged due to restricted oxygen and blood supply. Taking medication, emotional strain caused by pandemic, fatigue are also possible explanations of hearing dysfunctions during COVID-19. Even though none of our respondents reported having deafness or SSNHL, we obtained valuable data concerning the prevalence and features of HD among patients with confirmed COVID-19.

Since viral RNA have been found in the tears of infected people [22], ocular manifestations in confirmed COVID-19 patients attract an attention of scientists. In a systematic review [23] which included 16 studies reporting 2347 confirmed cases of COVID-19, the authors found that 11.64% of COVID-19 patients had ocular dysfunctions. The most common ocular abnormalities were ocular pain (31.2%), increased secretions (19.2%), redness (10.8%), conjunctivitis (7.7%). According to recent studies, the incidence of conjunctivitis among COVID-19 patients is ranged between 8 and 31.6% [24-25]. Probably, it happens because the ocular surface could be a portal of entry and a reservoir for viral transmission. Studies of ocular manifestation associated with COVID-19 are highly criticized. In the mentioned above systematic review, Aggarwal K et al. were skeptical about results, published earlier for "it is not clear whether these ocular features were pre-existing or occurred as a result of COVID-19 infection". According to our results, the majority of patients who had previously experienced ophthalmopathy did not have any complaints on eye disorders during infection. So that ocular manifestations reported by the patients can be considered as nonspecific evidence of SARS-Cov-2 along with other sensory impairments.

CONCLUSIONS

1. The axiomatic statement concerning the necessity of early recognition of SARS-Cov2 and strict adherence to the patient's roadmap requires the visualization of markers that herald the disease onset. Which, according to the obtained results, can be the appearance of signs of sensory dysfunctions.
2. Analysis the frequency and mechanism of sensory impairments in COVID-19, highlights one of the potential cornerstones of understanding the infectious process. Obtained results can be used to develop guidelines for personal protective equipment during a pandemic.
3. Significant prevalence of quantitative and qualitative manifestations of chemosensory dysfunctions requires further study of the virus penetration. An activation of a large number of neurovegetative impairments confirms the possibility of neurogenic pathway of virus.
4. Patients with all four sensory dysfunctions demonstrate significantly slower recovery time and need extended rehabilitation and psychological support. An inclusion of ophthalmologists and otolaryngologists in the early treatment stages is necessary for early recognition and better therapy of sensory dysfunctions.

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ORIGINAL ARTICLE

THE IMPACT OF MENTAL HEALTH, SUBJECTIVE HAPPINESS AND RELIGIOUS COPING ON THE QUALITY OF LIFE OF NURSING STUDENTS DURING THE COVID-19 PANDEMIC

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ABSTRACT

The aim of this study is to examine the effect of psychological distress and religious coping in quality of life of nursing students during the second wave of the pandemic in Greece.

Materials and methods: A cross-sectional online survey was conducted among nursing students. Data were collected via an e-survey consisting of five parts including HADS Questionary, SF-36, B-RCOPE and Subjective Happiness scale.

Results: From the total of 200 nursing students the 86.5% were female, 35.5% were in their first year of study, 54% were single and 65.5 were urban residents. 51.9% of the students were experiencing anxiety and 31,5% were depressed. In regard to subjective happiness, the mean value was 4.51±1.27. In addition, the majority of the students consider themselves unhappy (67.5%). Finally, in regard to SF-36 scores, we observed that PCS mean score was 68.49±13.19, MCS56.12±24.23. Depression, as well as negative religious coping, can have a negative effect on both physical and mental health components of quality of life.

Conclusions: Nursing students experience very high levels of stress and anxiety during the COVID-19 pandemic and need support and guidance to better manage stress and fear in this unusual situation.

KEY WORDS: mental health, quality of life, religious coping, nursing students, COVID-19

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INTRODUCTION

COVID-19 PANDEMIC PERIOD

COVID-19 is an infectious disease that can spread among humans. It first appeared in the city of Wuhan in China in late December 2019, when cases of pneumonia of unknown etiology were reported. Following the outbreak of the virus, it spread worldwide and led the World Health Organization (WHO) to declare a global pandemic. The compulsory quarantine that followed had a serious impact on the psychological state of the citizens. A recent review study showed that the negative psychological effects of quarantine typically include confusion, anger, and symptoms of post-traumatic stress, as well as an increase in the prevalence of anxiety and depression symptoms in the general population. The quarantine that was imposed changed the people's way of life [1].

With the introduction of recommendations for the non-spreading of COVID-19 such as frequent hand washing, use of a face mask outside the home, avoiding the touching of one's face, nose, eyes with hands, social distance has resulted. Ahorsu et al have shown that fear of COVID-19 is positively associated with depression, anxiety, perceived infectivity, and microbial aversion in Iran [2]. Compared to the activity of Weibo users (popular social media in China) before and after the outbreak of COVID-19 in China, Li et al found an increase in negative emotions such as anxiety, depression, and resentment as there was a decrease in positive emotions and life satisfaction [3].

PANDEMIC AND QUALITY OF LIFE OF STUDENTS. NURSING STUDENTS

In addition, the changes that the pandemic brought to higher education and the implementation of online educational activities further isolated students globally. To date the are few studies examining the effect that COVID-19 pandemic and quarantine have on mental health and quality of life of nursing students and the role that negative/positive religious coping can on these variables.

According to a cross-sectional study among nursing students in West Virginia, 21% to 54% of nursing student QoL scores indicated poor QoL. While resilience, having online experience, and being well prepared for online learning were found to affect various QoL domains [4]. Increased stress and poor mental health have been associated with poor quality of life among nursing students in Poland and

Spain revealing both the effect that the covid-19 pandemic had on those variables as well as the association between mental health and Quality of life [5].

MENTAL HEALTH, SUBJECTIVE HAPPINESS AND RELIGIOUS COPING. PANDEMIC PERIOD

According to a recent cross-sectional study in Colorado in which 222 nursing students participated, 25% of students reported moderate to severe levels of psychological distress while 23.8% of students scored within the area of clinical concern for the presence of posttraumatic stress disorder [6]. Similarly, in a larger scale study in the U.S.A among 2031 college students, 48.14% reported moderate-to-severe levels of depression, 38.48% reported moderate-to-severe levels of anxiety while 18.04% stated that had suicidal thoughts. According to the majority of participants (71.26%), their stress/anxiety levels had increased during the pandemic while less than half of the students (43.25%) indicated that they were able to cope adequately with the stress related to the current situation [7].

According to a recent qualitative study that aimed to explore the coping strategies of nursing students during the COVID-19 pandemic, three main effective strategies were used from the students staying positive, use of infection prevention measures and religion were identified as effective coping strategies [8]. In the context of another study among 331 students in Brazil religious practice, was found to be associated with lower scores of depression, anxiety, and stress symptoms, as well as with coping strategies that can act as protective factors [9]. Similar results were reported by Rahimi, Bakar, and Yasin in their study in which 450 students participated. According to the researchers, the level of psychological disorder among university students has been high during the pandemic. In addition, higher negative religious coping was significantly associated with psychological disorders, while higher positive religious coping was found to be protective against psychological disorders [10].

THE AIM

Although the are some international studies examine the effect that religious coping can have on mental health in nursing students the effect that religious coping and psychological distress can have on the quality of life is not fully investigated, especially in Greece. Thus, the aim of this study is to examine the effect of psychological distress and religious coping on the quality of life of nursing students during the second wave of the pandemic in Greece.

MATERIALS AND METHODS

STUDY DESIGN AND PARTICIPANTS

A cross-sectional online survey was conducted among nursing students during March 2021 in two university nursing departments in Greece. Participation was voluntary. The Nursing students of the two universities were invited to participate in the online survey. The participants provided informed consent anonymously on the survey platform before they could proceed to the completion of the questionnaire. A structured questionnaire was used for data collection.

ETHICS

The study protocol was approved by the Ethics Committee of the participated Departments. Students were informed that their participation is voluntary, anonymous, the collected data would be used for the purposes of the study. In addition, they were informed that they could withdraw from the study at any point they wished. This study was in adherence with the Helsinki Declaration as well as with the ethical standards of the responsible institution on human subjects.

DATA COLLECTION

Data were collected via an e-survey using specially designed five-sections questionnaire.

The *first section* collected socio-demographic and academic information through standard questions.

The *second section* contained Hospital Anxiety and Depression Scale (HADS) [11,12]

In the *third section* we used Greek version of the Short Form 36 (SF-36) questionnaire (self-administrated questionnaire that evaluates health related Quality of Life) [13,14].

In the *fourth section* we assessed religious coping with the Brief Religious Coping inventory (B-RCOPE) (two score Positive Religious Coping (PRC) and Negative Religious Coping (NRC) styles) [15,16].

The *fifth section* contained Subjective Happiness scale [17,18].

STATISTICAL ANALYSIS

Data is presented as mean±SD or as percentages. Dichotomous variables are expressed as percentages. Normal distribution was assessed by the Kolmogorov-Smirnov test. Univariate correlations were performed by Pearson's correlation coefficient. Linear regression analysis was performed to estimate the impact of Mental health, Subjective Happiness, and Religious coping on the Quality of life of nursing students. The coefficient of determination (r2) was used to estimate the percentage of effect explained by the model. A p-value of <0.05 was considered to be statistically significant. Analysis was performed using the SPSS 25 statistical package (SPSS Chicago, IL).

RESULTS

From the total of 200 nursing students the 86.5% were female, 35.5% were in their first year of study, 54% were single and 65.5 were urban residents. Detailed information

Table I. Sample demographic and academic characteristics (n=200), n (%)

Sex	Female	173 (86.5)
	Male	27(13.5)
Age		22.8±12.2
Sexual Orientation	Heterosexual	177(88.5)
	Homosexual	8(4)
	Other/No responce	15(7.5)
Year of Study	1o	71(35.5)
	2o	81(40.5)
	3o	31(15.5)
	4o or more	15(8.5)
Classes	0	127 (63.5)
	1	30 (15)
	2	9 (4.5)
	3	7 (3.5)
	4 or more	25 (12.5)
Family status	Single	108(54)
	Married	7(3.5)
	Cohabitation agreement	3(1.5)
	In relationship	59(29.5)
	Other	23(11.5)
Area of Residence	Rural	37(18.5)
	Semi urban	33(16.5)
	Urban	130(65.5)
Monthly family income	Low income	43(21.5)
	Middle income	71(35.5)
	High income	76(38)

regarding sample demographic and academic characteristics are presented in table I.

Table II presents the results the SF36 questionnaire, HADS and subjective happiness scale. Briefly, the HADS score for anxiety was found to be 8.71 ± 4.32 and for depression was 7.03 ±3.26. Taking 8 as a cut-off value the 51.9% of the students were experiencing anxiety and 31,5% were depressed. In regards to subjective happiness, the mean value was 4.51±1.27. Taking into consideration as a cutoff point the value 5 then the majority of students consider themselves as unhappy (67.5%). Finally, in regards to SF-36 scores, we observed that PCS mean score was 68.49±13.19, MCS56.12±24.23. The greater score in SF-36 was observed in PF 87.30±18.86 while the lowest was observed in RE 41.16±40.51 which was the only value below 50 that is considered a normative value of SF-36 scores. Detailed descriptive statistics for variables of the study are presented in table II.

Bivariate analysis between sample demographic characteristics and QoL domains revealed that there is a statistically significant difference [F(4,195)=2.753, p=0.029] in PF between married participants (69.5±30.7) compared to singles (87.3±), in relationship (88.3±13.8) and other relationship status (88.2±18.4). In addition, a significant difference [F(4,195)=2.527, p=0.042] was observed in the BP score of the participants that were in cohabitation agreement (50±50) as compared to singles (75.7±20.7) and other relationship status (78.7±20.7). Statistically significant differences [F(2,187)=3.625, p=0.029] also observed in RP in participants with low family income(47.09±38.6) compared with middle (64.0±40.25)and high (66.1±37.7) family income. Moreover, statistically significant differences [F(2,187)=4.558, p=0.012] were also observed in PCS in participants with low family income (63.9±12.5) compared with middle (71.4±14.7)and high (68.6±10.9) family income.

The association of QoL with students' mental health, subjective happiness and religious coping was estimated with the Pearson correlation coefficient. Negative significant correlations were observed between almost all of QoL subscales with HADS Anxiety, HADS Depression and Negative Religious Coping. Subjective Happiness exhibited significant positive correlations with almost all of QoL domain and significant negative correlations with HADS Anxiety, HADS Depression and Negative Religious Coping. The detailed results of the Pearson correlation test are presented in Table III.

Table IV show the results of multiple regression with enter method results with QoL Summary scores as dependent variables and Mental health, Subjective Happiness and Religious Coping as the independent variables, adjusted for demographic and work-related characteristics. We further found that HADS Anxiety (<0.001), HADS Depression (0.003), and Negative Religious Coping (0.001) were independently predictive of PCS of SF-36. While HADS Anxiety (<0.001), HADS Depression (0.003) Positive Religious Coping (0.013), and Negative Religious Coping (0.036)) were independently predictive of MCS of SF-36.

DISCUSSION

The present study examined the effect of mental health, subjective happiness, and religious treatment on the quality of life of nursing students during the Covid-19 pandemic in Greece.

The mean HADS score for anxiety was found to be 8.71 ± 4.32 and for depression 7.03 ± 3.26. Taking 8 as the cut-off value, 51.9% of students experienced anxiety and 31.5% depression. An online survey of the general population of Wuhan, China, found that the prevalence of stress was 22.6% during COVID-19 [19]. According to Huang and Zhao [20] the prevalence of stress in general population during the onset of the COVID-19 pandemic was up to 35%. In addition, as was reported by Liang et al [21], 40% of Chinese people were reporting psychological problems from the begging of the pandemic. Zhang and Ma found that the general population in China reported mild stress due to COVID-19 epidemic with 7.6% of participants to report moderate to severe stress according to the study [22]. According to recent review of the literature the most common psychological problems that were reported during

Table II. Descriptive statistics for variables of the study

	min	max	mean	SD
HADS Anxiety (HA)	0.00	18.00	8.71	4.32
HADS Depression (HD)	1.00	15.00	7.03	3.26
Happiness (HP)	1.25	7.00	4.51	1.27
Possitive Religious Coping (PRC)	7.00	28.00	13.54	6.29
Negative Religious coping (NRC)	7.00	28.00	9.74	4.32
Physical Functiong (PF)	0.00	100.00	87.30	18.86
Role Personal (RP)	0.00	100.00	61.62	39.29
Bodily Pain (BP)	0.00	100.00	73.12	22.29
General Health (GH)	0.00	97.00	62.82	17.89
Vitality (VT)	0.00	95.00	50.75	19.92
Social Functioning (SF)	0.00	100.00	68.68	27.90
Role Emotional (RE)	0.00	100.00	41.16	40.51
Mental Health (MH)	4.00	92.00	53.04	20.16
Physical Component Summary (PCS)	0.00	94.25	68.49	13.19
Mental Component Summary (MCS)	5.00	98.00	56.12	24.23

Table III. Correlation between SF-36 scores, HADS, Subjective Happiness and Religious Coping

	HA	HD	SHP	PRC	NRC	PF	RP	BP	GH	VT	SF	RE	MH	PCS	MCS
HA		0.573 ***	-0.538 ***	0.058	0.358 ***	-0.328 ***	-0.252 ***	-0.177 *	-0.301 ***	-0.484 ***	-0.249 **	-0.426 **	-0.642 **	-0.477 **	-0.485 **
HD	0.573 ***		-0.629 ***	-0.020	0.191 **	-0.291 ***	-0.289 ***	-0.145 *	-0.286 ***	-0.565 ***	-0.321 ***	-0.368 ***	-0.619 ***	-0.476 ***	-0.492 ***
SHP	-0.538 ***	-0.629 ***		0.025	-0.302 ***	0.232 ***	0.100	0.008	0.418 ***	0.501 ***	0.328 ***	0.358 ***	0.646 ***	0.416 ***	0.419 ***
PRC	0.058	-0.020	0.025		0.442 ***	-0.099	0.050	-0.086	-0.005	-0.051	0.017	0.119	-0.053	-0.093	0.064
NRC	0.358 ***	0.191 **	-0.302 ***	0.442 ***		-0.290 ***	-0.148 **	-0.187 **	-0.303 ***	-0.217 **	-0.160 *	-0.129	-0.347 ***	-0.367 ***	-0.232 ***

*p<0.05, **p<0.01, ***p<0.001 . HADS Anxiety (HA),HADS Depression (HD), Subjective Happiness (SHP), Possitive Religious Coping (PRC), Negative Religious coping (NRC), Physical Functiong (PF), Role Personal (RP), Bodily Pain (BP), General Health (GH), Vitality (VT), Social Functioning (SF), Role Emotional (RE), Mental Health (MH), Physical Component Summary (PCS), Mental Component Summary (MCS)

the COVID-19 pandemic were anxiety and depression (16–28%) and self-reported stress (8%).

In terms of subjective happiness, the mean value was 4.51 ± 1.27. Considering the value 5 as a cut-off point, the majority of students consider themselves unhappy (67.5%). In a survey conducted on 59 senior Turkish nursing students to assess subjective happiness, the majority of them (69.5%) showed sufficient subjective happiness. However, 55.9% of participants were worried about the future of the current COVID-19 pandemic [23].

Regarding the assessment of the quality of life related to health (SF-36), the average PCS score was 68.49 ± 13.19 while the average MCS score was 56.12. 24.23. The highest score on SF-36 was observed at PF 87.30 ± 18.86 while the lowest was observed at RE 41.16 ± 40.51 which was the only value below 50 which is considered the regulatory value of SF-36 scores. As evidenced by other studies, students faced emotional and mental health challenges during the pandemic. The psychological effects of COVID-19 on students vary between countries and regions due to differences in COVID-19 infection (24-27). A recent quantitative in Spain in regards to psychological distress experienced by faculty members as an effect of the COVID-19 pandemic indicated that lockdown severely affected both students (76.8%) and staff (23.2%). More specific showed that 87, 5%, 48.1%, 35.2%, and 40.3% of the respondents reported symptoms of post-traumatic stress disorder, depression, anxiety, and stress. Furthermore, the same study highlighted that nursing students were experiencing higher levels of depression, anxiety, and stress [28]. Similarly, in a study conducted

Table IV. Multiple regression results with SF-36 summary scores as dependent variables and HADS, Subjective Happiness and Religious Coping as independent, adjusted for demographics and other sample characteristics (n=200).

Quality of Life		β	SE	95% CI		p
				Lower	Upper	
Physical component Summary	HADS Anxiety	-0.830	0.212	-1.248	-0.412	<0.001
	HADS Depression	-0.923	0.305	-1.524	-0.322	0.003
	Happiness	0.798	0.781	-0.742	2.338	0.308
	Positive Religious Coping	0.117	0.136	-0.150	0.385	0.388
	Negative Religious Coping	-0.697	0.215	-1.121	-0.272	0.001
	adjusted R^2=31.3%,F=12.577,p<0,001					
Mental component Summary	HADS Anxiety	-1,491	0,396	-2,271	-0,711	<0,001
	HADS Depression	-1,687	0,569	-2,809	-0,566	0,003
	Happiness	1,836	1,458	-1,037	4,709	0,209
	Positive Religious Coping	0,635	0,253	0,137	1,134	0,013
	Negative Religious Coping	-0,847	0,402	-1,639	-0,055	0,036
	adjusted R^2=29%F=11.353,p<0,001					

Notes: Regression coefficient (standard error) adjusted for sex, age, marital status and family income.

at Texas A&M University14, USA, found that 80.6% and 71.8% of respondents reported symptoms of depression and anxiety. While according to a similar study in Bangladesh among quarantined students, in regards on the impact of the COVID-19 pandemic on students' mental health showed that 69.3%, 46.9%, 33.3%, and 28.5% of students were reporting symptoms of post-traumatic stress, depression, anxiety, and stress [29]. Finally, in a large contemporaneous survey that was conducted among students in China's Guangdong Province which aimed to assess psychological impact of the COVID-19 pandemic indicated that half of the participants (50.9%) had abnormal scores on the health assessment scale and 0.5% them reported poor mental health and 3.2% reported poor sleep quality [30].

The average score of the PRC scale was calculated at 13.54 ± 6.29 while that of the NRC was 9.74. 4.32. It has been found that during times of crisis, people often engage in religious activities [31]. During the COVID-19 pandemic, people around the world pray for an end to the crisis, as it concerns people from all walks of life [32]. In a Community survey [33] during the COVID-19 pandemic in India, more than half of the participants agreed that there has been an increase in spirituality during the ongoing pandemic among the general population. Studies (34) support that a positive religious attitude helps to combat stress, while a negative religious attitude can worsen stress and guilt. Previous studies [35] with chronic diseases have shown that positive religious treatment is associated with a better overall quality of life in patients. A study conducted in India and Nigeria found that significant proportions of people after the COVID-19 pandemic took religious measures to overcome their problems. During this pandemic, positive religious treatment among the communities of India and Nigeria is more prevalent than negative religious treatment [36].

The bivariate analysis between the demographic characteristics of the sample and the QoL domains revealed that there is a statistically significant difference in the PF between the married participants compared to not married, in the relationship and other relationship status. In addition, a significant as observed in the BP score of the participants who were in a cohabitation agreement compared to the unmarried and the status of another relationship. In addition, an online survey conducted in Saudi Arabia reported mild to moderate levels of anxiety among the general population and a significantly higher level of anxiety among married respondents [37]. Statistically significant differences were also observed in the RP in participants with low family income compared to middle and high family income. In addition, statistically significant differences were also observed in PCS in low-income participants compared to middle-income and high family income. A similar studies concluded that the increased stress is due to reasons related to financial and labor market problems for Polish sudents [38], reduced psychological well-being is associated with low level of mental health literacy for Indonesian students [39] due to the COVID-19 pandemic.

LIMITATION OF THE STUDY

The present study includes some limitations. Although the sample size in this study is representative of the university, its results cannot be generalized to the entire population of university students in Greece as well as in other countries. Greater and more generalized research is needed on the impact of mental health, subjective happiness, and religious attitudes on the quality of life of nursing students during the Covid-19 pandemic worldwide.

Future research should include students from different types of universities and colleges (eg medical, social sciences, technical sciences, theoretical sciences). The research is about starting the general quarantine for the coronavirus, when the level of stress and anxiety may be the highest.

It is possible that over time and gaining the ability to deal with coronavirus quarantine, stress will gradually decrease. Future research should be conducted prospectively at different stages of coronavirus spread. Finally, this study did not examine variables related to stress and anxiety, such as contact with family, place of permanent residence (urban center or province), and time spent on social networking sites. Further research should include these co-variables in the examination of stress and anxiety during COVID-19 among college students.

CONCLUSIONS

The main conclusion of this research is that university students experience very high levels of stress and anxiety during the COVID-19 pandemic and need support and guidance to better manage stress and fear in this unusual situation. Participating in healthy activities such as physical exercise can improve the ability to cope with this dramatic situation. In addition, the university administration should organize strategies to prevent and deal with the stress and stress of their students. Finally, the information and education of students are considered necessary to be able to respond to the outbreak of the disease.

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ORIGINAL ARTICLE

BIOFLAVONOIDS AS AGENTS FOR CORRECTING NITRO-OXIDATIVE STRESS AND SALIVARY GLAND FUNCTIONS IN RATS EXPOSED TO ALCOHOL DURING MODELED LIPOPOLYSACCHARIDE-INDUCED SYSTEMIC INFLAMMATORY RESPONSE

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ABSTRACT

The aim: To investigate the effects of bioflavonoids (curcumin, epigallocatechin-3-gallate and quercetin) on nitro-oxidative stress and the functions of submandibular SGs in rats under alcohol exposure during SIR.

Materials and methods: The studies were conducted on 35 rats of the Wistar line weighing 205-220 g, divided into 5 groups of seven animals in each: the 1st group, control group I, included animals receiving isotonic sodium chloride solution intragastrically twice a day; the 2nd group, control group II, included rats exposed to alcohol (in a dose of 24 mg/kg intragastrically through gavage a twice a day) for last 2 weeks during lipopolysaccharide (LPS)-induced SIR; the rats of the 3rd, 4th and 5th groups exposed to alcohol during LPS-induced SIR, which also received bioflavonoids. The bioflavonoids ("Sigma-Aldrich, Inc.", USA) were as following: curcumin (in a daily dose of 200 mg/kg), epigallocatechin-3-gallate (in a daily dose of 40 mg/kg), and quercetin (in a daily dose of 200 mg/kg), respectively. SIR was induced by intraperitoneal administration of *Salmonella typhi* LPS (during the first week a dose of 0.4 μg/kg of body weight was administered 3 times a week; during the next 7 weeks of the experiment rats received 0.4 μg/kg of body weight once a week. The formation of superoxide anion radical ($O_2^{\cdot-}$), activity of NO-synthase – total (NOS), its constitutive and inducible isoforms (cNOS, iNOS), and concentration of peroxynitrites and S-nitrosothiols were evaluated spectrophotometrically. To assess the functional status of submandibular SGs in their homogenate we determined α-amylase activity (spectrophotometrically) and the aquaporin-5 concentration (by enzyme-linked immunosorbent assay). through gav-age with orogastric cannul.

Results: When applying bioflavonoids under the conditions of alcohol administration during SIR, NADH-induced $\cdot O_2^-$ production decreased and yielded to the result in the control group II by 36.8% under administering curcumin, by 34.5% under administering epigallocatechin-3-gallate, and by 41.3% under administering quercetin. The total NOS activity in SGs tissues was inferior by 42.8% to the relevant data in the control group II (under curcumin administration), by 33.7% (under epigallocatechin-3-gallate administration) and by 46.6% (under quercetin administration); and the iNOS activity decreased by 47.0, 38.3 and 52.0%, respectively. Under the administration of bioflavonoids peroxynitrites concentration in the submandibular SGs tissues was inferior to the control group II by 35.6% (under curcumin administration), by 37.4% (under epigallocatechin-3-gallate administration), and by 39.3% (under quercetin administration); the content of S-nitrosothiols was lower by 34.5, 31.1 and 35.3%, respectively. The administration of bioflavonoids led to the changes in α-amylase activity in the submandibular SGs tissues: its values exceeded the relevant data in the control group II by 40.4% (under curcumin administration), by 38.2% (under epigallocatechin-3-gallate administration), and by 34.1% (under quercetin administration); under those conditions aquaporin-5 concentration grew in 2.66, 2.61 and 2.55 times, respectively.

Conclusions: The use of bioflavonoids (curcumin, epigallocatechin-3-gallate, and quercetin) under the combined administration of 40% ethanol solution and LPS considerably limits the development of nitro-oxidative stress in the tissues of the submandibular SGs. The administration of the bioflavonoids increases the level of cNOS coupling, and improves the functional status of the submandibular SGs under the combined administration of alcohol and LPS enhancing the activity of α-amylase and concentration of aquaporin-5.

KEY WORDS: bioflavonoids, curcumin, epigallocatechin-3-gallate, quercetin, lipopolysaccharide-induced systemic inflammatory response

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INTRODUCTION

More than a half (57%, or 3.1 billion people) of the global population aged 15 years and over have abstained from drinking alcohol in the previous 12 months. Some 2.3 billion people are current drinkers. Alcohol is consumed by more than a half of the population in only three World Health Organization regions – the Americas, Europe and Western Pacific [1].

Recent experiments on white rats exposed to systemic inflammatory response (SIR) have revealed metabolic and functional disorders of SGs that are supposed to occur due to increasing concentrations of reactive oxygen and nitrogen species [2, 3]. Numerous studies have found out the relationship between inflammatory and dystrophic SGs diseases and systemic diseases, pathogenesis of which is accompanied by the SIR devel-

opment (metabolic syndrome, cardiovascular diseases, diabetes, etc.) [4, 5, 6, 7].

Promising approaches in the SIR pathogenetic correction may consist in applying bioflavonoids capable of modulating the activity of redox-sensitive transcription factors NF-kappa B and Nrf2 with subsequent inhibition of expression of genes encoding pro-inflammatory cytokines, acute phase proteins, nitro-oxidative stress markers, etc. [8, 9, 2].

However, the efficiency of polyphenols as agents for pathogenetic therapy of alcoholism against the background of systemic inflammatory response has not yet been elucidated.

THE AIM

The aim of this study is to investigate the effects of bioflavonoids (curcumin, epigallocatechin-3-gallate and quercetin) on nitro-oxidative stress and the functions of submandibular SGs in rats under alcohol exposure during SIR.

MATERIALS AND METHODS

The studies were conducted on 35 rats of the Wistar line weighing 205-220 g, divided into 5 groups of seven animals in each: the 1st group, control group I, included animals receiving isotonic sodium chloride solution intragastrically through gavage twice a day; the 2nd group, control group II, included rats exposed to alcohol for last 2 weeks during lipopolysaccharide (LPS)-induced SIR; the rats of the 3rd, 4th and 5th groups exposed to alcohol during LPS-induced SIR, which also received bioflavonoids intragastrically through gavage. The bioflavonoids ("Sigma-Aldrich, Inc.", USA) were as following: curcumin (in a daily dose of 200 mg/kg), *epigallocatechin-3*-gallate *(in a daily dose of* 40 mg/kg), and quercetin (in a daily dose of 200 mg/kg), respectively.

To simulate the pattern of alcohol consumption, 40% ethanol solution in a dose of 24 mg/kg was administered intragastrically through gavage a twice a day for 14 days [10]. SIR was induced by intraperitoneal administration of *Salmonella typhi* LPS (pyrogenalum, "Medgamal", RF) according to the following scheme: during the first week a dose of 0.4 μg/kg of body weight was administered 3 times a week; during the next 7 weeks of the experiment rats received 0.4 μg/kg of body weight once a week [2].

The research is consistent with the standards and policies of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes drawn up by the Council of Europe (Strasbourg, 18.III.1986). The rats were decapitated under ethereal anesthesia.

The production of superoxide anion radical (O_2^-) was evaluated by a test with nitroblue tetrazolium using spectrophotometry of the tissue homogenate with following inductors: nicotinamide adenine dinucleotide reduced (NADH) was used to assess $\cdot O_2^-$ production by the mitochondrial electron transport chain, nicotinamide adenine dinucleotide phosphate reduced (NADPH) was used to evaluate $\cdot O_2^-$ production by endoplasmic reticulum and NO-synthase (NOS), and *S. typhi* LPS was used to assess $\cdot O_2^-$ production by phagocytic NADPH oxidase [11].

The NOS activity was determined by the difference between the concentration of nitrite ions before and after the incubation of homogenate into the medium containing L-arginine and NADPH [12]. To evaluate the activity of constitutive isoforms (cNOS), we added 1% solution of aminoguanidine hydrochloride (98%, "Sigma Aldrich") [13]. The activity of inducible NOS (iNOS) was evaluated by subtracting the cNOS activity from the overall NOS activity.

The cNOS coupling index was calculated as the ratio between the cNOS activity and the $\cdot O_2^-$ generation rate by the NADPH-dependent electron transport chains. This index points out the presence of substrates (L-arginine, O_2) and tetrahydrobiopterin for NO production, but not for $\cdot O_2^-$ generation under oxidative metabolism of L-arginine [14].

Peroxynitrites of alkali and alkali-earth metals concentration was measured by using their reaction with potassium iodide under pH 7.0 in 0.2 M phosphate buffer with the same pH [12]. The content of low molecular weight S-nitrosothiols was determined by the difference between the concentration of nitrites before and after oxidation of nitrosothiol complexes with a mercury chloride solution [15].

To assess the functional status of submandibular SGs in their homogenate we determined α-amylase activity (spectrophotometrically) and the aquaporin-5 concentration (by enzyme-linked immunosorbent assay using the Rat Aquaporin 5 ELISA Kit, MyBioSource, USA).

The findings obtained were statistically processed using Microsoft Office Excel software pack and Real Statistics add-in. To verify the normality distribution, the calculation of the Shapiro-Wilk test was applied. When the ordered sample values corresponded to the normal distribution, then the Student's t-test was used to compare independent samples. When the result ranges were not subject to normal distribution, statistical processing was performed using a non-parametric method, the Mann-Whitney test.

RESULTS

Reactive oxygen species (ROS) production in the tissues of submandibular SGs significantly elevated under the administration of alcohol during LPS-induced SIR. At that $\cdot O_2^-$ production (Table I) by mitochondrial respiratory chain exceeded the relevant parameters in the control group I by 84.6%; $\cdot O_2^-$ production by microsomal oxygenases and NOS grew by 70.2%, and by phagocyte NADPH-oxidase by 74.1%.

Significant growth in $\cdot O_2^-$production under these conditions is apparently due to the emergence of additional ways of ROS generation under the combined action of ethanol and the SIR progression. On the one hand, alcohol causes 1-electron O_2 reduction in the mitochondrial and microsomal electron transport chains. On the other hand, the influx of LPS as a pathogen-associated molecular pattern through the activation of Toll-like receptors 4 and their dependent NF-kappa B- and AP-1-associated signaling

Table I. Effect of bioflavonoids on the production of superoxide anion radical in submandibular salivary glands under alcohol exposure during lipopolysaccharide-induced systemic inflammatory response (M±m, n=35)

Experiment conditions	Sources of the superoxide anion radical production		
	NADH-dependent (mitochondrial) electron-transport chain	NADPH-dependent electron-transport chains	NADPH-oxidase of white blood cells
Administration of isotonic sodium chloride (control group I)	17,97±1,02	14,55±0,82	1,74±0,10
Administration of ethanol during SIR (control group II)	33,17±1,49 *	24,77±0,37 *	3,03±0,07 *
+ Curcumin	20.95±0.81 *,**	16.94±0.66 *,**	1.86±0.07 **
+ Epigallocatechin-3-gallate	21.74±0.60 *,**	17.57±0.46 *,**	1.92±0.07 **
+ Quercetin	19.46±1.05 **	15.79±0.84 **	1.70±0.10 **

Note (in table 1-4): * – p<0.05 compared with values in the control group I; ** – p<0.05 compared with values in the control group II.

Table II. Effect of bioflavonoids on the activity of NO-synthase isoforms in submandibular salivary glands under alcohol exposure during lipopolysaccharide-induced systemic inflammatory response (M±m, n=35)

Groups of test animals	NOS activity, μmol (NO2-) / min·g of protein			cNOS coupling index
	Total	cNOS	iNOS	
Administration of isotonic sodium chloride (control group I)	7,67±0,39	1,76±0,09	5,91±0,34	0,122±0,007
Administration of ethanol during SIR (control group II)	16,41±0,71 *	1,04±0,23 *	15,37±0,53 *	0,042±0,009 *
+ Curcumin	9.39±0.53 *,**	1.25±0.18 *	8.14±0.59 *,**	0.073±0.010 *,**
+ Epigallocatechin-3-gallate	10.88±0.51*,**	1.40±0.14	9.48±0.46 *,**	0.080±0.008 *,**
+ Quercetin	8.76±0.46 **	1.38±0.16	7.38±0.40 *,**	0.089±0.011 *,**

Table III. Effect of bioflavonoids on the reactive nitrogen species content in submandibular salivary glands under alcohol exposure during lipopolysaccharide-induced systemic inflammatory response (M±m, n=35)

Groups of test animals	Peroxynitrites of alkali and alkali-earth metals concentration, μmol/g	S-nitrosothiols, μmol/g
Administration of isotonic sodium chloride (control group I)	0,91±0,04	0,75±0,02
Administration of ethanol during SIR (control group II)	1,63±0,06 *	1,19±0,03 *
+ Curcumin	1.05±0.03 *,**	0.78±0.02 **
+ Epigallocatechin-3-gallate	1.02±0.04 **	0.82±0.03 **
+ Quercetin	0.99±0.05 **	0.77±0.04 **

pathways promotes ROS formation by various sources and enables to induction other pro-inflammatory mediators in an easier way [16]. The change in the redox potential under these conditions, in turn, further activates redox-sensitive transcription factors, and in particular, NF-kappa B [17]. At the same time, moderate oxidative stress becomes intensified and enhances more intense formation of pro-oxidant and inflammatory mediators.

When applying bioflavonoids under the conditions of alcohol administration during SIR, NADH-induced $\cdot O_2^-$production decreased and yielded to the result in the control group II by 36.8% under administering curcumin, by 34.5% under administering epigallocatechin-3-gallate, and by 41.3% under administering quercetin.

The administration of polyphenols during the experiment also led to a considerable decrease in NADPH-induced $\cdot O_2^-$production in the tissues of the submandibular SGs compared to the control group II. Accordingly, NADPH-induced production of $\cdot O_2^-$by microsomal monooxygenases and NOS in the tissues of the submandibular SGs went down by 31.6% when applying curcumin, by 29.1% when applying e*pigallocatechin-3*-gallate, and 29.1 % when applying quercetin. LPS-induced generation of this radical by phagocytes fell by 38.6, 36.6 and 43.9%, respectively.

The exposure to alcohol during SIR resulted in an increase in NO-synthase activity (Table II) as evidenced by the homogenate of submandibular SGs tissues. At the same time, results of the total NOS activity and the activity of NOS inducible isoform exceeded the values in the control group I in 2.14 and 2.6 times, respectively, while the cNOS activity lowered by 40.9%.

Among the mechanisms ROS generating along with such sources as mitochondria, microsomal monooxygenases and NOS, NADPH-oxidase of white blood cells, xanthine oxidase, lipo- and cyclooxygenase, considerable attention has recently been paid to the functioning of non-conjugated cNOS [18]. Under the alcohol administration during SIR

Table IV. Effect of bioflavonoids on parameters of functional state of submandibular salivary glands under alcohol exposure during lipopolysaccharide-induced systemic inflammatory response (M±m, n=35)

Groups of test animals	α-Amylase activity, mg/min × g	Aquaporin-5 concentration, pg / ml
Administration of isotonic sodium chloride (control group I)	68.18±0.95	0.51±0.02
Administration of ethanol during SIR (control group II)	44.42±0.95 *	0.18±0.01 *
+ Curcumin	62.38±1.55 *,**	0.48±0.02 **
+ Epigallocatechin-3-gallate	61.37±0.80 *,**	0.47±0.02 **
+ Quercetin	59.55±1.45 *,**	0.46±0.02 **

modelling, cNOS coupling index was 65.6% lower than in the control group I. This indicates that cNOS produces $\cdot O_2^-$, instead of producing NO, thus creating a vicious circle of mutual strengthening between the level of oxidative stress and cNOS uncoupling.

When applying bioflavonoids under the alcohol administration during modeled SIR, the indicators of nitrosative stress significantly changed. The total NOS activity in the submandibular SGs tissues was inferior by 42.8% to the relevant data in the control group II (under curcumin administration), by 33.7% (under epigallocatechin-3-gallate administration) and by 46.6% (under quercetin administration); and the iNOS activity decreased by 47.0, 38.3 and 52.0%, respectively.

However, the bioflavonoids used in the study did not significantly change the cNOS activity in submandibular SGs compared with the control group II. The calculation of cNOS coupling index revealed that applying these polyphenols notably improved the cNOS coupling in the tissues of the submandibular SGs. The value of cNOS coupling index exceeded the values in the control group II by 73.8% (under curcumin administration), by 90.5% (under epigallocatechin-3-gallate administration) and 111.0% (under quercetin administration).

The alcohol administration during modeled SIR resulted in the considerable growth in the content of important effectors of nitrosative stress, peroxynitrites and S-nitrosothiols, in the tissues (Table III) by 79.1 and 58.7%, respectively, compared with the control group I.

Under the administration of bioflavonoids peroxynitrites concentration in the submandibular SGs tissues was inferior to the control group II by 35.6% (under curcumin administration), by 37.4% (under epigallocatechin-3-gallate administration), and by 39.3% (under quercetin administration); the content of S-nitrosothiols was lower by 34.5, 31.1 and 35.3%, respectively.

We investigated the activity of α-amylase and the concentration of aquaporin-5 as markers reflecting the functional state of submandibular SGs tissues. Aquaporin-5 in SGs is known to form water channels transporting fluid through biological membranes [19]. The alcohol administration during LPS-induced SIR considerably restricts α-amylase activity and lowered the aquaporin-5 concentration (Table IV) in the submandibular SGs homogenates by 34.8 and 64.7% compared with control group I. That is, the level of functional impairment of SGs is consistent with the above demonstrated indices of nitro-oxidative stress progression.

The administration of bioflavonoids led to the changes in α-amylase activity in the submandibular SGs tissues: its values exceeded the relevant data in the control group II by 40.4 % (under curcumin administration), by 38.2 % (under epigallocatechin-3-gallate administration), and by 34.1 % (under quercetin administration); under those conditions aquaporin-5 concentration grew in 2.66, 2.61 and 2.55 times, respectively.

DISCUSSION

The previous works have reported on the dependence of between the development of nitro-oxidative stress in the SGs under SIR and the activity of the NF-kappa B-dependent signaling pathway. The administration of pyrrolidine dithiocarbamate, a potent NF-kappa B inhibitor, lowers NOS activity, $\cdot O_2^-$ production, and the level of lipid peroxidation in SGs, and enhances antioxidant protection [3].

It has been shown that SIR modeled in the SGs tissues also manifests by impaired functioning of the nuclear factor erythroid 2–related factor (*Nrf2*), a transcription factor, which binds to the antioxidant response element (ARE) and thus regulates the expression of a large battery of genes involved in the cellular antioxidant and anti-inflammatory defence, and mitochondrial protection as well [2].

Reactive oxygen and nitrogen species formed are the means of redox-sensitive transcription factors (NF-kappa B, in particular) regulation, whose alterations in the activity affect not only oxidative metabolism in SGs, but also in other organs through the SIR development. The latter is known as an important mechanism of damaging SGs tissues because it induces nitro-oxidative stress [3]. Many studies suggest the main physiological function of bioflavonoids is to correct this process. Among the principal mechanisms, which provide protective effects of polyphenols, along with their high antiradical activity, their ability to interact with the Nrf2 / ARE signaling system is known to play a critical role [20, 21]. Nrf2 regulates the expression of ARE, which is an enhancer for a number of genes including genes of most antioxidant enzymes and genes of many enzymes of phase II metabolism of xenobiotics, in particular, NAD(P)H-quinonoxidorefutase, hemoxigenase-1, glutathione transferases, UDP-glucuronyltransferase that are important for antioxidant cell protection.

Moreover, curcumin and quercetin serve as inhibitors of NF-kappa B activation demonstrating different mechanisms of action: the first is able to block the phosphorylation and degradation of the inhibitory IκB protein [22], while the latter inhibits the proteasome formation [23]. Curcumin can also impact the activity of the AP-1 transcription factor (activator protein 1) by inhibiting c-Jun N-terminal kinases that is explained by the predominant inhibitory effect on c-jun gene expression [24-27].

Our study has evidenced that applying bioflavonoids for the correction of nitro-oxidative stress in SGs results in an improvement of their functions, therefore, further in-depth investigation of these polyphenols as agents to prevent and treat SG diseases under the conditions accompanied by SIR seems to be very promising.

CONCLUSIONS

1. The use of bioflavonoids (curcumin, epigallocatechin-3-gallate, and quercetin) under the combined administration of 40% ethanol solution and *S. typhi* lipopolysaccharide considerably limits the development of nitro-oxidative stress in the tissues of the submandibular salivary glands. This is confirmed by a significant decrease in the superoxide anion radical production by microsomal monooxygenases, mitochondrial respiratory chain, phagocyte NADPH-oxidase, lowered activity of inducible isoform of NO-synthase and concentration of reactive metabolites of nitrogen (peroxynitrites and S-nitrosothiols).
2. The administration of bioflavonoids (curcumin, epigallocatechin-3-gallate, and quercetin) under the experimental conditions increases the level of cNOS coupling in the tissues of the submandibular salivary glands.
3. The investigated bioflavonoids (curcumin, epigallocatechin-3-gallate, and quercetin) considerably improve the functional status of the submandibular salivary glands under the combined administration of alcohol and *S. typhi* lipopolysaccharide, enhancing the activity of α-amylase and concentration of aquaporin-5, essential for water transport through biological membranes in salivary glands.

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ORIGINAL ARTICLE

THE POTENTIAL ROLE OF EPSTEIN BARR VIRUS IN MULTIPLE SCLEROSIS MOLECULAR AND SEROLOGICAL STUDY

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ABSTRACT

The aim: To identify the relation between EBV infection/reactivation and multiple sclerosis, compared to the normal controls.

Materials and methods: A case-control study conducted in 120 MS patients, aged between 12-42 years, and 120 apparently healthy age- and sex-matched volunteers as controls. Viral DNA was extracted from 100µl of plasma samples, and then viral DNA was detected and quantified by quantitative real time-polymerase chain reaction (q-PCR). Serum samples were used for the detection of anti-EBNA-1 IgG.

Results: Quantitative polymerase chain reaction of EBV showed absence of EBV viremia in all MS patients and control. However, anti EBNA-1 IgG antibody was positive in 51.7% (62/120) of MS patients and 39.2% (47/120) of controls, (P=0.035). The median of anti EBNA-1 IgG level in MS patients and controls were 81.08 U/ml and 67.73 U/ml, respectively (P=0.043). Additionally, EBNA-1 antibody was significantly higher in younger age groups. Patients with the first-line and second-line treatment showed no significant differences in anti EBNA-1 IgG levels, while the median level in patients without treatment (newly diagnosed) was higher.

Conclusions: EBNA-1antibody could play a significant role in development of MS, as it is significantly higher in MS patients than in controls, especially at younger age groups, at early stages of the disease and in female patients.

KEY WORDS: Multiple sclerosis, EBV, EBNA-1 IgG, Real time PCR

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INTRODUCTION

Multiple sclerosis (MS) is a central nervous system disease characterized by demyelization, inflammation, and neuronal destruction. Genetic and environmental factors are coupled with the danger of developing MS, other than the precise reason still remains undisclosed. Among the well-recognized environmental hazard factors in MS were EBV, smoking, and vitamin D deficiency. The risk of rising MS is enlarged by infectious mononucleosis, which is caused by delayed primary infection with EBV. Potentially the EBV acts together with both genetic and additional environmental risk factors to amplify receptiveness and severity of MS disease [1]. EBV is a ubiquitous gamma-herpesvirus universally found in every geographic place and more frequently in developing countries [2]. Infection with EBV is very much common worldwide and around 90% of adults become antibody-positive before 30 years of the age [3]. Primary acute infection with EBV usually occurs during childhood sub-clinically, and subsequently a latent infection of B lymphocytes is established by the virus as the EBV continues for life [4]. In all actively dividing EBV-infected cells, EBNA-1 is expressed and is responsible for fusion of the viral episome to the mitotic cellular DNA, confirming duplication and transport of virus genome to all daughter cells [5]. EBNA-1, the vital EBV antigen for virus latency, makes up a principal antigen for both cell-mediated and humoral immune responses to the virus, and in MS the deregulation of immunity specific for EBV has been reported principally for this antigen [6]. Antibody responses specific for EBNA-1 may furthermore foretell alteration from clinically isolated syndrome (CIS) to MS [7]. Additionally, an enlarged occurrence of EBV DNA in serum for the duration of re-lapses, compared to periods of remission, has been reported [8]. Also, increased risk of MS has been associated with the presence of EBV DNA in the plasma [9].

THE AIM

This study aimed to compare the seroprevalence of Anti-EBNA among MS patients and controls and to study the frequency of active EBV in patients' plasma in comparison to the controls and, finally, to find out whether there is a relation between the disease severity and anti-EBNA titer and/or the level of EBV viremia.

MATERIALS AND METHODS

A case-control study was conducted in 120 patients with multiple sclerosis, aged 12-42 years, from November 2020

to June 2021. Blood samples were obtained from MS patients in the clinic of multiple sclerosis in Baghdad Teaching Hospital of Medical City, and from 120 controls, who were noticeably healthy age and sex-matched volunteers collected (their blood was obtained from blood donation centers). From all subjects we got informed consents before taking the samples. This study was approved by the ethical committee of the Al-Nahrain University, College of Medicine (No.20200980 on 17/11/2020).

The study was conducted in the labs of microbiology department in the College of Medicine-Al-Nahrain University. From all MS patients and controls, 3 ml of whole blood were collected and then divided into two parts: the first part was put in EDTA tubes and the second one (1.5ml) - in gel tubes. The blood samples in each tube were centrifuged at 5000 RPM for 5 minutes to get plasma from EDTA tubes and serum from the gel tubes. Plasma and serum were preserved in (-20 ºC); plasma was used for viral DNA extraction and serum for ELISA. Viral DNA was extracted from 100µl of plasma using WizPrep™ Viral DNA/RNA Mini Kit (V2) (South Korea). EBV Real-TM Quant Kit (Sacace, Italy) was used for detection of *LMP*-gene of EBV. EBV *LMP* DNA amplification was detected on JOE (Yellow) channel, whereas the IC glob gene DNA amplification was revealed on FAM (Green) channel and Master mix. 15 µl was added to all PCR tubes and 10µl of (DNA sample, negative control, positive control and standards) were added to master mix. The real time-PCR instrument used in the study was SaCycler-96 open (Sacace, Italy). The thermal protocol for Quantification Kit of Sacace consisted of an initial step of denaturation at 95 °C for 15 min, for activation of the Hot Star Taq DNA Polymerase, followed by five cycles of thermal cycling at 95 °C for 15 s, and 60 °C for 20 s, and 72 °C for 15 s, and lastly 40 cycles of 95 °C for 10 sec, and 60 °C for 40 sec, and 72 °C for 15 sec. The ELISAs kits (Abnova /Taiwan) for anti-EBNA-1 IgG antibodies measurement, depended on the binding of antibodies in the sample with EBNA-1antigen that coat the wells of ELISA plate and the antibodies being in complexes with antigen are later recognized by animal anti-human IgG antibodies labeled with horseradish peroxidase. The labeled antibodies are revealed by an enzymatic reaction with a Chromogenic substrate. For quantitative evaluation the sample antibody titers in artificial units (AU/mL) were computed as follows: 1. A calibration curve was constructed by plotting the units of Standards (x-axis) to absorbance of Standard (y-axis). The place, where the absorbance of tested samples intersect calibration curve were found and the corresponding values (AU/mL) were marked on the X axis.

STATISTICAL ANALYSIS

SPSS version 21 was used for statistical analysis, categorical data were formulated as count and percentages, and Chi-square test was used to describe the association of these data. Numerical data were described as the mean with standard deviation. And independent sample t-test was used for comparison between two groups. The lower level of statistical significant difference was regarded as ≤ 0.05.

RESULTS

Among the 120 MS patients; 48(40%) were males and 72 (60%) were females. The median age of MS patients was 32 years (Percentile 25=26, Percentile 75=38). There was no statistically significant difference between the median age of the MS patients and controls (*P*=0.594), and no statistically significant difference between the age of the MS patients and controls for the different age groups indicating that they were of a comparable age (*P*=0.465). Most of MS patients (76.67%) 92 out of 120 were having a low number of relapses of equal or less than 3 relapses, and the lower percentage of patients (23.33%) 28 out of 120 were having a higher number of relapses of more than 3 relapses. The treatments used by MS patients included Avonex (5%) (β-interferon-1a), Betaferon (28%) (β-interferon-1b), Rebif (8%) (β-interferon-1a), Gilenya (7.5%) (Fingolimod), Natalizumab (41.5%) (Tysabri), and the last group without treatment with newly diagnosed cases (10%). Large number of MS patients (49%) treated with the second line therapy that included (Natalizumab or Gilenya). The results of quantitative real-time PCR showed that all the 240 samples were negative for EBV *LMP*-gene. However; all of them were positive for internal control, and the positive control gave positive results also. The results of ELISA showed that anti EBNA-1 IgG antibody was positive in 51.7% (62/120) of MS patients and 39.2% (47/120) of controls. The median of anti EBNA-1 IgG level of MS patients was 81.08U/ml (Percentile 25=57.88, Percentile 75=101.40), and of control was 67.73 U/ml (Percentile 25=54.62, Percentile 75=103.93), table I. Statistically, the sero-positivity and median level of anti EBNA-1 antibodies (IgG) were significantly higher in the MS patient than in the controls (*P*=0.035). The rate of sero-positivity and median of EBNA-1 IgG level of the MS patients were significantly higher than the controls in relation to the age groups: (<21 years) ((*P*=0.050,0.006), and (21-30) (*P*=0.003, <0.001) respectively; whereas in the remaining age groups (31-40), (>40), there was no significant differences in the median level and sero-positivity rate of EBNA-1 IgG between MS patients and controls, table I. Statistically, in MS patients the frequency of positive EBNA-1 IgG and median level were significantly higher in the females compared to the males (p=0.004). Conversely, there was no significant differences in EBNA-1 IgG positivity and median level among the different sexes in controls (*p*=0.995), table II.

The rate of sero-positivity of EBNA-1 IgG is significantly higher in patients who have the disease for less than 2 years (83.33%), than in patients with a disease duration more than 2 years (41.11%) (*p*<0.001). In addition, the median of EBNA-1 IgG level in patients with a disease duration <=2 years is 82.81(Percentile 25=55.43, Percentile 75=126.14), which is significantly higher than in the patients with a

Table I. The association between EBNA-1 IgG serology results and age groups

		MS		Control		P value
<21 years	Negative	6	42.9%	10	83.3%	0.050*
	Positive	8	57.1%	2	16.7%	
	Median	80.85		54.42		0.006
	Percentile 25	55.23		45.73		
	Percentile 75	94.58		63.84		
21-30 years	Negative	20	42.6%	30	75.0%	0.003
	Positive	27	57.4%	10	25.0%	
	Median	94.83		63.59		<0.001
	Percentile 25	55.63		54.92		
	Percentile 75	102.32		79.94		
31-40 years	Negative	27	55.1%	30	49.2%	0.569
	Positive	22	44.9%	31	50.8%	
	Median	73.62		80.62		0.755
	Percentile 25	59.32		56.24		
	Percentile 75	104.99		112.46		
> 40 years	Negative	5	50.0%	3	42.9%	0.995
	Positive	5	50.0%	4	57.1%	
	Median	86.66		81.31		0.894
	Percentile 25	61.81		59.53		
	Percentile 75	99.43		126.14		
Total	Negative	58	48.3%	73	60.8%	0.035
	Positive	62	51.7%	47	39.2%	
	Median	81.08		67.73		0.043
	Percentile 25	57.88		54.62		
	Percentile 75	101.40		103.93		

Table II. ENBA-1 IgG result in relation to sex among MS patients and controls

EBNA-1 IgG	Study groups			
	MS		Control	
	Female	Male	Female	Male
Negative	30	28	44	29
%	40.5%	60.9%	61.1%	60.4%
Positive	44	18	28	19
%	59.5%	39.1%	38.9%	39.6%
Median	94.58	63.91	66.67	67.84
Percentile 25	64.12	53.81	53.40	55.84
Percentile 75	104.99	97.88	109.79	101.79
P value	0.004		0.995	

disease duration >2 years - 64.22 (Percentile 25=53.81, Percentile 75=97.37), (p=0.029), table III.

On the other hand, results of this study observed no statistically significant association of EBNA-1 IgG sero-positivity with number of relapses (p=0.812). Finally, table IV illustrated that there was no statistically significant association of the EBNA-1 IgG results with line of treatment (p=0.549).

DISCUSSION

Multiple sclerosis (MS) is the most common chronic inflammatory autoimmune illness of the central nervous system (CNS). Although the pathological characteristics of this persistent demyelinating disease are well recognized [10], little is presently known regarding the complex mechanisms that guide to the inflammatory process related with MS. But similar to other autoimmune diseases, MS

Table III. The relation between EBNA-1 IgG serology and disease duration

EBNA-1 IgG	Disease duration		P value
	<=2 years	>2 years	
Negative	5	53	<0.001
%	16.67%	58.89%	
Positive	25	37	
%	83.33%	41.11%	
Median	82.81	64.22	0.029
Percentile 25	55.43	53.81	
Percentile 75	126.14	97.37	

Table IV. The relation between EBNA-1 IgG serology and line of treatment

EBNA-1 IgG	Line of treatment			P value
	No treatment	1st line	2nd line	
Negative	5	22	31	0.549NS
%	41.67%	44.00%	53.45%	
Positive	7	28	27	
%	58.33%	56.00%	46.55%	
Median	94.87	63.59	66.67	0.390NS
Percentile 25	64.17	54.42	53.2	
Percentile 75	144.13	101.26	102.32	

might be elicited by an infectious agent [11]. A number of studies communicate Epstein-Barr virus (EBV) with MS [12], while others locate no association [13]. In the current study, all the representative samples showed negative EBV viremia, these findings suggest that the existence of EBV DNA is not a familiar incident in plasma and/or serum of MS patients and do not boost a direct role for systemic EBV infection in the MS pathogenesis, which agrees with other studies (Franciotta D et al., 2009, Villegas E et al., 2011) that showed absence of EBV viremia in all plasma samples, both in MS patients and control with EBV DNA negative [14,15]. While other researchers (Cocuzza CE et al., 2014, Hollsberg P et al.,2005) showed only small percentage of EBV DNA in plasma of MS patients; in addition to that, the same studies also reported the presence of EBV DNA in control groups [16,17]. While another study that was conducted on the serum sample using nested PCR failed to detect any viral DNA in examined sample [18]. On the other hand, a number of studies reported that EBV DNA and high viral load were detected in the plasma or serum of MS patients during relapses or at the time of exacerbation [19]. Wandinger et al. in 2000 have revealed that active replication of the virus (increased IgA and IgM responses to EBV EA), and positive EBV DNA in the serum (by quantitative polymerase chain reaction, qPCR) could be found in more than 70% of MS patients with exacerbations through the study time, but not in patients with stable disease [8].

Also Villegas E et al. in 2011 mentioned that the investigations of plasma and serum would only reveal viral genome in currently ill patients or in those with a towering systemic load of EBV [15]. This finding explains the negative results for EBV DNA by q-PCR in plasma of MS patients in the current study, since nearly all patients were taken during remission stage or the period of clinically stable disease. Alternatively, absence of viremia may be owing to the low specimen size subjected for PCR, and/or to the extremely low viral load in these individuals who are asymptomatically infected (i.e. viral load could be lower than the detection limit) [20]. In the present study, anti-EBNA-1 IgG antibody was positive in 51.7% (62/121) of MS patients and 39.2% (47/121) of controls. To the best of our knowledge, there are two previous studies regarding the seroprevalence of anti EBNA-1 IgG in Iraq, the more recent study carried out by Abd WS and Abd Al Kareem RM in 2020 among the Iraqi female patients with MS who were admitted to Clinic of Multiple Sclerosis in neuro-science hospital in Baghdad and they reported that mean serum level was significantly higher in female patients than in healthy controls [21]; another study, conducted in Rizgary teaching hospital in Erbil /Iraq, also showed significantly higher level in MS patients than in healthy controls (Bakir SH et al., 2017) [22]. The significantly higher seropositivity of anti EBNA-1 IgG in the MS patients than in the controls, in accordance with other studies [8, 23], however, other study, which was carried out in children, failed to manifest any relationship between the virus and MS [24].

The presence of an antigen such as the myelin basic protein (MBP), peptide derived from the myelin, sheaths surrounding an axon having a homology to EBV viral proteins. Kumar et al have illustrated molecular mimicry of viral EBNA-1 to MBP that could prompt T-cell autoimmunity to myelin sheaths. For this reason, one of the most

relevant non-self-antigens that is thought to induce MS is EBNA-1[25]. The current study showed that the median of anti-EBNA-1 IgG titer was significantly higher in females compared to the males, MS is a disease of females, the females to males ratio is about 2:1 [26]. In addition, females have higher percentage of IgG seropositivity than males (59.5 % in females versus 39.1 in males), as shown in the table IV. Foroutan-Pajoohian1 Pet al. in 2018, also have reported higher seropositivity of anti-EBNA-1 IgG in MS females than males [27]. The above data may possibly be correlated with the more competent immune response advanced by estrogens compared to the immunosuppressive function of androgens. Females have a better humoral immune response than males, as manifested by higher titers of serum immunoglobulin, and a larger antibody response to a variety of antigens after immunization [28]. The current study revealed significantly higher anti-EBNA-1 IgG titer at young age group as compared to the controls could be explained by Levin LI et al. showed in 2005 that anti-EBV antibody titers among cases compared with controls were already significantly elevated for 5 or more years before the onset of MS. He suggests that the increased antibody response to EBV is not a consequence of MS, but rather may be an early event in the pathological process that leads to demyelization and clinical disease; he was also noted that this increase in EBNA-1 IgG occurred between the late teens and the mid to late 20s, independently from the age of MS onset. On the other hand, the incidence of infectious mononucleosis peaks at this age (29) that clarified the relation between EBV infection and MS.

In support of these theories, this study found that the seroprevalence is significantly higher at early stages of the disease both qualitatively and quantitavely as shown in table III; in addition, table IV revealed that the median IgG titer is higher in patients at early diagnosis (i.e. before starting therapy). Drosu et al. in 2018 found that Zidovudine (a nucleoside analogues), a component of combivir, is known to inhibit EBV DNA replication and recommended in accordance with standard of care and well-established guidelines for Combivir treatment in newly diagnosed MS cases [30].

CONCLUSIONS

In conclusion, anti-EBNA-1 antibody could have an important triggering role of MS because of significantly higher levels both quantitatively and qualitatively in MS patients than in controls, especially at younger age groups, and at early stages of the disease (in those who haven't start treatment yet), and also is higher in females who are well known to have risk factor for MS.

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List of abbreviation: EBV = Epstein-Barr virus, ELISA = Enzyme-linked immunosorbent assay, LMP = Latent membrane protein, CSF= Cerebrospinal fluid, IgA=Immunoglobulin A, IgM=Immunoglobulin M, EA=Early antigen, EBNA-1= Epstein- Barr virus nuclear antigen-1.

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ORIGINAL ARTICLE

TRAINING OF FUTURE PROFESSIONALS FOR SUSTAINABLE DEVELOPMENT

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ABSTRACT

The aim of the research is to develop the educational recommendations for acquiring environmental knowledge of future specialists on the basis of the analysis of the educational state of ecology in the theory and practice of higher education.

Materials and methods: During the research, a group of methods was used. It consisted of the theoretical (specific search) methods i.e. analysis, comparison and generalization of scientific literature on the problem of research to systematize and summarize the facts, information, materials on the problem under study and determine the essence of basic concepts; structural and functional methods i.e. analysis of the content of curricula, textbooks, manuals, dictionaries, directories, etc.).

Results: Future experts were interviewed to evaluate their environmental and professional orientation and level of environmental knowledge. The questionnaire was aimed at clarifying the following problems: determining the level of environmental knowledge of future specialists for sustainable development; attitude of students to environmental problems; determination of the level of ecological consciousness, environmental activity of students. The questionnaire was created on the Google Forms online resource and was used both online and in the standard standard face-up form in the test form filling at the classrooms. The respondents were students receiving engineering, biological and pedagogical education. The age of the participants in the experimental study reached 18-22 years. The ratio of men and women at this stage of the experiment varied within the following limits: 34.66% of male and 65.34% of male (150 persons in total).

Conclusions: The great responsibility for the environment and the future gene pool of the nation rests not only on state structures but also on educators, the public, the church and other factors. We suggest, the path to the ecological culture lies through effective environmental education, through the greening of citizens' consciousness. Therefore, in time, the reorientation of the educational system content in the dimension of sustainable development, which envisages its greening in the first place i.e. the development of environmental thinking, the acquisition of environmental knowledge, the formation of environmental behaviors so that children and adolescents can nowadays and in the future be able to satisfy their needs in safety of nature, without harming it, took care of the environment, about their own health and safety, acted in the environment and made appropriate decisions not as consumers but as conscious citizens.

KEY WORDS: nature conservation, health care, education greening, environmental issues

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INTRODUCTION

At the turn of the millennium, all of humanity has been united around the idea of sustainable development: it is viewed as the ideology of the survival of human civilization. Traditionally, sustainable development is understood as systematically managed development, the basis of the controllability of this complex process i.e. a systematic approach, modern information technologies that allow not only creating different models and variants of directions of development quickly, but also making forecasts of their results and choose the most optimal variant of economic development with high accuracy. Sustainable development is a complex and multidimensional concept. It is an economic development that is comprehensively balanced, with high requirements for environmental protection, environmental security, social justice, ensuring the elimination of exploitation, poverty and discrimination, etc. In the 80's of the XX century the world community is raising the issue of sustainable development, eco-development as development without destruction, the need for sustainable development of ecosystems. For example, in 1987, the International Commission on the Environment and Development (ICSID), chaired by ex-Prime Minister of Norway H. Brundland, in his report, "Our Common Future," raised the issue of "sustainable development" and clearly defined its content: it such a development that not only provides the needs of today's generation of people, but also does not impede the ability of future generations to meet their own needs [1].

These ideas, formally proclaimed in the 21st Century Agenda 21 (Agenda 21), approved at the 1992 International Conference on Environment and Development in Rio de Janeiro (United Nations Conferenceon Environment and Development (Earth Summit)) and socio-economic de-

velopment, high quality of the environment and a healthy economy for all peoples of the world, meeting the needs of people and maintaining sustainable development for a long period [1]. All subsequent definitions of the concept were based on this interpretation of sustainable development. Thus, environmental security qualifies as part of the national and global security of mankind. However, after all the Rio de Janeiro Conference, despite all the declarations to achieve a balance between economic growth, social community and the environment, this has not happened. On the contrary, the situation has worsened and is becoming more and more complicated every day, and the question of the conservation of the biosphere and of civilization, in general, remains open. We consider the urgency of the problem not only in the planetary context, but also in the state dimension.

Greening education, which involves the formation of a person with a high level of environmental culture, is an important pedagogical problem; it is one of the leading areas of the profession of future specialists' formation. It can be received by mean of education at the higher schools. Therefore, it is important that students acquire knowledge in the general laws of the development of nature and society, nature conservation and management, correct understanding of the relationship between the existence of a human and nature, to be able to subordinate their activities to the requirements of rational nature management. Meanwhile, the national higher educational system requires a thorough, deep (rather than formal / superficial) greening of content. On this basis gradual transition to education can be pursued for balanced development, which implies a higher level of consciousness, spirituality and ecological culture. In today's consumer society, we must oppose the alternative of a high-spirited, educated generation of young people who care about their future, their children, and humanity in general [2]. We are convinced that in such circumstances we can positively solve the global environmental problems that face not only Ukraine but also human civilization. In order to address global environmental problems, Ukraine must contribute to the implementation of the Balanced Development Concepts productively, where the environmental education is a top priority. The current Environmental Education Concept (discussed below) and the mechanisms that have been implemented give reason to suppose that Ukraine has sufficient potential (environmental educational experience, educational system, relevant regulatory framework, qualified personnel, etc.) that is capable and must be activated in terms of implementation Sustainable development concepts.

Since the beginning of the XXI century environmental problems in particular have worsened due to the deterioration of the environmental situation in the world and regions of Ukraine, the question of nature conservation, nature protection, the formation of nature conservation behavior skills has become acute, since it started in the family, preschool, and therefore continued in school. Environmental education should naturally and logically be continued in higher education. The complexity of this process is due to the fact that adolescence enters an independent life in an era of not only the rapid development of innovation, science, technology, but also those challenges that are associated with the risks that are triggered by the negative effects of the scientific and technological revolution, the demographic explosion, etc. Therefore, the young man of the 21st century needs a new philosophy of life, a high level of ecological culture, and formed environmental skills. Formation of personal ecological culture is caused not only by the need of time, it is also an important pedagogical problem. However, as we will show below, higher educational institutions do not fully provide such training today.

Basic educational documents also emphasize the importance of environmental education for the growing generation. «Environmental Education Concept», adopted in 2001 in Ukraine, which is based on the Law of Ukraine «On Environmental Protection» (in particular Article 7 «Education and Education in Environmental Protection») [3] emphasizes: "The ecological world, as a holistic cultural phenomenon, including processes of education, upbringing, development of personality, should be directed to the formation of ecological culture… greening of educational disciplines and training programs, as well as to professional ecological training through basic ecological education" [3]. Also important for us is Article 71 of this Law «Participation of Ukraine in international cooperation in the field of environmental protection» (section XVI.International relations of Ukraine in the field of environmental protection), since it reflects the international context of environmental protection of our country [4]. However, there are still no effective mechanisms for greening education, so a number of recommendations from the Environmental Education Concept and the Law of Ukraine "On Environmental Protection" on practical implementation in the field of greening education are not fully implemented to date, in particular in accordance with the main provisions of international instruments i.e. sustainable development. We see this as a problem, because Ukraine, by declaring its course on European integration, has joined international forums that recognize education as «the basic element of society's transformation to sustainable development» and the «foundation of sustainable development». The Strategy of the European Economic Commission for Education for Sustainable Development, adopted in 2005 in Vilnius (Lithuania), emphasized the actualization of the issue of sustainable development and obliged all alliance members to intensify the practical measures for the implementation of the Decade of Education for Sustainable Development (until 2014). The document (Strategies of the United Nations Economic Commission for Europe (ECE) on Education for Sustainable Development (ODA)) The UNECE Strategy for Education for Balanced Development, in particular, states that national Action Plans should be a key element of its implementation. the state of affairs of the country and the basic documents on CSF for all levels of education have been developed and approved. The UNECE strategy at that time directed the Ministry of Education and Science, the Ministry of Nature of Ukraine to create a thorough research

on strengthening the links between the natural, economic, social and political sciences, maximizing their greening, taking into account the principles of balanced development in educational curricula teacher training courses, creation of interdisciplinary projects, etc. Ukraine, as early as 2001, adopted the Concept of Environmental Education, the very beginning of the 21st century is a successful and productive period of realization of the defined goals and educational policy. However, after 20 years, there is a certain setback for the development of a legal framework in the field of greening education: unfortunately, the Law on Environmental Education has not yet been adopted, neither the Concept of Education for Sustainable (Balanced) Development, nor the Concept of Sustainable (Balanced) development.

We are now following the serious steps of Ukraine at the state legislative level. In 2019 the Law «On the Fundamental Principles (Strategy) of the State Environmental Policy of Ukraine for the Period up to 2030» [5] was adopted, which is the state's answer to the challenges that led processes of globalization and social transformation. Ukraine, on the one hand, clearly stated the priority of environmental protection, environmentally balanced use of nature, on the other hand, outlined the root causes of environmental problems, including the subordination of environmental priorities to economic feasibility; *low level of understanding in society of the priorities of environmental protection and the benefits of balanced (sustainable) development, the imperfection of the system of environmental education and education (highlighting is ours. - Ed.);* unsatisfactory level of compliance with environmental legislation and environmental rights and obligations of citizens; insufficient funding from state and local budgets for nature conservation measures, funding for such measures on a residual basis, etc. [5].

The analysis of this Law gives grounds to state that the State Environmental Policy Strategy of Ukraine until 2030 is in line with the ideas of ensuring environmentally balanced use of nature, in particular, the United Nations General Assembly Resolution «Transforming our world: a 2030 Agenda for Sustainable Development».

According to the above state documents the drama and complexity of the situation, among other things, is that the leading tasks of environmental education are the formation of ecological culture by means of formal and non-formal education of all segments of the population, especially heads of different ranks, production managers; training environmental specialists for all sectors of the economy without exception; the strategic task is to develop its scientific foundations.Meanwhile, the theoretical, methodological and educational foundations of environmental education at the higher educational establishments require a thorough analysis of the problem with training a specialist (teacher, ecologist, engineer, diplomat, etc.) as a person of a new type, new environmental thinking, capable of developing an eco-friendly society, capable of solving the problems of nature.

Therefore, despite the fact that greening is one of the strategic directions of education, the results of the analysis of the current state of the educational environment at thehigher schools indicate that the content and structure of the environmental component are insufficiently implemented. Here are the arguments. For example, the subject «Fundamentals of ecology» is one of the optional disciplines for the students, and environmental knowledge is not a necessary component of competencies. Moreover, the introduction of only this discipline into the curricula of the universities is not able to realize the environmental education of future specialists.

The problem of the current educational situation is that the actual termination of teaching «Fundamentals of environmental knowledge» in general education institutions is a prerequisite for a critical situation, which can predict the lack of means of ecological consciousnessformation, the preservation behavior of students with a form of education with a form of education. nature, responsible for the environment, cares about the future of his family, his state, the world community. In such circumstances, the sustainable development of Ukraine may be in danger.

THE AIM

The aim of the research is to develop the educational recommendations for acquiring environmental knowledge of future specialists on the basis of the analysis of the educational state of ecology in the theory and practice of higher education.

MATERIALS AND METHODS

During the research, a group of methods was used. It consisted of the theoretical (specific search) methods i.e. analysis, comparison and generalization of scientific literature on the problem of research to systematize and summarize the facts, information, materials on the problem under study and determine the essence of basic concepts; structural and functional methods i.e. analysis of the content of curricula, textbooks, manuals, dictionaries, directories, etc.) to substantiate the structure, content, principles of construction of the methodology for the formation of environmental knowledge; scientific and pedagogical examination of educational materials, with the help of which the conceptual and terminological apparatus, content and structure of environmental protection knowledge of future specialists were defined and elaborated, the method of their formation in the process of studying individual disciplines, extra-curricular work was developed; experimental-empirical methods i.e. pedagogical observation, conversation, interviewing, questioning and testing of students and teachers to determine the state of the problem and substantiation of methodological approaches for acquiring students' environmental knowledge; statistical and mathematical methods i.e. mathematical and statistical processing of data of pedagogical experiment, systematization and generalization of its results.

Experimental research base. The researches were conducted during 2017-2019 on the basis of the State Higher

Educational Institution "Vasyl Stefanyk Precarpathian National University". The experiment involved 150 students.

RESULTS AND DISCUSSION

The study was conducted in several stages. At the theoretical and diagnostic stage the state of development of the problem in the scientific literature was studied; the international documents on environmental protection and sustainable development were researched; the national pedagogical experience was analyzed and summarized; the ascertaining experiment carried out through interviews, questionnaires, observations, conversations, which resulted in obtaining baseline data on the state of environmental knowledge of future specialists; it was analysed the results of students learning environmental knowledge in the course of study at the university, their attitude to environmental conservation activities. It was also developed the theoretical and methodological principles for creating an ecologically rich environment for the formation of environmental knowledge and ecologically appropriate behavior of specialists in the educational process of higher education and in non-audit work, formulated conclusions and developed methodological recommendations.

The results of the theoretical and diagnostic stage of the study give grounds for the following conclusion: the issue of ecological education of children and adolescents has always been in the field of view of Ukrainian researchers, in particular, scientists have notbest studied the environmental education of students in teaching Geography [6; 7; 8]. It was revealed the general pedagogical and psychological aspects of ecological education of children and adolescents, issues of theory and practice of formation of responsible attitude to the environment during the lessons of geography. The scientists mentioned above, referring to the creative achievements of famous scientists-psychologists A. Leontiev, S. Rubinstein, emphasize that only a person who has understood himself as part of the universe is psychologically prepared for future environmental activity.

A special place in the source database belongs to the property of S. Sovgira, who devoted his works to the formation of ecological culture of students-biologists (the criteria for selection of content and construction of environmental education and upbringing of students of higher educational institutions of biological profile, as well as the problems of ecological studies). H. Bilyavsky, T. Sajenko, O. Pashchenko [2], V. Korneev [9] and others who have researched the problems of greening school education and also analyzed the issues of greening of higher education. Foreign experience of ecological education was studied by N. Bidiuk, I. Zadorozhnaya, T. Kuchay, G. Marchenko, Y. Polyakova (Great Britain), D. Kvasnychkova (Czech Republic), O. Romanov (Belarus), I. Rudkovskaya (Germany), D. Cikhi (Poland) and others.

In the context of our study concept, the opinions of scientists from the United States, the United Kingdom, and Europe, which actualize the problem of environmentally sound behavior (Brennan, 1986; Cunningham, 2005; Judy, 1993; James, 1974, etc.) are rather important as their aims were needs for human communication with the environment, protection from pollution and destruction; the person in his activities and relations with nature shows concern for the possible consequences of its transformation, feels responsible for the decisions that he makes. This is the key conclusion of these studies [10, p. 49]. Therefore, it focuses on the legal aspect of environmentally sound behavior and value-oriented knowledge as important components in the system of environmental training for children and adolescents.

In general, the global and European dimensions of environmental education for sustainable development are quite representative. Let's mention, for example, the environmental educational guide "The Handbook 01 Environmental Education" (PalmerJoy & NealPhilip, 1994), which is to be taken as an environmental educational textbook that provides the reader with clear guidelines for environmental conservation, development, implementation and evaluation of cross-curricular programs operating in the UK. Authors Joy Palmer (Senior Lecturer in Education at Durham University and Former Chairman of the National Association for Environmental Education (UK)) and Philip Neal (Former Secondary School Principal and current Secretary General of the National Association for Environmental Education (UK)) offer analysis of global crises, international environmental education, policy emphasize that environmental education is a process that is interdisciplinary and lifelong in nature and application; consider environmental education holistically, which includes social, political, economic, technological, moral, aesthetic and spiritual aspects, emphasize the value approach to solving environmental problems in educational institutions. This is a step-by-step practical guide, an effective resource that helps educators create an effective environmental educational program for their schools, answering the question "What is environmental education and how to implement it?" [11].

The reference document "Environmental education and training in Europe.

Background paper for the European Union Conferenceon EE&T in Europe" has not lost its conceptual significance. It outlines the European environmental education vectors that Ukraine is focusing on today. It should be noted that the research of Gratiela Dana Boca and Sinan Sara"Environmental Education and Student's Perception, for Sustainability" [12] is rather interesting in the context of our problem; it characterizes environmental education and expresses its role for sustainable environmental development. On the example of the Northern Center of the University of Baia Mare (Romania), which surveyed 358 students, studied the state of environmental education, its relationship with sustainable development, the students' perception of ecology among future engineers, mechanics and economists, their relationship to sustainable development, etc., analyzes measures to protect the environment, which involve students. It should be noted that Ukrainian scientists have not conducted a similar study.

At the initial stage (theoretical and diagnostic) of the experimental study, we made the following scientific steps. It was analyzed the source base on the problem of research, current normative international environmental documents, national legislative framework on the greening of education, teaching and methodological support of this process in higher educational institutions, environmental component in the content and structure of the higher schools. It was defined the level of knowledge of environmental knowledge, the level of formed environmental protection skills to the environmental activities, the willingness of future professionals to carry out environmental activities under conditions of sustainable development by questioning the students at the higher school.

The obtained results served as a material for the development of methodological recommendations for the formation of environmental knowledge, skills and abilities of future specialists, which consisted of special measures aimed at creating an environmentally rich educational environment. It provided optimal conditions for greening education (search-theoretical stage). Let's consider the individual results of our study in details.

It should be noted that in our study we will be talking about specialists with non-specialized higher environmental education, since under the present conditions (declaring Ukraine's course for integration into the European community), sustainable development implies, in particular, the development of higher education based on a comprehensive, technology-based higher education, economic, legal and socio-cultural approaches. Greening should cover all levels of training, taking into account the needs of the individual, region and state. Therefore, in the curricula of non-professional higher schools (which do not train environmental specialists) at the bachelor level, according to the Environmental Education Concept [3], there should be an ecology course that includes the necessary theoretical and practical aspects, as well as corresponding to each individual university course from the block of applied environmental disciplines. For this purpose, one of the obligatory courses "Fundamentals of ecology" (basic ecological knowledge) and courses of the block "Applied ecology" (depending on the profile of the university i.e. "Agroecology", "Urboecology", "Landscape ecology", "Military ecology", "Geoecology", "Environmental problems of energy", "Environmental problems of transport", "Ecological law", "Economics of nature management", etc.).

Although ecological education is of particular importance for students of pedagogical higher schools and it is supposed that future teachers must master the methodology of ecological educational work along with the general high level of ecological culture, as it is shown by the results of analysis of educational and methodological support of educational process of several universities, greening education has not got a sufficient basis for implementation nowadays: in bachelor's degrees, the basics of ecology is optional everywhere. Nowadays, for example, at the National Pedagogical Universities, the course of choosing "Elements of Ecology" is provided by the greening of teacher education. The future teachers' environmental culture is being taught with some other disciplines through cross-curricular communication. For instance, in the course of Natural Science for this purpose, the topic "Ecological education of elementary school students at the lessons of Science" (2 hours of lectures, 2 hours of practical classes). In the course of studying pedagogy students' methods and forms of younger student ecological education are introduced to the content. The discipline "Methods of educational work" provides curriculum time to familiarize students with the content, directions, forms of environmental education in extracurricular work.

However, this is not enough. We can say that the process of greening higher education does not meet the requirements set by the international community and the above-mentioned Ukrainian documents: analysis of current educational courses gives grounds to say that the curricula do not thoroughly and comprehensively address the problems of sustainable development, environmental problems in the region, where the students live or study, and similar global environmental problems, ways to solve them.

By the way, the same situation can be observed at the level of school environmental education. Thus, the announced inclusion in the invariant part of the school component of the third stage of the course "Fundamentals of Environmental Knowledge" [3] is often now only a written declaration as nearly since 2008 or 2010 this subject is optional. Besides, the schools choose such subjects as Christian Ethics, Logic, Choreography and so on. Certainly, the educational and professional programs of the bachelor and master preparation in the sphere of Ecology provide a wide range of professionally oriented disciplines, for instance, General Ecology, Plant Ecology, Animal Ecology, Human Ecology, Landscape Ecology, Environmental Monitoring, Applied Ecology, Environmental Audit, Environmental Management, Environmental Security Management, Fundamentals of Sustainable Development, Environmental Policy, Environmental Economics and Environmental Activities, Anthropogenic Rationing Modern Environmental problems, Ecological economy, etc.

Thus, the analysis of educational and methodological support for the process of greening education for sustainable development in the environmental protection of non-environmental profile showed that the range of educational disciplines is insufficient ("Fundamentals of Ecology" is not the only optional course for students; the cross-curricular nature of environmental knowledge is taken into account (separate problems are considered in particular the course of study of pedagogical disciplines), under such conditions, the acquisition of environmental knowledge with a projection for sustainable development is insufficient. In the course subjects Fundamentals of Ecology / Elements of Ecology) it is fragmentary and episodic, mainly at the level of generalizations about natural complexes of different rank, emotional and value expression of judgments about the need for their protection [13-18]. Thus, there are reasons to say that the higher education

Table I. Participants of the experimental study

The name of the specialty	Number of respondents at 1-5 courses of the State Higher Educational Institution "Vasyl Stefanyk Precarpathian National University"					Total number of respondents
	1stcourse	2nd course	3d course	4th course	5th course	
Engineering	28	3	0	20	5	50
Bioligy	3	3	10	22	12	50
Pedagogy	2	26	0	16	6	50
Total	33	32	10	58	23	150

system needs further serious greening-improvement in the direction of formation of ecologically competent person who has the proper environmental knowledge about sustainable development, problems of conservation of the planet / state / region, personal conservation, has the skills of environmental behavior in nature [19].

We came to this conclusion by analyzing the problem of the higher education greening in practice, based on the observation of the educational process, and the results of surveys, questionnaires of students and teachers.

To determine the basic levels of student environmental knowledge formation among young people of certain specialties at the Vasyl Stefanyk Precarpathian National University, the following evaluation criteria were developed: knowledge about sustainable development, global environmental problems; professional knowledge of students in natural sciences, botany, pedagogy, teaching methods of science; attitude of students to ecological situation, problems of nature management, nature conservation; environmental consciousness; students' motivation for environmental conservation; environmental protection activities, etc.

Future experts were interviewed to evaluate their environmental and professional orientation and level of environmental knowledge. The questionnaire was aimed at clarifying the following problems: determining the level of environmental knowledge of future specialists for sustainable development; attitude of students to environmental problems; determination of the level of ecological consciousness, environmental activity of students. The questionnaire was created on the Google Forms online resource and was used both online and in the standard standard face-up form in the test form filling at the classrooms. The respondents were students receiving engineering, biological and pedagogical education. The age of the participants in the experimental study reached 18-22 years. The ratio of men and women at this stage of the experiment varied within the following limits: 34.66% of male and 65.34% of male (150 persons in total). General characteristics of the sample of students are presented in Table I.

At the beginning of the experiment 150 questionnaires were issued; the purpose of the questionnaire was to determine the level of student ecological culture. The most active students were fourth-year students (Table 1). For various reasons, third-year students of engineering and pedagogical specialties abstained from voting. Therefore, biology students have been taken into account because of their sufficient theoretical level and the highest aspiration and goal setting for self-development and self-improvement. It should be noted that students with a pedagogical education showed higher results in answering questions of a general cultural nature. In turn, engineering students showed the lowest results and the least interest in this survey. About 31.33% of students did not want to indicate their identity. In the personal data column, they only identified themselves as "anonymous".

Let us analyze the data of the fourth-year students' questionnaires, since they were the most active in the study and in general almost the same number of students participated in the survey (20 future engineers; 20 future biologists; 16 future teachers).

Here are some specific questions.

Having asked "What is the place of environmental knowledge in your profession?" we obtained the following results: 94% of future engineers, 100% of future biologists, 100% of future teachers classify them as an important and necessary component of the profession. This indicates that the respondents are aware of the importance of mastering knowledge about nature conservation. However, there is no understanding of the nature of the environmental conservation problem under conditions of sustainable development. This is evidenced by the answers to the following two questions. First of all, the question "What do you mean by" sustainable development"? as it should be noted that only 10% of the students of the Faculty of Education gave definitions of the term "Sustainable development" (typical answers - "stable balanced development", "development that meets the needs of the present time, but brings the danger to the ability of people in the future to meet their own needs", 37% of respondents associate this concept with economic development, 43% answered that they do not know what it is about.It is interesting to note that the students of the Pedagogical Faculty formulated the concept of environmental activity in the conditions of sustainable development as the environmental protection, care and conservation, in determining the directions of environmental activity of human beings, only 10% of future teachers identified protection against negative impacts of humans, most instead called environmental protection activities called flora and fauna protection. Thus, future experts identify the ecological activities of a human in terms of sustainable development with the protection of

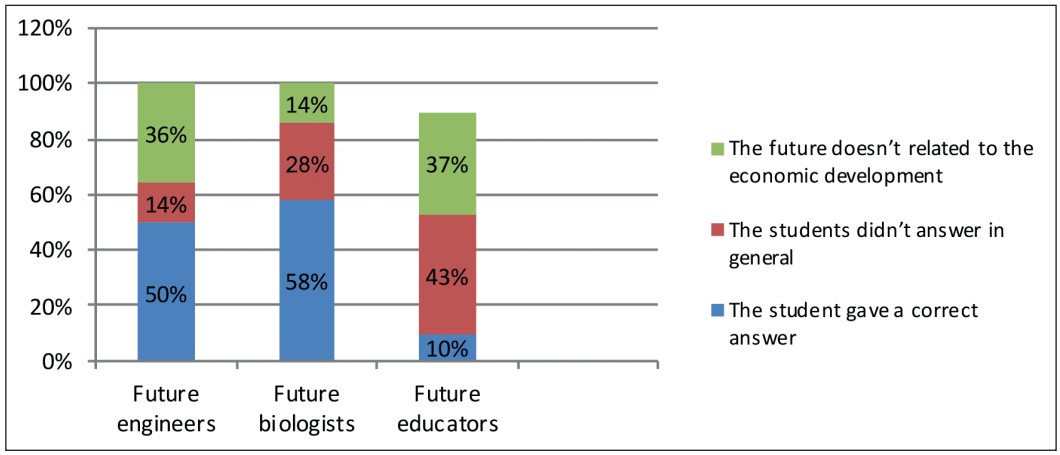

Fig. 1. The results of the answers to the question "What do you mean by sustainable development?"

flora and fauna, they have formed a somewhat one-sided understanding of nature protection as protection and conservation of wildlife.

The students of the Faculty of Natural Sciences showed a greater awareness of the problems of environmental management for sustainable development: 58% identified the main directions of sustainable development in the field of environmental management and conservation; 14% connected environmental development to sustainable economic development; 10% were not aware of this problem; 18% said they did not know what they were talking about.

The answers of future engineers were as follows: 50% answered about sustainable development problems; 36% embodied sustainable development with economic growth; 14% could not answer. Thus, future experts are not sufficiently informed about the content of the concept of "sustainable development", problems of environmental management, sustainable development is usually identified with stable economic growth (Fig. 1).

We found out the respondents' level of knowledge about international environmental activities. Respondents' answers to the question «What are your famous organizations, structures, institutions involved in environmental activities? a) in their local region; b) in Ukraine; c) in the world"showed that, on the one hand, almost all students are aware of the global level of environmental protection (for example, most questionnaires make a sure mention of UNESCO, UNEP, WHO, IUCN as international environmental organizations. On the other hand, at the level of Ukraine, with the exception of the corresponding ministries (76%), students could not name such institutions, 24% supposed "Green party" to be environmental institution. A similar situation is in giving similar examples at the level of their region. So, it turned out, students' level of knowledge and interest in global environmental manifestations are higher than at regional and national levels.

One of the questions of the survey was aimed at revealing knowledge about the environmental activities of the students themselves. We offered the students the following situation: "After your picnic in the forest, there is a lot of litter (bottles, food waste, household waste (paper), plastic utensils and plastic bags. What would do you do with the garbage?" 10% said they would burn paper, 46% said that they would bury food waste, 84% said that garbage should be taken away, not left in the forest, and then thrown away at home. We emphasize that none of the students indicated that they would burn plastic utensils. However, while respondents are generally aware of the inadmissibility of leaving garbage in the forest and the harmfulness of burning (except for those who are willing to burn paper), they are prepared to take the garbage home, but they did not indicate environmental practices that are consistent with the principles of sustainable behavior in the environment (refusing plastic utensils and polyethylene in general, sorting garbage, etc.).

To summarize, let us note that the greening of higher education is insufficient (this process is not supported by the relevant disciplines). The future specialists will master the environmental knowledge on the global level and at the level of operation of the conceptual apparatus, they are best acquainted with the problems of environmental protection in the conditions of sustainable development students of the Faculty of Natural Sciences. The future teachers practically don't know the problems of sustainable development, they do not distinguish between bioecological and environmental knowledge, insufficiently aware of modern environmental education.In general, respondents mistakenly represent sustainable development, not in the dimension of environmental management and conservation, but in the sphere of sustainable economic development; in higher education, insufficient attention is paid to empirical experience and theoretical generalizations, to the development of environmental knowledge skills for sustainable behavior in the environment. Unfortunately, the students do not distinguish rational use of nature as a direction of nature conservation for sustainable development.

So, despite the fact that greening is declared as one of the strategic directions of higher education, the content and structure of the environmental component is insufficiently implemented. In this situation, we believe, it seems most paradoxical to us that all 100% of students from different specialties on the question «Do you think that the periodic training and advanced training of specialists in various fields of ecology and environmental protection is necessary?» answered positively. This demonstrates the students' interest in the issue of environmental culture among other

things and, probably, their willingness to increase their own level of environmental culture in the future.

Based on the analysis of the problem of greening education in the conditions of sustainable development in theory and practice, we distinguish a number of measures / methodological recommendations that can optimize this process at the higher educational establishments.

First of all, all components of the standards of higher education should be updated and supplemented by new requirements for the content of training, social ordering, diagnostics of the quality of environmental and creative competence of future specialists during the decade of education for sustainable development. The content of environmental education should be reflected in the National Standards of Higher Education in all areas of preparation in accordance with the provisions of this Concept. There is an urgent need to substantiate, develop and specify the content and structure of empirical and theoretical environmental knowledge and methods for their formation in higher education. The problem is also seen in the fact that the current system of environmental education in Ukraine, unfortunately, does not have the proper level of educational management. The Ukrainian higher educational system must take decisive steps towards the management of environmental education.

We consider that higher education is gradually losing its former advantages in the formal sphere through the imitation of other's educational schemes. The informal educational activities remain underdeveloped, and their importance can hardly be overestimated in the successful achievement of educational goals for a balanced information society.The latest telecommunication technologies have not yet been widely implemented in the educational processes of most higher educational institutions of the state, which calls into question the practical implementation of the basic provisions of the Agenda for the 21st Century [2].In addition, in the public consciousness, the understanding of environmental education often comes down to teaching only the Natural Sciences (Biology, Geography, etc.), while forgetting the great potential of pedagogical subjects, subjects of the linguistic and literary cycle, etc., as any ecological form values, the ecoculture of the student body, it is only necessary to isolate it in the course of one or some disciplines and then use it as a didactic tool.

Therefore, in the current situation (due to a number of reasons related to the reduction of training load, saving material resources, reducing the amount of training load, etc.) in the Ukrainian realities of the higher educational establishments it is advisable to ask the question not about the expansion of environmental education, but rather about its renewal, innovation content and forms, its new status and importance in the context of building an education system for sustainable (balanced) development of Ukrainian society.

Thus, natural resources, inherited by the current generation of Ukrainians from thousands of previous generations, are testimonies to the high level of their ecological consciousness, where the knowledge of nature as a gift of God was passed from the generation to generation, so it should be appreciated and preserved. That is why the folk-pedagogical knowledge of Ukrainians in the field of nature conservation should be updated and used creatively in higher education. So, on the one hand, there is a great historical and pedagogical potential of Ukrainian folk pedagogy and academic pedagogy, on the other hand, it is not fully used and actualized (works of S. Rusova, V. Sukhomlynsky, and other educators, the experience of the Ukrainian school of the early twentieth century, the heritage of teachers of the Soviet era, the practice of environmental education in the second half of the twentieth century, etc.).

We believe that the analysis of pedagogical experience of Ukraine in the field of environmental education, as well as a comprehensive assessment of national environmental potential will make an important step towards the implementation of a balanced international policy of ecological and economic progress. In this regard, it is advisable not only to solve environmental problems in the Ukrainian dimension, but also to offer the international community educational achievements in shaping the environmental consciousness of the individual, the Ukrainian experience in solving global and regional environmental problems.

Nowadays, humanity has entered the stage of continuous environmental education, lifelong environmental education, and the problem of formal and non-formal education is becoming more relevant, in particular the issue of adult environmental education in Ukraine is in its infancy. Without obtaining the proper environmental knowledge of all citizens, it is problematic to talk about conditions for sustainable development. Moreover, international documents focus on global environmental problems, sustainable development, and in Ukraine sustainable development problems are often interpreted in a distorted light, linked to economic development, which, among other things, is caused by environmental ignorance, lack of knowledge about the international context of education greening. Making environmental knowledge accessible to everyone is one of the goals of greening education in Ukraine.

In this context, close cooperation between teachers, families, community activists, professional organizations and the church is rather important in the field of greening and ecology. In other words, it's about creating a so-called environmentally saturated environment, which we consider as one of the effective conditions and a factor of sustainable development. The heritage of the Ukrainian system of ecological education is the emphasis on the formation of ecological morality, ethics, culture, actualization of ethno-pedagogical knowledge of Ukrainian, formation of environmental consciousness by means of oral folk art, cultural and artistic means, Ukrainian song and more. This factor can become fundamental in building a model of formation of a personal ecological culture under the conditions of the higher educational establishments.

We consider education not only as a priority area of state economic development, but as a condition for ensuring its sustainable development through the introduction of a competent paradigm of learning (to replace the cog-

nitive-oriented) and focusing the content of education on the principles of environmentally sound, balanced development.

In our opinion, the legal aspect of shaping environmentally sound youth behavior should be important. The legal aspect is, first of all, that a person must be aware of his or her responsibility to the law for the destruction of nature, the harming of the environment, and unconditionally respect environmental laws. In our view, this aspect has not been sufficiently developed in theory or in practical terms in the context of greening education for sustainable development.

CONCLUSIONS

The content of environmental education of future specialists should provide theoretical training i.e. appropriate amount of environmental knowledge, proper management for sustainable development, knowledge of regulatory support of the process of greening education, information on international environmental documents, focused on sustainable development, orientation of the student; and practical training i.e. development of the necessary volume of practical ecological knowledge in the field of environmental protection and rational use of nature, ability to analyze and model environmental situations with an orientation to their management independently; developing an awareness of the reality of the environmental crisis and ways to prevent it; acquisition of environmental skills, ability to assess environmental situations and carry out environmental protection measures in the native region, to form an active public position to address environmental issues and preserve the biosphere, to be able to use modern information technologies actively to solve environmental problems (see Fig.2). Considerable importance is the actualization of environmental problems in the topic of diploma (qualification) works (projects) of graduates at pedagogical, natural, technical, agrarian, military and other areas of preparation, involvement of students in the implementation of research works on environmental topics, to participate in environmental trainings, competitions and conferences, introduction of laboratory and practical classes in ecology, field and industrial ecological practices into the educational process of educational institutions. The subjects of course and qualification works (projects) should be formed, first of all, taking into account the real needs of the region and the state for environmental protection and rational use of nature in the conditions of sustainable development.

Increasing the level of greening of education will be facilitated by the participation of specialists from different directions of educational training in the development of State standards for environmental education; liaison with NGOs; involvement of students in the implementation of joint environmental projects, research programs and the publication of textbooks and manuals; training and retraining of pedagogical staff of higher education institutions in the field of environmental education.

As there is no separate subject on ecology and rational environmental management in the higher school of Ukraine today, the problem of student environmental knowledge formation needs to be solved on a cross-curricular basis, with the main didactic task which has to be solved by teachers of pedagogical, natural science, and literary cycles. The ways to increase environmental education within the higher educational establishments are to increase the weight of environmental issues, both within specific subjects and through the establishment of internal and cross-curricular links; creation in educational establishments of appropriate educational and material base: corners of nature protection and pets' corners at schools, etc.; improvement of forms and methods of environmental education, active involvement of the student in environmental work; the formation of motives for a responsible attitude to nature, the desire to know it more deeply, to multiply its riches. To do this, it is necessary to develop pedagogical conditions for cooperation between students and teachers in the context of environmental education. The leading methodological principles are the principles of environmental imperative (environmental responsibility of the individual), scientific-theoretical (ecological way of thinking), humanitarian (ecological culture), economic and legal (ecological reasonableness, ecological expediency, leading principle methodological principles of introduction of ecological education and upbringing at the higher schools; applied (environmental safety) and pedagogical (environmental education). In other words, environmental knowledge is an important basis for shaping the environmental culture of future professionals.

It is advisable to implement the greening of the higher educational system through a holistic educational system of formal and non-formal education on the basis of a cross-curricular ecological-educational model, creation of an ecologically saturated environment on an activity basis. An important condition for the effectiveness of this process is the introduction of innovative teaching methods, project technologies, game techniques, field trips to nature protection objects, the very environmental activities of students, etc., which are designed to develop cognitive interest of future professionals in environmental issues, environmental activities.

Emphasizing on the cross-curricular nature of environmental knowledge, we have developed a methodology for their formation in the process of educational and extra-curricular activities on the basis of a competent approach with a focus on sustainable development. This methodological system has three main components: the target, the cognitive, and the behavioral components. Unlike the traditional approach (the purpose of education is to master the knowledge system), under a competent approach, not only is the mastery of relevant environmental knowledge, but students acquire knowledge about nature conservation in the context of sustainable development, they acquire the ability to solve environmental problems on the basis of the acquired knowledge. Therefore, we do not diminish the value of knowledge, but emphasize the priority to apply and use it.

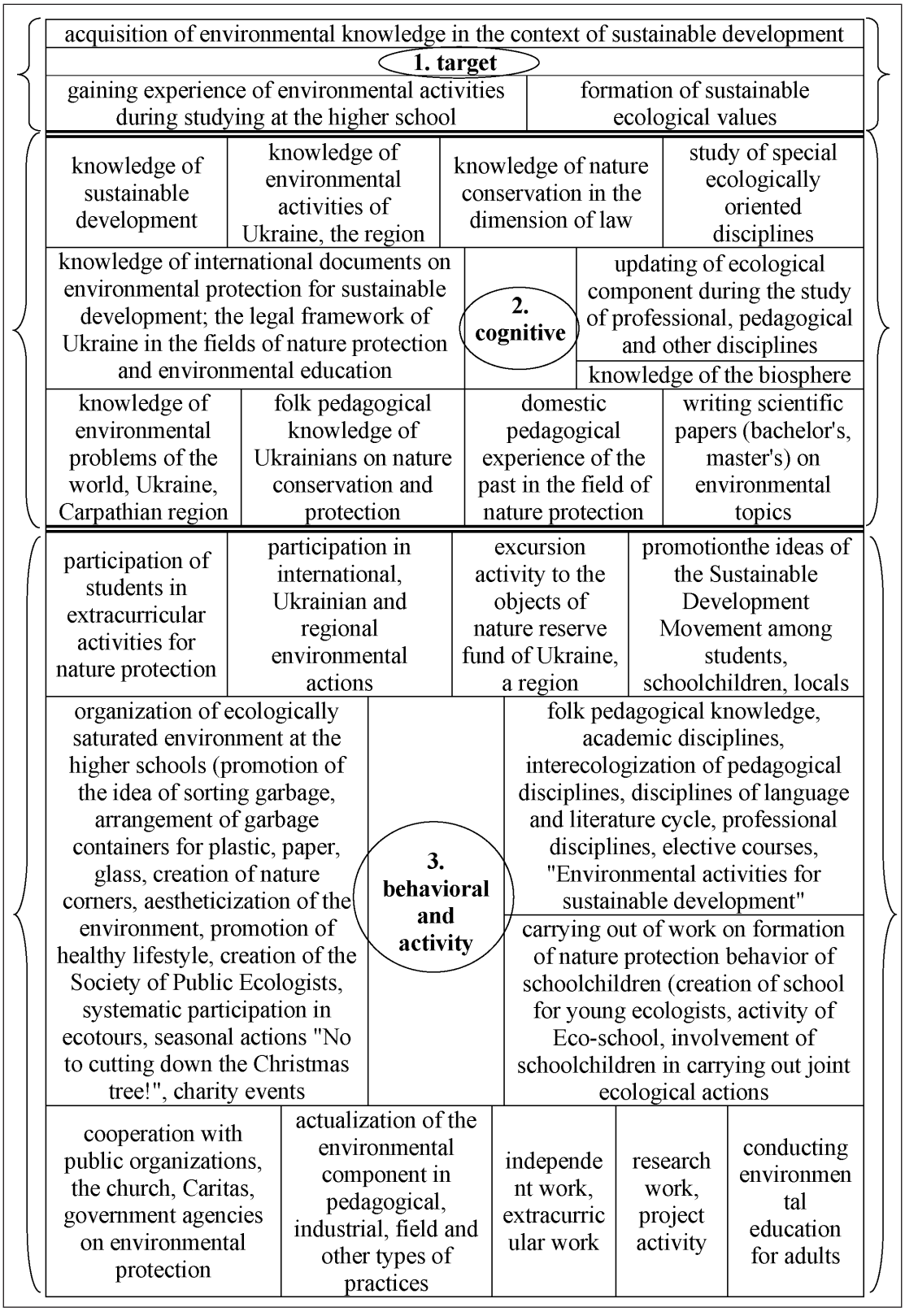

Fig. 2. Components of the process of formation of environmental knowledge of future specialists on the basis of a competent approach

The objective will be to develop the ability of future professionals to address environmental issues through nature conservation based on environmental information for sustainable development, and to gain real environmental experience while studying at university. The cognitive (perceptive) component involves the students' educational and cognitive activity aimed at assimilation of environmental knowledge in the context of sustainable development, information on environmental problems of the world, Ukraine, its region, etc. It is carried out in the course of studying relevant disciplines, as well as on a cross-curricular basis. The behavioral and activity component involves students' participation in nature conservation activities, their involvement in university, city, state, international

environmental actions, the ability to organize environmental activities with schoolchildren, the organization of environmental education for adults, and others.

In further studies, we will analyze the results of the implementation of our methodology in the educational process of the higher educational establishments, which we have been conducting since 2017 at Vasyl Stefanyk Precarpathian National University.

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REVIEW ARTICLE

PROGNOSTIC SIGNIFICANCE AND LIMITATION OF VISIT-TO-VISIT BLOOD PRESSURE VARIABILITY IN PATIENTS WITH REDUCED EJECTION FRACTION: A MINI-REVIEW

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ABSTRACT

Visit-to-visit variability (VVV) of blood pressure (BP) can facilitate in predicting future reduced ejection fractions cases. In the recent past, the prognostic significance of visit-to-visit variability of BP has been examined widely in patients with a high risk of cardiovascular disease. The findings of numerous investigations have indicated that increased visit-to-visit variability of blood pressure can lead to better estimation or proper treatments that can minimize blood pressure variability and associated risks while enhancing clinical outcomes. However, inconsistent data of the visit-to-visit hypothesis in the post-hoc analysis have also been explored. Therefore, this review discusses recent analysis, background, and reports of the limitations of visit-to-visit blood pressure variability (VVV-BP) and the prognostic significance of visit-to-visit blood pressure variability in populations at high risk of reduced ejection fractions in predictions of future vascular diseases. The role of the antihypertensive drugs is highlighted while describing the clinical implications and future research directions.

KEY WORDS: VVV of BP, antihypertensive drugs, cardiovascular disease, systolic heart failure

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INTRODUCTION

Multiple visit-to-visit variabilities of blood pressure have been evaluated concerning mortality and reduced ejection fractions outcomes in various researches, including several patient populations [1–8]. There is a high reduction in associated cardiovascular deaths due to cardiovascular risk detection and management advancements. However, elevated blood pressure has been investigated as a significant risk factor for heart-related diseases. Few controversial studies have claimed that it is not yet well understood whether variation in blood pressure can accurately predict future heart complications and other associated diseases [9]. In addition, current researches indicate that various outcomes in populations with stroke or with systolic heart-elevation myocardial infarction are independently related to VVV of BP [10, 11].

Kobalava et al. reported little relation between VVV of BP and reduced ejection fractions. VVV of BP was recorded between 2.3 and 20 mmHg in a total of 47 endpoints in 37 patients [2]. The findings demonstrated that the populations with endpoints possessed maximum systolic VVV of BP between (11.2+-4.0 *vs*. 9.5+3.5 mmHg), which was considerably higher. In addition, the logistic regression analysis suggested that the risk of reduced ejection fraction outcomes did not correlate with adverse results in persons with stable reduced ejection records. More analysis on chronic heart failure data with reduced ejection fraction (HFrEF) provided empirical evidence that HFrEF is recognized as a condition of "paradoxical epidemiology" whereby higher blood pressure is connected with the more beneficial outcomes [11–13]. Partly, their results can be sustained by the understanding that in chronic heart failure (CHF), higher blood pressure is indicated probable improved cardiac output [14].

Conducted research on blood pressure variability (BPV) in patients with CHF proposed that data taken during a limited period, especially short-term BPV monitored within 24-hours with low BP and the level of NTproBNP recorded in 24-hours were associated with more severe heart failure cases and showed minimal prognostic significance [15]. For a better treatment of hypertension, it is recommended that "usual blood pressure" determined as the mean of the total blood pressure records over multiple occasions should be constantly monitored because it accounts for several risks of cardiovascular and associated events. Moreover, it is used to settle antihypertensive drugs to administer [16].

Hypertension is classified among the independent risk factor for various fatal or nonfatal heart failure cases such as vision loss, dementia, stroke, heart failure, renal disease, and myocardial infarction (Fig. 1). A meta-analysis of patient's data for one million adult populations in more than 61 potential research has determined that elevated systolic blood pressure (SBP) up to 20 mm Hg or 10 mmHg of diastolic blood pressure (DBP) from 115 to 75 mmHg is related with an advanced risk of cardiovascular disease or cardiovascular-related deaths as well as other multiple risk factors as shown in Figure 1 [18]. Supportive meta-analysis

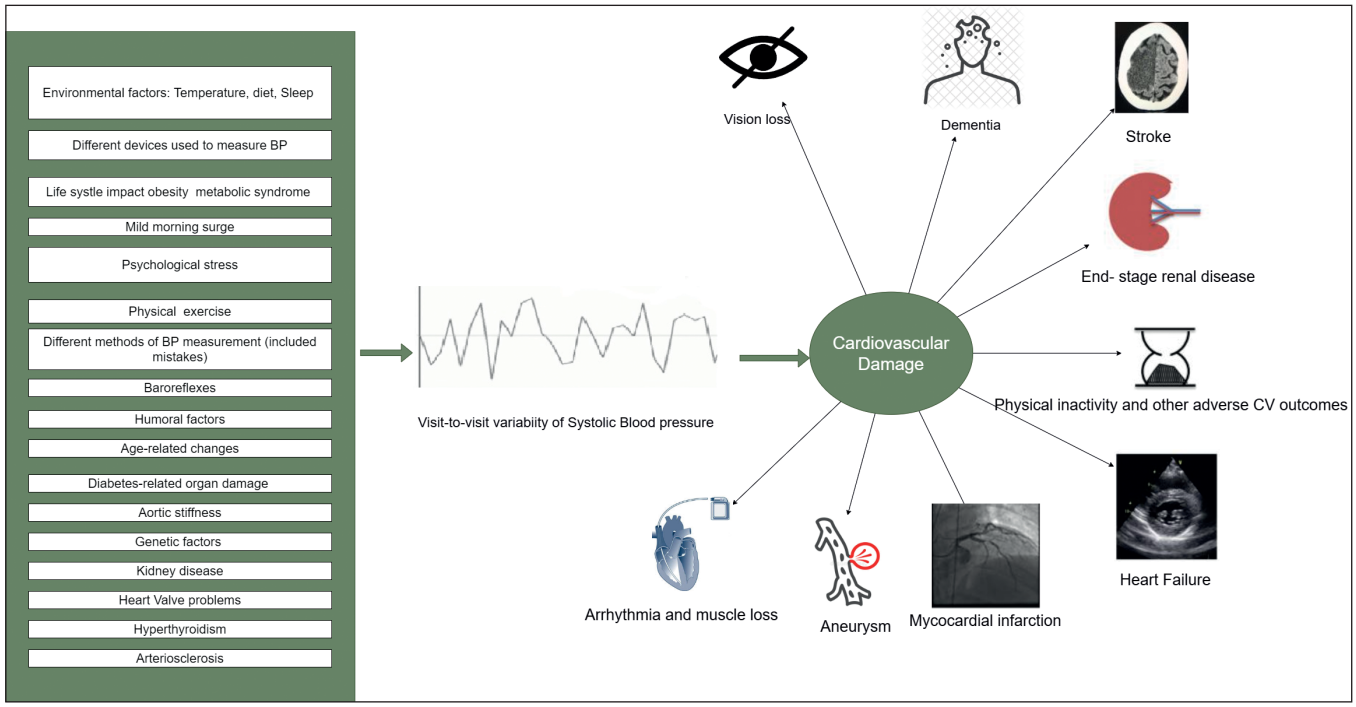

Fig. 1. Several causes of VVV of BP and the related organ damage. Information adapted from [17] with minimum modifications.

studies recommended that minimizing systolic blood pressure below 130 mmHg can notably lower cardiovascular disease risk [17]. The use of antihypertensive drugs was also recommended in patients at high risk of heart failure disease [19, 20].

The novelty of this review includes combining together the significance and limitations of VVV of BP in an attempt to provide clinicians and researchers with a combined assessment that might find solutions to overcome challenges associated with blood pressure variability in patients with high-risk systolic heart failure, such as the implementation of research results data into proper health measures and policy; identifying an accurate VVV of BP that pauses a greater risk of suspicious cardiovascular events; determining the recommended time and number of visits to measure blood pressure and establishing long-term BP variability clinical relevance. Moreover, our review has discussed the recommended behavior and medications for populations at high risk of cardiovascular disease.

METHODOLOGY

This study literature search was conducted in the PubMed, Embase, and Google Scholar databases. Our search terms were: visit-to-visit variability of blood pressure (VVV of BP), heart diseases, heart failure, cardiovascular disease, reduced ejection fractions, coronary heart disease, BP, hypertension. Our search was limited on the importance of BP-VVV and the shortcomings discussed in previous studies. Several articles assessing these mentioned keywords were identified. The studies with relevant data were retrieved and discussed in this review.

REVIEW AND DISCUSSION

THE SIGNIFICANCE OF INCREASED VVV OF BP IN PATIENTS WITH A HIGH RISK OF CARDIOVASCULAR DISEASE

Considerable evidence has been collected on the significance of increased VVV of BP as a significant risk in patients with a high risk of myocardial infarction. Worrying patterns of blood pressure were reported with increasing age. Studies found that systolic blood pressure (SBP) progresses to vary with increasing age. In contrast, diastolic blood pressure (DBP) rises at the age of 50 and reduces after one decade and, in most cases, remains at the same level throughout life [21]. DBP and SBP prevail in patients of age 50 and above. Studies have shown that DBP has a great influence as a cardiovascular risk factor than SBP [21, 22]. Clinical trials have shown that managing SBP can minimize death cases associated with SBP, stroke, and heart failure cases [23]. Clinical trials, as well as observational trial data, might result in poor systolic blood pressure, which causes low rates of blood pressure. However, more findings show that poor SBP also correlates with the physician's antihypertensive and lipid-lowering treatment. Attack trial (ALLHAT) and verapamil to understand more about cardiovascular end points (CONVINCE) Trial treatments show both treatments can effectively control DBP at a 90% rate. In comparison, SBP was controlled between 60-70%. More studies are needed to understand how to manage systolic hypertension, especially in this aging world population (Fig. 1).

Ajs and Pm investigated the effect of hypertension in end-organ damage and its correlation in causing other vascular events [24]. It is widely accepted that high BP

is the cause of all BP-associated risk of vascular events. Therefore, the importance of drugs that reduce high blood pressure and other safety guidelines on diagnosis and better hypertension treatments should be properly implemented. For better treatment, it is advised that various informative measures such as VVV of BP in clinics should not be neglected. More attention should be given to understand the long-term effects of antihypertensive drugs. Even though clinical guidelines suggest that episodic hypertension is not treatable, it can be monitored and regulated. The possible risks of residual variability in a patient with hypertensive risks should be carefully monitored [25]. The recently documented BPV over several hours by ambulatory BP morning (ABPM) showed

standard deviations between 10-15 mmHg during daytime and 5-10 mmHg at night. Nevertheless, numerous analyses of the prognostic value of 24-hours ABPM have determined mean blood pressures, or at day-night dissimilarity in mean level, and it was noticed that patients with higher night-time mean BP than the one found at night time are more exposed to experience target organ damage, vascular death, vascular events, however, the definite correlation is inaccurate, reverse dippers incline to be greater, several times is more observed in diabetic patients with a record of a previous vascular occurrence.

More studies by Mehlum et al. highlighted BPV and risk of heart failure events and deaths in populations with hypertension risks and various established baseline conditions at different risk levels [16]. To understand if an associated risk of valsartan antihypertensive long-term use combined with amlodipine affects BPV, patients with hypertensive risk of various cardiovascular events were investigated. The timeline for the experiment was 4.2 years. The systolic VVV of BP mean standard deviation was calculated from six-month visits on the ward in patients who showed up at least ≥ 3 visits and showed no cases during the first six months of a visit to the hospital. Comparing the highest and lowest quintile of normal blood pressure variability using cox regression of 13803 patients, 1557 (11,3%) had a cardiovascular event, and 1087 (7.9%) deaths were reported as BP-related cases. Patients with the highest VVV of SBP had the highest risk of cardiovascular cases. The calculated hazard ration of 2.1, 95% confidence interval (95% Cl) 1.7-2.4; p <0.0001], the valsartan of 5 mmHG augmented in SD of systolic blood pressure which had correlation with a 10% rise in death risk (HR 1.10,95% Cl 1.04-1.17; p =002). The correlations were particularly higher among younger adult patients and populations with minimum SBP. The association was also recorded in patients with various baseline risks except for a higher death risk in patients with well-determined cardiovascular disease. Therefore, it was established that higher VVV systolic blood pressure is related to elevated cardiovascular disease risk in populations with hypertension and other cardiovascular events [26].

It has been established that non-controlled BP increases cardiovascular risk, despite the type of medication used. To understand the influence of verapamil SR-trandolapril on the consistency of blood pressure and whether there is any adverse outcome closely related to taking that treatment, 22576 patients with known hypertension and coronary artery complications were separated into four groups depending on the number of visits at the hospital for blood pressure record as well as the consistency of their BP (<140/90 mmHg): <25%, 25% to <50%,50% to <75%, and >75%. Several primary cardiovascular outcomes were registered (nonfatal stroke, nonfatal myocardial infarction, first incidence of mortality, myocardial infarction, and reduced stroke). It was observed that from the group of <25% BP to the group of <75% BP, the number of strokes was reduced progressively. More findings showed that the risk of primary outcomes such as nonfatal stroke (heart rate:0.50;95% CI:0.37 TO 0.67) was minimal in the group with >75% with BP under control in comparison with the group with <25% of the visit of BP, nonfatal myocardial infarction (heart rate:0.58;95% CI:0.48 TO 0.70), the first occurrence of death, myocardial infarction and stroke was reduced significantly in <25% BP to the group of <75% BP. It was demonstrated that baseline of blood pressure could not predict the risk of primary outcomes. However, comparing the proportion of visits with blood pressure control, they were related to the mean follow-up systolic blood pressure (r^2=0.64), and both independently associated with the outcomes.

In contrast, in cases where the proportion of visits with blood pressure reduces the rise, a steep depletion in heart diseases was reported associated with independent of baseline characteristics and mean on-treatment blood pressure. Reports show that consistency of blood pressure control could provide additional information during treatment using protective antihypertensive treatment. It is recommended that doctors or types of medications should be altered when blood pressure is not controlled at each visit [27].

Ferrari and Fox reviewed the importance of heart rate reduction in decreasing the risk of myocardial ischemia [28]. Understanding how elevated rates affect cardiovascular disease can provide critical information in the reduction of cases. It has been established that a high heart rate can stimulate myocardial ischemia in patients with known CAD. Among the preventive measures to minimize myocardial ischemia and other chronic heart failures (HF) include the use of the antianginal effect of β-blockers (bisoprolol and metoprolol) and other calcium blockers (diltiazem and verapamil) that reduce heart rate. Reducing heart rate is an established method to ease prognosis in patients with heart failure conditions. Comparative analysis between SHIFT AND SIGNIFY indicated different results, whereby SHIFT findings showed that reducing heart rate enhances prognosis while SIGNIFY findings argue that heart rate variability is a non-modifiable risk factor in patients with cardiovascular disease (CAD). However, further studies showed that heart rate reduced blood flow in coronary arteries and played a significant role in determining cardiac arrhythmias, while low heart rate can be correlated with atrial [29, 30]

Masugata et al. explored the relationship between systolic blood pressure (SBP) variability and cardiac infarction in hypertensive patients [22]. Their study directly contrasted VVV in systolic BP and left ventricular (LV) diastolic dysfunction to understand their correlation with the mean SBP value and other cardiac parameters in patients under various treatments. For one year, forty treated patients with hypertensive conditions (69±9 years of age) recorded their BP every one or two months at the outpatient clinics. Their findings showed that the standard deviation of systolic blood pressure demonstrated some critical difference between the high and low SBP, especially during the assessed VVV period. The mean of SBP was also analyzed despite the limitation (Table 1). Left ventricular diastolic function was analyzed using (E/A) ratio of early (E), early diastolic mitral annular velocity(é), and late (A) diastolic transmittal flows. The ratio (E/é) of E to é employing echocardiography/A was only associated with the standard deviation of systolic blood pressure (r=-0.327, p=0.040), on the contrary, it was associated with a standard deviation of systolic blood pressure (r=-0.496, p=0.001) and maximum-minimum SBP difference (r=-0.490, p=0.001). E/é correlated with a standard deviation of SBP (r=0.384, p=0.014), the recorded high-low SBP difference was between (r=0.410, p =0.009), and the mean value of SBP (r=0.349, p=0.028). Multiple regression calculation determined that only the maximum-minimum SBP difference independently correlated with E/é (β=0.410, p=0.009). Therefore, it was concluded that VVV of SBP demonstrated a better association with left ventricular diastolic dysfunction than the mean values of SBP. Elevated VVV was lined with left ventricular diastolic dysfunction and may thus, present high damage for diastolic heart failure in patients with the hypertensive condition. These results correlated with the findings from different studies that visit-to-visit variability in systolic blood pressure can predict stroke occurrence [7, 17].

In addition, more convincing evidence in a meta-analysis of 77299 patients has confirmed that VVV of SBP, regardless of age, could be used to alert cardiovascular, mortality, and stroke. The meta-analysis study of 13 potential types of research was performed to assess the prognostic significance of VVV of SBP by various parameters in 77299 patients with a mean follow-up of 6.3 years. The findings showed that the pooled age and mean SBP-recorded hazard ratios (HRs) for all-cause of fatality rate were 1.03 (95% confidence interval [CI],1.02-1.04; ≤0.010) per 1-mmHg, while the SBP standard deviation (SD) was 1.04 (1.02-1.06, p≤0.001) per 1% SBP coefficient of variation, the associated values of heart failure mortality were 1.10 (1.02-1.17, p≤0.001). The results showed that an increase of 1 mmHg in SD was related to the occurrence of stroke. Therefore, the above clinical analysis demonstrated that VVV of SBP could be used to estimate the future occurrence of cardiovascular events [31].

LIMITATION OF VISIT-TO-VISIT VARIABILITY OF BLOOD PRESSURE HYPOTHESIS

Rossignol et al. examined the limitations of the VVV of BP hypothesis in various patients [32]. They highlighted the significance of the normal BP variability in anticipating the future occurrence of cardiovascular cases. Their investigations used HEAAL (Angiotensin II Antagonist Losartan), whereby 3834 patients with underlying heart failure records and reduced ejection fraction were given 150 mg or 50 mg of losartan daily in a double-blind, supervised, and randomized trial. The patients were monitored for up to 6.8 years during a randomized experiment, and their blood pressure was taken at least three times points in the first year and at a semi-annual visit in the years afterward. During the patients' three-time visit to the hospital, their VVV of BP standard deviation was calculated. The average absolute for each patient visit-to-visit variation and the coefficient of variation. Their study used cox proportional hazard models to understand the associations between variation in SBP, time to death, heart failure cases, hospitalization, and baseline covariates. In a complete multivariate analysis which correlated with BP baseline, the subjects with relatively higher VVV of BP showed adverse consequences; their average absolute variation in systolic blood pressure in mmHg was 1.023 (95% CI (1.013,1.034), Pb 0.0001, these results were separate of the administered extra dose of losartan which was considered enhancing the outcomes. The above assessment concludes that in chronic myocardial infarction patients with reduced ejection fraction, there was an elevated VVV of BP. This is highly confirming some reported cases of more inadequate cardiovascular events; therefore, more clarifications and analysis should be prioritized in patients with congestive cardiac failure diseases to minimize CHF cases and increase testing prevention strategies.

Similar studies by Muntner et al. seek to analyze whether the VVV of BP was associated with coronary heart diseases, stroke, mortality, or heart failure [33]. Their assessment monitored 1194 fatal chronic heart diseases or nonfatal myocardial infarction, 1948 deaths,921 heart failure cases, and 606 strokes. Their study conducted a multivariable analysis whereby the mean of systolic blood pressure, the ratio was recorded comparing the highest against the lowest quantile of participated patients of SD of SBP (≥14.4 mmHg *vs.* ≤6.5 mmHg) was found to be 1.30 (95% Cl,1.06 to 1.59) for fatal chronic heart diseases or nonfatal myocardial infarction, 1.58 (Cl,1.32 to 1.90) for all-cause mortality, 146 (Cl,1.06 to 2.01) for stroke, and 1.25 (Cl, 0.97 to 1.61) for heart failure. The results showed that the higher VVV diastolic BP highly correlated with chronic vascular diseases cases and mortality. However, their study has not assessed the long-term impact. Their study recommended that future studies should determine whether reducing VVV of BP lowers the BP events.

In a study by Vishram et al., 8505 patients were subjected to losartan and atenolol medicament in the LIFE study, and their blood pressure was monitored over 24,6,12,18 months [34]. It was found that antihypertensive treatment is associated with the mean value of BP measurements. Nevertheless, it is unknown whether high VVV of BP is beneficial or detrimental in patients with complications in the left ventricular hypertrophy (LVH).

Table I. Experienced limitations encountered during different visit-to-visit blood pressure variability researches.

Limitations	Explanations	Reference
Inaccurate recordings, substandard peripheral circulation, elevated ectopy, or atrial fibrillation during the measurement. Statistical strength to understand various restrictions of BPV was limited by the minimum cases of repetitive vascular events. BPV was assessed after the inception of administering antihypertensive drugs, which may influence BPV and its relationship with repetitive events. Repeated assessments for estimation of repetitive conditions were conducted after the commencement of treatment.	This indicates the weakness of the investigation; however, it might also show the robustness of the investigation, which include a continuously enrolled, random old population with acute events. The investigation is among the broadest investigation of the prognostic effect of visit-to-visit SBP fluctuation in patients with stroke . This, however, basically eliminates the perplexing impacts of deficient mean BP control. This is required to cause a disparage of reproducibility.	[35]
A post hoc investigation of the TOPCAT trial, incomplete variables, and residual confounding factors can affect the outcome. Various point characteristics were self-declared and may therefore get disqualified due to some bias. Data on using BP-lowering medication on top of which the drug survey can be provided was not obtainable. An extensive investigation of the TOPCAT publications research did not describe precise effectiveness in controlling BP measurements in the TOPCAT trial. The number of people who participated in clinical trials was limited by the need to examine the validity of the observations in comparison with data from the community.	The use of antihypertensive drugs and other medications as a time-dependent co-variable. The assumption can fairly have made that the BP records in TOPCAT, a trial concentrated on HFpEF, were of clinical grade. Despite the evidence that the BP readings in TOPCAT were sub-optimally standardized across centers, this has diminished the study's strength and correlations between different difficulties regarding BP level and variability. Future studies should focus on the same topic.	[13]
The study was post-hoc and exploratory. Their results outcome was delivered from patients with HFrEF, and light symptoms and generalized to different patients with cardiovascular failure was not possible. The mechanisms behind the connection between SBP-CoV and results (particularly for the relationship between low SBP-CoV and worse results) were not perceived, and a threshold SBP value was utilized to decide to participate in the trial which could affect SBP-CoV results partially Due to the multi-centric design of the investigation that incorporated endpoints not centered around BP records, various devices for BP measurement were utilized These results were for hypothesis testing only, as various interactions tests are statistically unsatisfactory, and these records were not aligned for multiplicity	Information was extracted from an enormous randomized controlled trial, allowing adequate statistical capacity to assess the connections between SBP-CoV and risk. Further studies were recommended. All BP estimations were made utilizing approved semi-robotized BP machines with alignment records checked during each clinical exploration monitoring visit. Besides, having various devices decrease the likelihood of a systematic error occurring, reinforcing the relationship validity because they were not involved by random vacillations in the estimation measurements	[36]
This was a post-hoc exploratory analysis	Patients were not exposed to randomization. All things considered, the huge survey data and the thorough analysis of HR and SBP information data give the statistical backup to permit an accurate investigation of the associations to risk. These results might have clinical significance because they demonstrate that doctors that low SBP variation and low HR variation over several medical checkups indicate significant clinical data on future predictions in HF patients. It has to be established that this information is useful to HF with systolic dysfunction; on the contrary, in the HF population with preserved ejection fraction, no information was accessible	[37]
Their results could not represent all types of HF. For instance, a low pacing rate could be destructive in patients with extreme or decompensated HF, just as in serious heart failure problems Since the majority of the medical cases were registered with biventricular pacing (during the two pacing time frames), direct impacts of ventricular resynchronization as indicated by various pacing rates were not barred		[38]

It is widely recorded that patients with causal hypertension in the clinic are at high risk of experiencing a transient ischemic attack or recurrent attack regardless of careful control of mean blood pressure [24]. A study by Webb et al. assessed whether regular BPV could determine whether they will be an increased risk of the cardiovascular case [35]. However, it has some limitations: it can only reflect one type of VVV of BP, requires continuous monitoring to provide accurate results, requires a particular statistical analyst, needs validation from other cohorts, requires normative values and thresholds for pathological BPV. Webb et al. used 520 patients, 22 patients with atrial fibrillation and 26 patients with an irregular beat-to-beat history. Among 520 patients, 400 of them had consistency in all kinds of monitoring. In six weeks of the regular transient ischemic attack, BPV was recorded every 5 minutes, day-to-day for one week on home follow-up with at least three readings per day with a sphygmomanometer and measuring awake ambulatory blood pressure. It was found that beat-to-beat BP predicted recurrent stroke and cardiovascular cases with no correlation with mean SBP. Therefore, beat-to-beat BPV should be considered as an essential predictor of cardiovascular events. In addition, more data analysis of VVV in SBP patients, conducted to understand its relationship with the rise in the number of deaths in the general population, has concluded that VVV for DBP is not associated with mortality. However, VVV of SBP can be found in clinical practice, which is assumed to be the effect of measurement error [7, 21].

The relationship of elevated VVV of SBP in comparison with variability and all-cause of death were also assessed using medical records on the US adult population of > 20 years of age from the 3rd National Health and Nutrition Examination Study. The survey used three consecutive BP records registered during three different regular checks up from 1988 to 1994. According to the mean results of the second and third evaluations from the medical checkup, the VVV of BP for every patient was determined using the coefficient of variation and standard deviation between visits. The mortality rate was evaluated on the 31st December 2006 (the median follow-up was 14 years while the n=240 deaths). The findings showed that the mean, standard deviation for systolic blood pressure registered in-between visits was 7.7 mmHg. However, more analysis of multivariable adjustment such as female gender, older age, the records of myocardial infarction, elevated mean SBP, utilizing angiotensin-converting enzyme inhibitors, and pulse pressure were closely related with maximum standard deviation in SBP. The assessment found that the multivariable aligned hazard ratios for all-cause mortality correlated with a 4.80 to 8.34 and 8.35 mmHg systolic blood pressure, and the standard deviation were 1.57(95% CI),1.07 to 2.18), and 1.50 (95% CI,1.03 TO 2.18) respectively (Table I) [7].

CONCLUSION

Elevated VVV of BP is one of the causes of cardiovascular diseases. The researchers have inconsistently associated the increased blood pressure variability hypothesis with the epidemiology of hypertension, kidney, and stroke, and their clinical uses are still arguable, especially in patients with high BPV. However, there is some conclusive research on the role of blood pressure variability in increasing the risk of organ damage and stimulating cardiovascular events. Therefore, the prognostic significance of VVV of BP outweighs the limitations. The current hypothesis should be confirmed in future research to understand the cause, the mechanism, consequences, and the proper medication to regulate variability in blood pressure. In addition, future research should also focus more on verifiable predictions that can facilitate the treatment process of VVV of BP. Medical doctors and practitioners should be careful of the prognostic effect of VVV on BP and the effect of using the drugs recommended to control VVV of BP.

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REVIEW ARTICLE

HISTOINTEGRATION AND OTHER TERMS IN DENTAL IMPLANTATION

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ABSTRACT

The article describes biological and non-biological factors that affect the long-term stay of the implant in the bone under functional load, quality indicators of artificial teeth implantation. Instead of the term «osseointegration», the authors use the terms «histointegration» and «histodisintegration» defining them. The following concepts are used: time of histointegration achievement, histofunctional and histoaesthetic integration/disintegration, mechanical and biological stability/destability, integration/disintegration of implant and prosthetic structure, morphological and functional stability and destability of the implant.

KEY WORDS: primary and secondary histocontact, histointegration, histodisintegration, functional integration, implant stability, morphological and functional histostability, assessment of dental implantation

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INTRODUCTION

Modern medicine does not always restore the lost shape and function of a human organ. In such cases, we use rehabilitation and replacement therapy, devices and tools that fully or partially compensate for the lost and improve the quality of life. For example, there is a well-known prosthetics method of lost parts of the face or teeth based on implants (artificial tooth root substitutes) which provides food intake, speech, facial aesthetics, etc. [1-7]. This method has objective features, as well as risks and complications [8-15].

Creation of artificial support for dentures involves installation of an implant through a protective layer of the epithelium, disrupting separation of the internal environment from the external, and causing major biological problems of the method. The situation is complicated by the traumatic implant placement, excessive functional load of the supporting tissues of the implantation area, mainly bones, improper prosthetics and quality of materials used, the patient's health, the body's response to intervention, other factors, etc. It was described the phenomenon [11, 16, 17] of a long-term stay of an implant in the bone under functional loading named «osseointegration». In 1971, it was showed a tight fit of bone tissue to the implant without the appearance of a connective tissue layer and maintaining this contact under functional load [18].

In 1982, the conference in Toronto recognized the effect of «osseointegration» [4, 15, 17, 19-21]. However, some researchers recognize non-physiology, risks of the method and the harm from implantation of artificial teeth [7].

Today, we distinguish the following clinical problems, diseases and conditions of peri-implant tissues: 1) healthy tissues around the implant, 2) near-implant mucositis, 3) periimplantitis, 4) deficiency of soft and hard tissues around the implant [10].

The scientists study various biological and non-biological aspects to solve these clinical problems of dental implants. Biological ones include the patient's health, bad habits, local clinical conditions, quantity, structure and properties of the bone available for implantation, the condition of the tissues adjacent to the implant, method of implant use, primary implant stability, the quality of contact bone support with the implant, integration type, etc. [22, 23].

Non-biological issues include implant geometry, its thread, surface and anti-rotation quality, implant-superstructure connection, implant-bone interaction biomechanics, prosthesis and tool quality, role of CAD (Computer-Aided Design) / CAE (Computer-Aided Engineering) / CAM (Computer-Aided Manufacturing) technologies, use of guides, aesthetics, etc. Various clinical, radiographic, laboratory, computer and other methods are used to study them [5, 10, 24].

However, despite the achievements in the development and implantation of artificial teeth in humans, some issues remain uncoordinated, mainly regarding the biological aspects of the method, terminology.

THE AIM

The purpose is to clarify some formulations in the implantation of artificial teeth and insufficiently known indicators of implant quality, to assess the clinical situation more accurately and improve the use of the method.

MATERIALS AND METHODS

A comparison of some definitions and indicators of the implantation quality of artificial teeth known from the literature with our own clinical experience in the use of intraosseous dental implants.

REVIEW AND DISCUSSION

The term «integration» means «combination» – that is, the process of combining parts into a whole one that is used by many researchers. The available data in the literature show that the recognized definition of the term «osseointegration» is not definitively agreed, because there are many important features of this phenomenon. Thus, it is difficult to take them all into account or only the main ones in one sentence [2, 5, 24-27].

Osseointegration is a type of integration of an implant into bone tissue by way of direct contact (or strong «fusion» of metal with bone without an intermediate connective tissue layer, although living and non-living do not grow together) and functional connection between them.

Recently, instead of the term "osseointegration", the term "biointegration" of dental implants has been used, about which there is no common opinion yet [6]. The International Congress of Oral Implantologists experts believe that «biointegration» is the binding of living tissue to the surface of biomaterial or implant, regardless of any mechanical locking mechanism (2021), using it to describe adhesion to implants coated with hydroxyapatite. This term also refers to various things / processes: part of the basis of «sustainable development of forest ecosystems of the middle Russian forest-steppe» [28], «a step-by-step lifestyle changing system for «reference health, energy, activity and longevity», describing medical implant materials, etc. [29, 30].

We use the terms of preservation or destruction of biological substances-structures in various fields of human activity. These are, for example: biological stability of wine – resistance to the microflora which damages its consumer qualities, its appearance, and determines its degree; biostability and bioresistance of implants made of metal, cellulose, lead, polyacetal, polyurethane, ionic materials for humans [30]. There are also known terms bioerosion, bioresorption [31], which can be considered as possible in the implantation of artificial teeth. Given the unresolved issues of biology in the implantation of artificial teeth, scientists are conducting seminars on this topic, which emphasizes the lack of a single point of view on complex biological processes in the tissues around dental implants [3, 30, 32-34].

Considering the above, it is advisable to cite another definition of the complex phenomenon «integration» of artificial teeth adopted by experts, without going into a discussion about its essence. But, taking into account that the «integration» of the implant takes place in living tissues (histo) – in the bone, periosteum, there is contact with the mucous membrane, it is «histointegration». In addition, the implantation of any foreign body in the body will be an individual response of systems and tissues, which must be taken into account. Moreover, the created system «bone-implant-prosthesis» must have long-term stability – the ability to maintain proper condition under the external influence, functional load. Adding prefix «bio» to this term does not give additional and clear meaning to the general term because the integration of dental implants can happen after its introduction only into living tissues, as implants are not inserted into inanimate tissues.

Thus, «histointegration» is a direct, long-lasting, anatomically and functionally capable stable connection without the intervention of scar tissue between the functionally rebuilt support tissue and the foreign body, able to withstand long-term functional load without uncompensated damage to the patient's body.

Histointegration (formerly – osseointegration) can occur in conditions of partially open foreign body, it is open histointegration (after a single, direct implantation into the cavity of a newly removed tooth, or after delayed implantation). Closed histointegration occurs in conditions of a completely closed foreign body tissues (after a two-stage implantation). Histointegration can be complete (over the entire surface of the intraosseous part of the foreign body, the implant), or incomplete (over part of the intraosseous surface of the implant).

By supporting tissue we mean bone tissue and tissues that have arisen after an increase in bone volume in the implantation area, using biological and artificial grafts / materials (also scar mineral conglomerates, augmentates – the term needs to be agreed on). The mucous membrane and periosteum as well as scar tissue can not bear the mechanical functional load due to their structure, function and properties.

After placement of the implant into the supporting bone between them, there must not be any distance, the primary implant-bone gap. But such a gap sometimes occurs in some places, and it is filled with blood, bone substitutes, otherwise there will be no primary stability of the implant and subsequent histointegration. The surface of the implant is in direct contact with bone tissue, its deformed and damaged elements, destroyed bone beams and cells, blood, and often bone substitutes. This is the primary direct histocontact of the implant with the supporting tissues and their substitutes [35, 36].

Given that the properties of the supporting bone on the intraosseous surface of the implant, as a rule, are not the same along the entire length of the primary histocontact, the state of the supporting bone structures may differ in the areas of implant-bone contact. This can be: 1) deformation within the deformation capacity of bone, its Ewing module. Here, it is possible to provide rather fast adaptation of a bone to loading with compensation of deformation and preservation of available bone structures; 2) in areas where the load will exceed the deformation capacity of the bone, overloaded bone structures should be rebuilt with the emergence of secondary direct histocontact, adapted to the presence of a foreign body and restored bone structures; 3) destroyed bone structures were eliminated and replaced by new bone structural elements in accordance with the presence of a foreign body in the bone. However, we do not exclude the risk of incomplete osteogenesis in some cases / areas.

In the process of creating secondary direct histocontact between the support bone and the implant surface by removing destroyed or deformed over the level of elasticity of bone beams and other bone elements, there is a temporary

secondary implant-bone gap width according to biological needs, which is then filled with new elements. This secondary implant-bone gap, given the artificially uneven surface of the implant, should probably be less than the length / thickness of the primary bone structure – one collagen fiber (which will preferably turn into a bone beam) or one bone beam at once, and be filled with regenerating components of tissue fluid (glycosaminoglycans, etc.).

Obviously, the bone beams can contact pointwise with the implant on their end surface or planar lengthwise, or both. As a result of the completed osteogenesis, new normal bone structures should be created, old bone beams should be rebuilt and functionally oriented according to the new function, to which a new mechanical load is applied. This relationship between the implant and the bone means histointegration in this area of secondary histocontact.

If the distance between the implant and the bone is greater than the length / thickness of the bone beam and is filled with a layer of connective tissue several collagen fibers thick, then fibrointegration will be in this area of tissue contact.

It is clear that there must also be integration, i.e. periosseointegration, between the periosteum and the implant. Thus, osseointegration, fibrointegration, periosseointegration (and mucosal contact) are possible components of implant histointegration.

Implantation is considered successful when about 70% of the intraosseous surface of the implant has direct contact with bone tissue, i.e, osseointegration, which should be sufficient for physiological functional load on the implant. The other 30% of the implant surface may have fibrointegration, which should not adversely affect the overall functional properties of the support created for the denture.

These up to 30% of surfaces with fibrointegration are usually located near the crown of the implant, which may be due to the following: 1) osteogenic regenerative potential of alveolar bone is extremely low in the alveolar process of both jaws, and here it is programmed for regeneration with the cheapest for the body way, with most of the scar tissue or resorption; 2) the bone of the alveolar sprout will be resorbed in some diseases, in tooth loss and integral tooth-periodontal-alveolar-mucous complex, which has a system of mutual biological support of its structures; 3) there are major inflammatory complications in this area in the form of peri-implant mucositis, periostitis, osteitis, osteomyelitis and bone lysis (the accepted term is periimplantitis).

We know that there are both benefits to the body during implantation, and the harm from it. Damage is a surgical trauma, implantation of the internal environment of the body with the external (oral cavity), improper quality of foreign body material, penetration of infection into the bone wound, postoperative inflammation of the periosteum and mucous membranes (possibly septic), the need to restrain the cuff mucous membrane from microbial aggression of oral fluid, mechanical influences and temperature fluxes from hot food, which are more transmitted through the implant to the bone without its protection by the mucous membrane. Implantation also produces negative microwave fluxes from the alveolar bone to the implant, which also adversely affects physiology of the tissues adjacent to the implant. This damage must be eliminated or compensated by the patient's body, which begins immediately after the surgery and lasts throughout the stay of the implant in the tissues.

Considering the above definition of the histointegration phenomenon, it is advisable to add some concepts on the implantation of artificial teeth.

Time to achieve implant histointegration lasts from the moment of implant placement to the moment of supporting tissues adaptation to it, prosthesis and new function, mastering of the patient's ability to use dentures (period of habituation). It can last for different periods, 2-6 months or more, depending on the regenerative properties of the body and supporting tissues, used grafts and bone substitutes, and indirectly indicates the regenerative properties of the supporting bone. The shorter this time, the better the reparative adaptive response of the supporting tissues (bones) to the implant, and so on.

Implant histointegration ensures its functional and aesthetic (soft tissue) integration. Functional integration provides the implant, the implant-prosthetic structure of the ability to eat while maintaining the histointegration of the implant and its position in the jaw. Accordingly, there may be functional disintegration – loss of the achieved functionality.

Aesthetic integration (tissue and prosthesis) involves the normal appearance of soft tissues, the condition of the marginal mucosa, interdental papillae («red» aesthetics), as well as the appropriate shape and color of dentures, etc. Accordingly, there may be aesthetic disintegration of the implant (tissue and prosthesis).

Histodisintegration of the implant is partial or complete loss of positive morphological, functional and aesthetic effects of varying degrees, up to the loss of the implant.

The stability (resistivity) of the implant can be primary (mechanical or mechanical stability) and secondary (tissue or histostability), in the tissues rebuilt under the implant and the new functional load of the tissues. Mechanical stability should be achieved even when installing the implant in the bone due to the tight contact of it with supporting tissues that are injured and strain stress in the mismatch of the diameter of the implant and implant bed, bone compression. This is a manifestation of the primary direct histocontact of the implant. Mechanical stability is required for further histointegration of the implant and the achievement of secondary histocontact, i.e. histostability, when the elastic deformation of the bone disappears and becomes compensated, and damage to bone structure is eliminated by reparative regeneration.

Histostability of the implant (morphological and functional) means the long-term preservation of the supporting and adjacent tissues condition, their anatomical position, the quality of the achieved morphological and functional integration.

Accordingly, the histodestability of the implant can be morphological (partial or complete loss of morphological qualities of the supporting tissues) and functional (usually secondary and depends on the quality of morphological stability, partial or complete).

Stability of the implant and prosthetic structure is the preservation of their acceptable clinical-morphological and functional-aesthetic condition for a long time. Accordingly, destability of the implant and prosthetic structure may occur due to the loss of their biological and mechanical qualities.

Mechanical stability (mechanical resistivity) of the implant and implant-prosthetic structure is a long-term preservation of the properties of supporting tissues, mechanical components and the entire structure (implant, screw, superstructure, prosthesis). Accordingly, there may be mechanical destability – partial or complete loss of viability of adjacent tissues and the destruction of the implant-prosthetic structure with its preservation or loss. In addition, there may be only aesthetic destability of the implant-prosthetic structure (for example, breaking off the edge of the artificial crown).

The data on the implantation of artificial teeth in the literature and our own experience in the use of dental implants since 1977 indicate the need for more careful study and accurate determination of the processes that the doctor deals with when replacing lost teeth and restoring their basic functions. The authors substantiate the reason for this in the article.

The definition of the term «histointegration» of the implant refers to the compensation or subcompensation of the patient's body for damage and risks caused by dental implants. It is harmful as it determines the body's response to implantation, disrupts the continuous protective epithelial layer of tissues and combines the internal environment of the body with the external.

There is also a description of the terms in the article: time of achieving histointegration of the implant, functional integration and disintegration, aesthetic integration and disintegration, morphological and functional stability of the implant, mechanical stability / destability of the implant and prosthetic structure, aesthetic destability. These terms and their meanings can be discussed.

The use of these terms, indicators of the state of the implant and implant-prosthetic structure, comparison of their state over time clarifies the description of the clinical picture, the state of the implant and implant-prosthetic structure, expands clinical diagnostic capabilities. This more fully informs the physician about the dynamics of the processes involved in periimplant tissues, implants and implant-prosthetic design, allows you to take the necessary measures in advance to influence the clinical situation, and can be useful in the clinic.

CONCLUSIONS

1. The authors use the term «histointegration» of a dental implant which describes the essence of this phenomenon more accurately.
2. The article gives a more complete definition of the term «histointegration» of a dental implant, indicating the effect of the implant on the human body.
3. Histointegration can be open and closed, partial and complete, stable and unstable according to the method of implantation.
4. The article describes clinical components of histointegration and histodisintegration effect, stability and destability of the implant, the state of the implant-prosthetic structure, the use of which clarifies the clinical picture, condition and outcome of dental implantation.
5. The given data, terms and their values increase the number of indicators for a more complete assessment of the quality of dental implants, compare different components of dental implants and increase their efficiency.

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REVIEW ARTICLE

FINANCING HEALTH CARE FROM LOCAL BUDGETS OF UKRAINE AND THE REPUBLIC OF POLAND

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ABSTRACT

The aim: This article aims to show the role of local budgets in financing health care in communities, and to initiate a discussion on the possibility of expanding powers of local government bodies in the sphere of health care.

Materials and methods: This study is based on the normative acts of the Republic of Poland and Ukraine, conclusions, reports of the Regional Chambers of Audit (Regionalnych Izb Obrachunkowych) in the Republic of Poland, OECD Indicators and budget gminas. Dialectical, comparative, analytical methods and the method of system analysis were used.

Conclusions: Health care is among own tasks of gminas. Special legislation in the sphere of health care clarifies and narrows the tasks of gminas to monitoring and assessing public health, as well as to development, implementation and evaluation of special policy programs in the sphere of health care, according to identified needs of community residents and projects aimed at informing people about factors harmful to health and about their consequences. These programs can be financed from the budgets of gminas. This approach reduces and limits potential of local governments in the health care sector. Only in the event of an emergency gminas have the right to assign additional tasks to a medical entity and to finance these tasks from their own budget.

In order to improve the quality and clarity of legislation own tasks of gminas in the sphere of health care (point 5, Part 1 Art. 7 of the Law of the Republic of Poland dated 8 March, 1990 "On municipal Government") should be specified taking into account point 1 Art. 2 of the Law of the Republic of Poland dated 11 September, 2015 "On Health Care".

KEY WORDS: financing of the health care system, expenditures of local budgets, own and delegated tasks of gminas

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INTRODUCTION

In modern conditions, when the global pandemic has suddenly entered our everyday life, the value of human existence and health are gaining great importance. This problem has become extremely topical and has made the issue of health care in many countries the most discussed among multidisciplinary experts.

Successful functioning of the health care system in any country is assessed by specialists on several important factors, including: qualification level of physicians, availability of modern medical equipment and medicines, the development of an appropriate infrastructure, etc. At the same time, financial provision of health care is the most important condition, which guarantees efficiency of functioning of medical branch in general, as well as quality and accessibility of medical services for citizens, in particular.

The global pandemic has demonstrated those significant volumes of monetary means needed to purchase antiviral vaccines, other drugs and materials required to overcome the acute respiratory illness COVID-19 caused by the SARS-CoV-2 coronavirus and its consequences. Moreover, at the beginning of the pandemic, these funds were not provided for in the budgets, but they had to be quickly allocated and spent, despite the fact that each country legislatively approved a certain multi-link procedure for their use. At the same time, the existing hospital infrastructure, existing equipment and even the level of physicians' salaries were tested for compliance with the new challenges.

It should be noted that in the field of health care, as a rule, there is a direct relationship between the wealth of the state including the degree of development of institutions involved in financing medical expenditures, and the status of branch infrastructure including quality of medical services provided to the population. The richer and more socially responsible the country is, the higher is the percentage of funds from the state budget that this country can spend on financing the medical sector. Thus, according to the OECD Club of Developed Countries which includes 34 countries (the Republic of Poland has been a member of this club since 1996), "health spending by government schemes and compulsory insurance stood at around 15% of total government expenditure across the OECD. In Japan, Switzerland, New Zealand, the United States and Germany more than 20% of public spending was dedicated to health care" [1].

The indicators are quite eloquent and for Ukrainians they are the goal to which Ukraine aims to move gradually. It should be noted that the Law of Ukraine "On State Financial Guarantees of Medical Service to the Population" dated 19 October, 2017 No 2168-VIII provides annual expenses for implementation of the program of medical guarantees not less

than 5 percent of the gross domestic product of Ukraine [2]. In general, health care in the structure of expenditures of the State Budget of Ukraine in 2021 is 11%, which is a significant growth in comparison with previous years.

But does the mentioned level of financing of the health care system correspond to the existing demand in this sphere? Despite the large difference in the amount of funds allocated in Poland and Ukraine, the answer is the only one – No, it doesn't. Problems of balance or compliance of resources with the needs of society, as well as their sources, are currently widely discussed in the world.

It should be noted that scientists engaged in research of health care resources emphasize the importance of preserving namely public financing of this sphere in contrast, for example, to private health insurance. "Publicly generated finance contributes to efficiency and equity by providing protection from financial risk and by detaching payment from risk of ill health. It also ensures that resources are allocated on the basis of need, that is on the basis of where they can do the most good, rather than on the basis of ability to pay" [3, 95]. Public financing here should be understood in a broad sense, including local budgets.

According to Article 9 of the Law of the Republic of Poland "On Public Finances" the public financial sector in the Republic of Poland includes, among others, local government units and their associations, state trust funds, the National Health Fund, independent state health care institutions, etc. [4]. Note that a well-chosen model of health care financing: from state or local budgets, from certain special funds, or through mandatory state health insurance - may be of great importance for provision of appropriate medical services, even in conditions of limited financial resources in developing countries.

THE AIM

This article aims to show the role of gminas' budgets in financing health care in communities, to analyze the own and delegated tasks of gminas in the sphere of health care, to compare the powers of gminas and united territorial communities (hereinafter referred to as UTC) formed in Ukraine, as well as to initiate a discussion on the possibility of expanding powers of basic local government bodies in the sphere of health care.

MATERIALS AND METHODS

This study is based on six normative acts of the Republic of Poland and three normative acts of Ukraine, activity reports of the Regional Chambers of Audit (Regionalnych Izb Obrachunkowych) in the Republic of Poland, OECD Indicators and budget indicators of gmina in KRYNICA MORSKA city for 2021. In total, 13 normative-legal acts and documents were used.

Dialectical, formal legal, comparative, analytical methods and the method of system analysis were used. The analytical method, the formal legal method and the method of system analysis were used to work with general and special normative acts in the sphere of activity of local government bodies and health care bodies, as well as with the materials of activity reports of the Regional Chambers of Audit in the Republic of Poland and indicators of gmina's budget. The comparative-legal method was used to identify similar and different principles, rules of health care financing, and directly legal norms regulating relations in the sphere of health care financing in the Republic of Poland and Ukraine. The dialectical method revealed certain contradictions and shortcomings that exist in legislation and practical activities. The complex of the specified scientific methods of research gave an opportunity to achieve the stated aims of the article.

REVIEW AND DISCUSSION

When studying problems of financial support of the health care sector from budgets of various levels, it is necessary to emphasize importance of a clear regulatory distribution of tasks in this area among central, regional and local authorities. According to the current legislation, health care activities are included in the scope of powers of authorities at all levels both in Poland and Ukraine. Note that in the Law of the Republic of Poland "On Health Care" dated 11 September, 2015 even the essence of health care is disclosed through a list of tasks to be performed by authorized entities. These tasks include: monitoring and assessment of public health, threats to health and quality of life related to public health; health promotion; disease prevention; activities aimed at identifying, eliminating or limiting threats and harm to physical and mental health in the environment of living, studying, working and leisure; analysis of adequacy and effectiveness of medical services provided in relation to the identified needs of the society in the sphere of health care; reduction of health inequalities due to social and economic conditions, etc. [5]. But the specified normative act does not clearly distribute the specified tasks among branches of power, and in part 1 Art.3 the need for their implementation in cooperation with public administration bodies, in accordance with the Law of the Republic of Poland "On Public Administration Departments" dated 4 September, 1997. The latter law refers the sphere of health care naturally to issues under control of the Ministry of Health, without detailing their content and determining the scope of competence of other authorities in this area (Art. 33.1.) [6]. Local government bodies should be guided by tasks of strengthening or protecting health, which are defined as their own tasks, and in the process of task planning and implementation they should also interact with state authorities and various levels of government.

Taking into account the fact that in this article we would like to focus on the role assigned in health care financing to the basic local governments, we will consider powers obtained in this sphere by gminas in the Republic of Poland and united territorial communities in Ukraine.

It should be noted that according to point 5 paragraph 1 Art. 7 of the Law "On municipal Government" dated 8 March, 1990 health care is among own tasks of gminas

[7]. Polish scientists, like us, have already noticed that health care is a very broad concept, and there is no clearly regulated legislative list, which should confirm the scope of own tasks of gminas in this area [8].

If we analyze the Law of the Republic of Poland "On Health Care", we can state that based on point 3 Art. 3 of this Law, the task set out in point 1. Art. 2, and namely "monitoring and assessment of public health, threats to health and quality of life related to public health" may be directly attributed to own tasks of gminas. We make this conclusion because point 3 Art. 3 of this Law says about implementation of this task by local authorities of gminas or powiats both independently and in cooperation with local self-government bodies of voivodeships. At the same time, it is not quite clear how the classical rule of financing own and delegated powers should be applied in this situation (according to this rule own powers are financed from local budgets and delegated powers are financed from the state budget).

The possibility of financing health care programs from own funds is provided for gminas in Art.13 of the Law of the Republic of Poland "On Health Care". In the event that local government health programs are in line with and included in the National Health Program (hereinafter referred to as NHP), gminas may receive targeted subsidies for their implementation. Moreover, we want to pay attention to the norm laid down in Art.18 of the Law of the Republic of Poland "On Health Care". It says that the monitoring and assessment of health of the society and other related studies (their list is given in Art.18) shall be financed in the amount of not less than 10% of the funds allocated for the fulfillment of tasks envisaged in the NHP as a whole. In this way the state guarantees financial support for implementation of the mentioned task, which can usually be financed on a residual basis.

Thus, the task in the sphere of health care, concerning monitoring and assessment of public health, threats to health and quality of life related to public health belongs to own tasks of gminas, and can be potentially financed both at the expense of gmina's own funds and at the expense of the state budget of the Republic of Poland, if the corresponding programs are included in the NHP. Under these conditions, the desire of gminas to initiate and implement relevant programs outside the NHP appears to be questionable.

At the same time, in our opinion, legislation of the Republic of Poland does not limit local government bodies, when implementing tasks in the sphere of health care according to the National Health Care Program. Based on the content of sub-paragraph b of paragraph 4 Art.18 of the Law of the Republic of Poland "On Health Care", which deals with assessment of effectiveness of public health objectives, such as ***health promotion or prevention of diseases*** other than those specified in the NHP, we can conclude that these events can be independently implemented and financed by local government bodies.

In order to confirm or refute this opinion, we will turn to the Laws of the Republic of Poland "On Medical Activity" dated 15 April, 2011 and "On Medical Services Financed from Public Funds" dated 27 August, 2004 [9, 10]. The latter law concerns the most important segment of the health care sector and it is a special normative act on the financing of health promotion measures.

Paragraph 1, Part 1 Art.7 of the Law of the Republic of Poland "On Medical Services Financed from Public Funds" dated 27 August, 2004 own tasks of gminas in the sphere of ensuring equal access to medical services include initiation, development, implementation and evaluation of special programs and projects. First, these are ***health care policy programs***, according to the identified needs of community residents, as well as assessing the impact of such programs on their health. This point is somewhat similar to the above mentioned paragraph. 1. Art. 2 of the Law "On Health Care", however, it can be much broader. Second, these projects are aimed at informing people about factors harmful to health and their consequences. It should be noted that ***health care policy programs*** are aimed at achieving intended goals (the detailed definition of the concept is contained in point 29a Art. 5 of the Law of the Republic of Poland "On Medical Services Financed from Public Funds" dated 27 August, 2004) and they may be financed by both the state and local government bodies. While health care programs that give an opportunity to achieve the goals set (point 30 Art. 5 of the Law of the Republic of Poland "On Medical Services Financed from Public Funds" dated 27 August, 2004) have to be a priori financed exclusively from the National Health Fund However, paragraph 1 Art. 48c of the same law contains a provision under which a local government body, within the framework of its own tasks, may co-finance other health care programs alongside with health care policy programs. Besides, it is important that paragraph 4, Part 1 Art. 7 of the Law of the Republic of Poland "On medical Services Financed from Public Funds" dated 27 August, 2004 allows carrying out other activities related to the revealed health needs of community residents. Such legislative nuances deserve a more detailed study and analysis of the practice of using the mentioned norms.

Przemyslaw Szetela also emphasizes the subjectivity of local government bodies in legal relations of health care financing. According to the scientist such subjectivity of local governments follows from Art.38 of the Law of the Republic of Poland "On Medical Activity". According to paragraph 2 Art.38 in emergency situations local government bodies may impose an obligation on a medical entity to perform additional tasks. Such emergency situations may occur in the event of a natural disaster, an epidemic or other global problems [11, 60]. The said norm is now topical due to the global pandemic related to the acute respiratory disease COVID-19 caused by coronavirus SARS-CoV-2. Pursuant to point 3 Art. 38 of the Law of the Republic of Poland "On Medical Activity", the body which imposes obligations to perform additional tasks has to provide funds for covering expenses related to fulfillment of such tasks. We agree with the researcher that the mentioned legislative norms give grounds to recognize the right of local governments (potentially the right of gminas) to finance the relevant health care expenditures. It

would only be desirable to add that the source of funding such expenses should be a special or reserve fund of the respective local budget.

There is no doubt that the vagueness of the legislative definition of sources of funding for health care tasks may pose certain problems in their practical implementation. Doctor of economic sciences, Malinovska-Misyingh points out some shortcomings in the system of distribution of public funds directed from the central budget to budgets of gminas and powiats to address the powers delegated by the state at the local level. Such distribution is carried out by voivodes, but principles and methods of its implementation are not clearly defined in the legislation. While monitoring the budget execution, the State Treasury and the Supreme Audit Office of Poland provide an assessment of the planning and distribution of these funds already ex post facto, which may entail various consequences. According to the scientist, amounts allocated under the section "Health care" make up approximately 27% of the total amount of subsidies and are directed exactly to the powiats [12, 74].

Thus, after considering the current legislation and after studying opinions of scientists, we can make a preliminary conclusion that the tasks of gminas concerning monitoring and assessment of public health, threats to health and quality of life related to public health can be implemented only within the framework of special programs of health care policy (health care policy programs) financed from the local budget of gminas as well as at the expense of the state budget or funds provided by the National Health Fund. Tasks concerning health promotion, prevention of diseases, reduction of inequalities in health status due to social and economic conditions as well as other tasks are legally permissible and, accordingly, possible to be implemented through special municipal programs at the expense of own funds of territorial communities, as well as in emergency cases – from the reserve fund or the special fund.

In Ukrainian legislation, in contrast to Polish legislation, tasks in the sphere of health care are not defined in detail by any special laws. Fundamentals of Ukrainian health care legislation enshrine the right to health care and outlines the areas of guarantees of this right. These guarantees include: 1- creation of a well-developed network of health care institutions; 2 - organization and implementation of a system of state and public measures for health care and strengthening; 3 - financing of providing all citizens and other legally-defined persons with guaranteed volume of medical and rehabilitation services and medical drugs in accordance with the procedure established by law, etc. [13].

The indicated guarantees concerning realization of the right to health care are to a certain extent correlated with powers of the executive bodies of village, settlement, city councils (including united territorial communities) in the sphere of health care. These powers are traditionally established as own (self-governing) powers and delegated powers. In accordance with Art.32 of the Law of Ukraine "On Local Governments in Ukraine" self-governing powers include the following: management of health care facilities owned or transferred to territorial communities, organization of their material, technical and financial support [14]. That is, local government bodies in Ukraine in the sphere of health care have their own rights and obligations only with regard to medical institutions, which are owned by or transferred to these bodies (the legislator does not interpret the content of the latter legal regime).

Delegated powers of local government executive bodies, including the united territorial communities in Ukraine, include the obligation to ensure availability and gratuitousness of medical care; to ensure the development of medical care, improvement of the network of medical institutions of all forms of ownership; to provide privileged categories of population with medicines and medical devices [14]. As can be seen from the contents of the above rule, delegated powers relate more to the organizational role in implementation of public health measures in communities. Of course, delegated powers should be exercised by local government executive bodies only on the basis of laws and at the expense of the state budget.

Based on the norms analyzed above, we can conclude that local health programs can be financed from the local community's own budget, only with respect to health care facilities owned by these communities. Other local health care programs that deal with a wider range of issues may be accepted, but they should be in line with national programs in this sphere and financed from the state budget. That is, despite the limited powers of gminas in the health sector they have much greater potential to implement their own programs in this sphere than united territorial communities in Ukraine.

As we have seen, in the powers of local governments in the sphere of health care the Ukrainian legislator emphasizes development of public health facilities. In the Republic of Poland, financing of health care infrastructure expenditures from budgets of gminas is possible only at the expense of development expenditures. These expenditures are usually funded by European funds, which thus make investments in municipal infrastructure projects.

From 2014 to 2020, the Republic of Poland as a whole had to receive 82.5 billion euros from the EU under the Cohesion Policy Program. Financial support is provided for implementation of national and regional development programs from the European Regional Development Fund (ERDF), the EU Cohesion Fund, and the European Social Fund (ESF). Regarding regional development, it should be noted that EU investments are primarily aimed at building social infrastructure, in particular, health, culture and education facilities [15, 124].

Ukrainian scientists studying experience of development of territorial communities of Polish gminas note that "in fact 60-70% of funds spent for capital investments in Polish gminas are European Union funds; respectively, for these funds a prerequisite for funding consists in availability of a strategic plan, and the compliance of the projects the gmina wants to receive funds for with the priority areas of the strategy" [16, 76].

At the same time, Polish scientists note a gradual reduction in development expenditures, both local budgets themselves and investment expenditures allocated to local governments of the Republic of Poland from European Union funds. A study by Doctors of Economics Ida Musiałkowska and Marcin

Wiśniewski shows that according to the trend of 2010-2015 (according to a retrospective analysis) in the structure of EU funds going to local budgets current expenditures were increasing and investment expenditures were declining. The corresponding trend was maintained in 2016 - 2019, and also such a financial instrument as repayable funds with equity participation of communities was increased [17, 92- 93]. According to the scientists, this situation did not contribute to active development of territorial communities.

However, despite the existing legal and resource problems in the financing of medical institutions, a number of experts still supported delegation of health care tasks to local governments, because at the local level it is possible to better assess needs of population in this area [11, 57]. After analyzing such documents as: The Law of the Republic of Poland "On Medical Activity", the Law of the Republic of Poland "On Public finance", Przemyslaw Szetela concluded on the possibility of financing health care institutions in Poland by providing targeted subsidies and credits from the founders of these institutions. We should recall that at a certain time medical institutions in Poland were on the initiative reorganized into economic entities, in which the founder is a local government body. Moreover, according to Polish legislation, a medical institution may potentially receive subsidies from any level of local government. However, the mentioned researcher of legislation draws attention to the legislator's warnings concerning the fact that funding for health programs, including purchase of devices and equipment, medical and other investments necessary to perform these tasks, as well as repairs, can be carried out only at the expense of public funds (NFZ and the state budget). And indicators of financing health care facilities today look as follows: 85% from the NFZ, 10% from the state budget and only 5% from the local budgets. Within the latter 5%, only 0.7% belong to gminas [11, 59-62]. That is, the real possibilities of gminas to participate in financing health care facilities are rather limited.

Despite the fact that when investigating the issue of health care financing some scientists, emphasize that financing of medicine from local budgets is a kind of fiction [18, 37-46], we should point out that the budget structure of gminas' expenditures still include expenses for medical services, both in current expenditures, and in capital expenditures. For example, 2,000.00 złotych are planned in the budget of Gmina Krynica Morska (in the section capital expenditures (code 4280)) for purchase of medical services in 2021 [19]. As we can see, the sum is extremely small, and one should also take into account that according to the information given in the report from Krajowa Rada Regionalnych Izb obracunkowych, in 2019 in Gmina Krynica Morska the share of property income was the highest in Poland and reached 52,8% of the total income [20]. In addition, the surplus of the previous years in the budget of 2021 in this gmina is 5,071,985.00 złotych (Appendix 4) [19]. As we can see, financial resources are available in this particular gmina, but they are not spent for health needs of the territorial community.

The problems we have addressed in this article are not purely theoretical. The global pandemic has shown that many lives depend on efficient governance in the health care system with minimal duplication of functions between central, regional and local governments, as well as with adequate funding for the branch. An example of this may be presented as significant problems in installation of stationary oxygen plants and provision of hospitals with portable oxygen concentrators (these problems emerged in Ukraine in the autumn of 2021). An awkward system of receiving targeted funds by local governments, lengthy tender procedures for purchase of goods at the expense of budget funds, lack of own targeted programs did not allow to quickly and efficiently address such issues.

CONCLUSIONS

As a result of analyzing legislation of the Republic of Poland, we can state that health care belongs to the own tasks of gminas. Alongside with this special legislation in the sphere of health care clarifies and narrows the tasks of gminas to monitoring and assessing public health, threats to health and quality of life related to public health; development, implementation and evaluation of special policy programs in the sphere of health care, according to identified needs of community residents and projects aimed at informing people about factors harmful to health and about their consequences. Therefore, we can recommend to specify own tasks of gminas in the sphere of health care (point 5, Part 1 Art. of the Law of the Republic of Poland "On Municipal Government" dated 8 March, 1990), taking into account point 1 Art.2 of the Law of the Republic of Poland "On Health Care".

Gminas can finance the mentioned measures from both the local budget and the state budget. Allocation of funds from the state budget is possible in case of introduction of municipal programs in the National Health Program.

However, there are legislative opportunities to approve, implement and independently finance municipal health programs, as well as preventative health care programs, reduction of inequalities in health status due to social and economic conditions and other health care programs in gminas; but these opportunities are extremely rarely used in practice.

Division into own and delegated powers of gminas and UTCs in the sphere of health care is quite conditional, given the fact that both groups of powers are financed mainly from the state budgets of Ukraine and Poland.

For Ukraine, it is recommended to use the experience of the Republic of Poland and expand own powers of UTCs in the sphere of health care.

As we have already mentioned above, at present, neither the richer nor the poorer countries keep a balance between the resources allocated to health care and the needs of the society. Therefore, we believe that the use of the potential of basic territorial communities in this sphere should be strengthened, especially since it is already established legislatively, but is rarely used in practice. Taking into account modern research, according to which prevention and early diagnostics of diseases are considered more effective and less expensive than, actually, treatment of diseases, we offer to gminas and united territorial communities to concentrate their attention and funds on preventative measures. Financing of health care activities aimed at prevention of diseases and their early diagnostics should be performed on the basis of special programs approved by local governments from special funds of local budgets.

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CASE STUDY

A CLINICAL CASE OF ARTERIAL THROMBOSIS IN A PREMATURE BOY WITH NEONATAL SEPSIS AND HIGH RECTAL ATRESIA

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ABSTRACT

Occlusion of the venous or arterial vessels in childhood is rather rare but dangerous complication. Occurrence of neonatal thrombosis is 0.5 per 10 000 of live newborns. Promoting factors include congenital defects of coagulation, maternal diabetes, neonatal sepsis, necrotic enterocolitis, asphyxia, and metabolic diseases. More than 90 % cases of neonatal thrombosis are associated with catheterization (umbilical arterial or venous, other central venous lines). Acute arterial thrombosis caused by a peripheral venous catheter is very rare complication, but it can result in severe consequences.

The article contains a clinical case of right brachial artery thrombosis in a premature boy with extremely low body weight complicated by development of dry gangrene and amputation of the limb. Complex risk factors promoting development of neonatal arterial thrombosis were found: preterm birth, neonatal sepsis, possible incorrect insertion of a peripheral venous catheter. Associative factors were congenital developmental defects of the intestine (high rectal atresia) and heart (bicuspid aortal valve, open oval foramen), and maternal factors. The authors emphasize their attention on the necessity to continuous education of the medical staff concerning the technique of catheter insertion and care of them, absolute implementation of safety policy concerning nosocomial infection, adequate provision of ultrasound examination devices with high rarefaction sensors, involvement of a multi-disciplinary team of specialists to manage complicated clinical cases.

KEY WORDS: thrombosis; amputation; premature infant; neonatal sepsis; anal atresia

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INTRODUCTION

Babies born before complete 37 weeks of gestation are considered to be premature. The WHO estimates about 15 million of children in the world to be born prematurely. Complications resulting from preterm delivery are the main cause of lethal outcome among children under 5 years. Thus, in 2015 they caused death of about 1 million of children. Three fourths of those deaths could be prevented by means of the existing means, even without an intensive care unit. The index of preterm delivery varies from 5 to 18 % of neonates. Premature babies are divided into categories depending on the terms of gestation: extremely premature (less than 28 weeks); considerably premature (from 28 to 32 weeks); moderately premature (from 32 to 37 weeks) [1].

Occlusion of the venous or arterial vessels in childhood is rather rare but dangerous complication. Occurrence of neonatal thrombosis is 0,5 per 10 000 of live newborns. Promoting factors include congenital defects of coagulation, maternal diabetes, neonatal sepsis, necrotic enterocolitis, asphyxia, and metabolic diseases. Though, more than 90 % cases of neonatal thrombosis are associated with catheterization (umbilical arterial or venous, other central venous lines). Acute arterial thrombosis is an extremely rare complication caused by a peripheral venous catheter. Its treatment requires involvement of a multi-disciplinary team of specialists [2-4]. Tsonis O. et al. (2020) indicate that neonatal arterial thrombosis can occur even during delivery; and cases with a low risk with the lack of visual promoting factors can be of a great danger for limb-sparing, in case appropriate measures are not initiated [5].

The main goal of this article is the presentation of clinical case of arterial thrombosis in a premature boy with neonatal sepsis and high rectal atresia.

Informed written consent about publication of case report and pictures was obtained from parents. This study was conducted in compliance with the basic provisions of the Good Clinical Practice (1996), Council of Europe Convention on Human Rights and Biomedicine (1997), Helsinki Declaration of the World Medical Association on Ethical Principles for Medical Research (1964 - 2008).

CASE REPORT

The boy's body weight at birth was 900 g, the body length 34 cm. The boy was born to X pregnancy (I degree anemia, vegetative-vascular dystonia, varicose veins of the lower limbs, respiratory infection at 20 weeks of gestation), X delivery in 29 weeks of gestation by means of caesarian section due to premature abruption of the normal placenta, and uterine bleeding. Waterless period was 15 days and 5 hours. The mother received antenatal prevention of respiratory distress-syndrome for her baby (24 mg of Dexamethasone). USD conducted at 29 weeks of gestation found low weight for the gestational term, the signs of

cardiomyopathy, hydropericardium, inconsiderable aortal stenosis and extreme oligo(hydr)amnios; there was no pathology found from the site of the digestive tract and urinary system.

Findings of the obstetrical anamnesis: I-IV pregnancy – mature babies with the body weight more than 3000 g, V pregnancy – mature baby with the body weight of 2480 g (antenatal death), VI pregnancy – 35 weeks of gestation with the body weight of 2500 g, VII pregnancy – 35 weeks of gestation with the body weight of 2500 g, VIII pregnancy – 34 weeks of gestation with the body weight of 2000 g, IX pregnancy – 34 weeks of gestation with the body weight of 2130 g.

The baby's condition at birth was assessed as severe at the expense of severe respiratory disorders, pronounced inhibition syndrome with low body weight and prematurity. Primary examination found the signs of congenital developmental defects of the intestine – anal atresia. The 1 minute Apgar score was 4 (2-1-0-0-1), the 5 minute score was 6 (2-1-1-1-1), the 10 minute score was 6 (2-1-1-1-1). A complex of resuscitation measures was initiated: lung inflation followed by tracheal intubation and mechanical ventilation through the intratracheal tube with 30 % oxygen, catheterization of the umbilical vein and injection of warm 0,9% NaCl solution 10 mg/kg. After the baby's condition was stabilized, natural pulmonary surfactant Poractant alfa in the dose of 200 mg/kg phospholipids was introduced by means of an invasive method.

The baby's condition remained extremely severe during the first day of life due to III degree respiratory failure, signs of hypoxic-ischemic damage of the nervous system and congenital developmental defect – anal atresia. Neurologically pronounced inhibition syndrome was detected. The skin and visible mucous membranes were rose-pink and warm by touch. Mechanical ventilation was conducted from both sides, rales were absent. Heart sounds were sonorous and rhythmical; HR was 120 beats per minute. Arterial BP corresponded to the gestational term. The tongue was moist and not coated. The abdomen was enlarged in the volume, bloated, symmetrical, participates in respiration by all the portions regularly, palpatory soft but tender. Symptoms of the peritoneal irritation were negative, peristalsis was intensified. Spontaneous and stimulated defecation was absent, urine with meconium admixtures. The baby was taken care of at the III level intensive care unit, in the infant incubator, under protective regimen. Traditional mechanical ventilation was carried out in the regimen of normal ventilation with FiO2 0.21. The baby received parenteral nutrition, empiric antibiotic therapy (penicillins and aminoglycosides in appropriate age doses) and anaesthetic therapy through the umbilical vein by means of an umbilical catheter. Continuous stomach decompression was performed through the orogastric probe. Results of laboratory findings were within physiological limits.

Provisional diagnosis: congenital developmental defect of the intestine: anal atresia. Low intestinal permeability. Primary lung atelectasis. III degree respiratory failure. 29 weeks prematurity.

On the *1st day of life* the baby was transferred to the surgical department of the children hospital, IV (specialized) level. According to clinical data and results of Wangensteen X-ray examination the surgical diagnosis was made: high rectal atresia with abscess into the urethra or urinary bladder. The pathology required immediate surgery after the baby's condition was stabilized, and starting adequate preoperative preparation.

On the *2nd day of life* surgery was performed keeping to the rules of surgical asepsis in the resuscitation room under conditions of the infant incubator and appropriate thermal conditions. The surgery consisted of left lateral laparotomy; separate sigmoid stoma was delivered (distal and proximal portions of the sigmoid colon were delivered as stomas).

The stoma started functioning on the 3rd day after surgery, but general baby's condition remained severe due to manifestation of the syndrome of multiple organ failure. In the dynamics of disease the child developed signs of severe respiratory failure and cardiovascular failure, which required invasive ventilation support and indication of inotropic support.

On the *10th day of life* the patient manifested signs of late neonatal sepsis associated with considerable increase of pro-inflammatory markers in the blood (leukocytosis higher $30*10^9$/L, neutrophil index more than 0.7, C-reactive protein more than 24 mg/L). Due to possible neonatal infection antibiotic therapy was corrected with step-by-step change of the combination of III generation cephalosporin and aminoglycoside, carbapenem and aminoglycoside, to fluoroquinolone monotherapy. The total duration of antibiotic therapy lasted 28 days. Echocardiography determined the signs of congenital heart defect – bicuspid aortal cusp and open oval foramen (d = 5.1 mm).

It should be noted that umbilical catheter was removed on second day of life, during admission to the surgical department. Attempts to find the central vein (subclavian, jugular or femoral) to give infusions by means of the technical means available failed. Catheters were inserted into the right ulnar vein and dorsal vein of the left foot. Then repeated venipunctures were made due to the lack of function of the peripheral veins punctured before.

On the *12th day of life* venipuncture was made in the right cubital fossa (catheter 24G Abbocatch) for continuous infusions. On the 13th *day of life* the right upper limb was noticed to become cyanotic and grey to the ulnar bend, nail bones of the right hand became black, but local skin temperature and pulsation of the major vessels remained normal. The catheter was immediately removed from the peripheral vein. *On the 14th day of life* cyanosis of the right upper limb spread to the upper third of the right upper arm.

First cyanosis and swelling of the right hand was observed with retained movements in the fingers and hand. Then against the ground of the above symptoms the nail bone of the II right finger was detected. On the *16th day of life* nail bones of I-V finger of the right hand became black, and the skin became cyanotic and grey. It spread to the upper third of the right upper arm, and pulsation of the arteries was not detected. On the *17th day of life* the

hand and forearm became black, vascular pulsation in the portion of the middle third of the upper arm was not found, though it was felt on the level of the axillary artery. Dry gangrene of the right upper arm was diagnosed. On the *19th day of life* the right upper limb till the level of the upper third of the upper arm is cold and dry by touch, black in color, insensitive; movements are possible due to mobile shoulder joint, bending of the hand and elbow joint is possible, but unbending is not, unclear demarcation line is determined (Fig. 1). Due to impossibility to restore blood supply in the limb, amputation was performed on the level of the upper third of the right upper arm.

It should be noted that the child presented the signs of marked hypocoagulation with underluing neonatal sepsis, which prevented from the possibility to initiate anticoagulation and/or fibrinilytic therapy with the aim to achieve conservative thrombolysis.

Nineteenth day of life. Progress of surgery. The surgery was performed in the resuscitation room under conditions of the infant incubator and appropriate thermal conditions. The baby was in the supine position, the upper part of the trunk was on the roller, and the upper arm was moved to the border of the infant incubator. First, the anterior-external flap was cut by means of semi-oval incision in order to retain m. deltoideus, playing an important role for the movements of the stump.

1. The cut was made along sulcus deltoideopectoralis from the clavicle to the inferior border of the greater pectoral muscle (*musculus pectoralis major*). The latter was cut transversally not far from the point of attachment to the humeral bone. розсікали поперек, недалеко від місця прикріплення до плечової кістки. The sheath of m. Coracobrachialis was opened, the muscle was drawn laterally; after the posterior wall of the sheath was opened *a*. and *v. axillaris* were secured.
2. The skin surrounding *m. deltoideus* anteriorly, inferiorly and posteriorly was cut. The muscle was cut out from the point of attachment to the humeral bone and drawn upward together with the skin. Special attention was paid to retaining *n. axillaris*, innervating the muscle.
3. The tendons of *m. latissimi dorsi* and *m. teretis majoris* were cut. After that a transverse cut was made on the level of the upper margin of the posterior incision of the skin surrounding the upper arm from the inside.
4. The soft tissues were cut till the bone. The soft tissues were drawn maximum upwards. The periosteum was cut moving it downward, and the humeral bone was sawn (Fig.2, Fig. 3).
5. Hemostasis was performed by means of electric coagulator. The nerve trunks were isolated and cut. Sutures were applied on the muscles and skin.

Considering high growth intensity of the humeral bone at the expense of the proximal germ cartilage, the cut-off part of the humeral bone was covered with a considerable amount of the soft tissues. Due to considerable contractile ability of the skin in children, the cut along the anterior surface was made 3 cm distally than that along the posterior surface.

The following principles were kept to while performing amputation: a) supply the stump with sufficient amount of the soft tissues; b) Fig. retention of every centimeter of the limb due to the lag of growth of the diaphysis stump as compared with the appropriate segment of a healthy limb (Fig. 4).

As a number of authors admit, arterial thromboembolism with a venous catheter is a rare complication [2-5]. Considering literature data, experience of the home and foreign specialists, as well as peculiarities of the given clinical case, we can suggest several causes promoting development of the complication.

One of the probable causes of arterial thrombosis of the newborn boy with an extremely low body weight were multiple punctures of the peripheral cubital vein and iatrogenic damage of the arterial branch located close, followed by thrombosis of the brachial artery and development of dry gangrene of the limb. Berzel S. et al. (2014) consider that similar situation can occur even without violation of the technique to insert a peripheral venous catheter, as it can be caused by traumatization of the adjacent artery due to the movements of the elbow joint without its artificial stabilization [2]. Complications can occur in case of direct puncture of the brachial or axillary arteries, since causative factors are condition of the vessels of a patient, anatomical peculiarities of the arteries, the point of puncture, success of the first attempt of puncture, and hermetic/sterile bandage [6, 7].

Bacciedoni V. et al. (2016) suggest that factors increasing development of neonatal thrombosis are premature birth and perinatal asphyxia. Premature babies are prone to clot formation due to peculiarities of coagulation system which is characterized by functional immaturity. Premature babies have low activity of antithrombin, and its normal degree of activity is achieved only at the age of 6 months. The children from this group have reduced activity of the fibrinolytic system resulting from decreased plasminogen activity and increased concentration plasminogen activator inhibitor [3]. This suggestion is confirmed by a group of researchers headed by Makatsariya A. (2020), who admit that babies with the gestation age of 22-27 weeks are the biggest risk group concerning development of neonatal thromboembolism [4].

It should be noted that in this particular clinical case, in addition to premature delivery, other provocative factors of neonatal thrombosis cannot be excluded including genetic disorders (congenital developmental defect of the intestine – high rectal atresia with an abscess into the urethra or urinary bladder; congenital heart defect – bicuspid aortal valve, open oval foramen, and prenatal factors which can be indicative of disorders in coagulation processes of the mother (varicose veins of the lower limbs, premature abruption of the normal placenta). The factors presented can be associated with pro-thrombotic gene polymorphism (F5Leiden, F2G220210A, PAI-1 etc). Belousova T.V. et al. (2018) recommend to examine all the children with thrombosis episodes irrespective of their origin concerning carriage of the major markers of thrombophilia and considering issues of the secondary prevention [8].

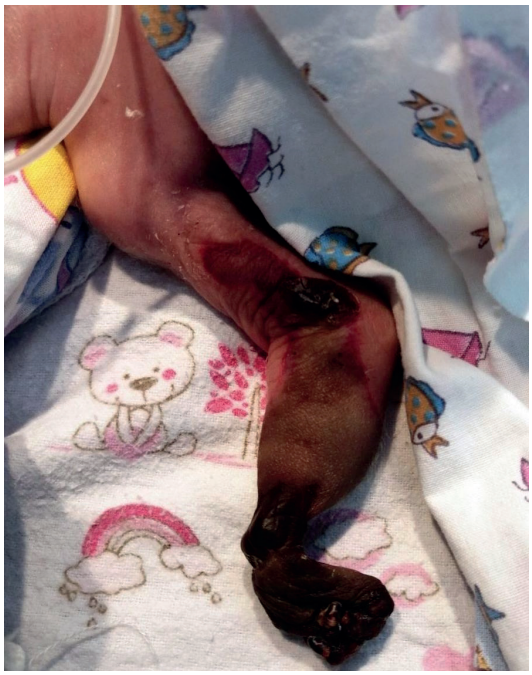

Fig. 1. The dry gangrene of the right upper arm (19th day of life).

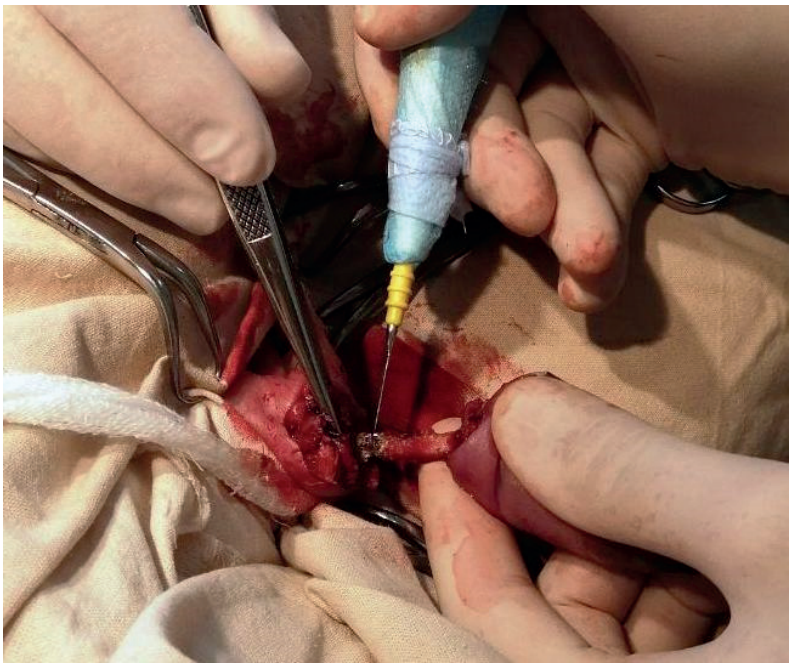

Fig. 2. The dry gangrene of the right upper arm; the stage of amputation; the humeral bone is sawing (19th day of life).

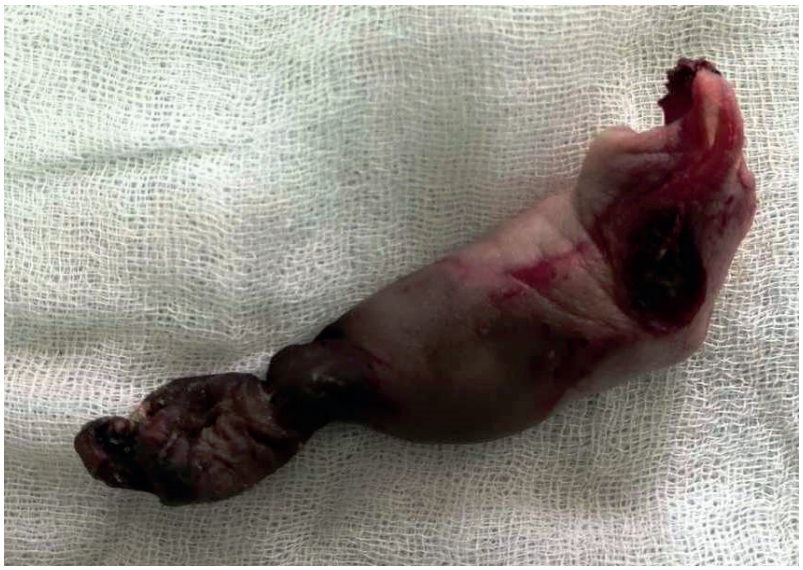

Fig. 3. The dry gangrene of the right upper arm; the right upper limb was amputated (19th day of life).

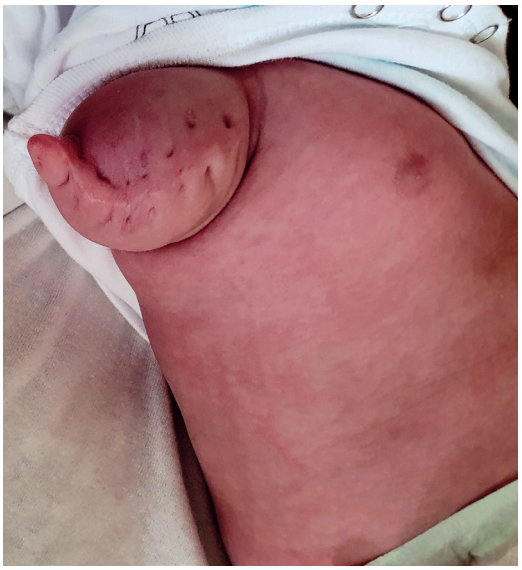

Fig. 4. The stump of the right upper limb; the stage after surgery (3rd month of life).

To our opinion, one of the possible factors promoting occurrence of thrombosis in this particular case is neonatal sepsis which might be complicated by clot formation in the deep veins of the lower limbs. We can suggest that a clot was isolated in a thrombophlebitic focus not clearly found, and it penetrated into the systemic circulation through the open oval foramen during the right-left bypass, and as a result, into the brachial artery (paradoxical embolism). Thrombus/embolus during its contact with the arterial wall caused a long spasm of the peripheral portion of the blocked artery, pathological changes of the endothelium of the vascular intima and secondary thrombosis spread along the brachial artery involving the collateral portions. Almuhyl R. A. H. (2020) admitted that it is an infectious factor that causes 7,3 % cases of deep venous thrombosis in children [9]. Makatsariya A. et al. (2020) consider septic processes and premature birth as two major trigger factors promoting the risk of neonatal thromboembolism [4].

The necessity to carry out Doppler technique with the use of high rarefaction sensors to control the blood flow along the suspected pathological vessel should be indicated [2, 5]. Berzel S. et al. (2014) presented a case of acute thrombosis of the brachial artery of a newborn caused by a peripheral venous catheter. The authors emphasize that it was timely

Doppler examination of the damaged limb that enabled to diagnose the level of occlusion of the brachial artery and find the signs of early collaterization. The latter fact was a reason to postpone surgery and apply conservative therapy (non-fractional heparin and low molecular weight heparin) [2]. Unfortunately, in our clinical case we were not able to carry out Doppler examination of the patient due to his extremely low body weight and the lack of sensors with appropriate frequency detection.

CONCLUSIONS

Acute arterial thrombosis caused by a peripheral venous catheter is very rare complication, but it can result in severe consequences. The provocative factors during the neonatal period are premature birth, sepsis, violation of the technique to insert catheters, genetic disorders of the hemostasis system etc. We consider that to reduce the frequency of complications after catheterization of the central and peripheral vessels of neonates is possible by means of continuous education of the medical staff concerning the technique of catheter insertion and care of them, absolute implementation of safety policy concerning nosocomial infection, adequate provision of ultrasound examination devices with high rarefaction sensors, involvement of a multi-disciplinary team of specialists (pediatric surgeons, anaesthesiologists, neonatologists, hematologists, sonologists) to manage complicated clinical cases.

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CASE STUDY

ULTRASONIC DECALCIFICATION OF AORTIC VALVE IN MODERN ERA: A CASE REPORT

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ABSTRACT

Aortic stenosis (AS) – a constantly progressing disease characterized by thickening and calcification of leaflets of the valve, which leads to obstruction of the blood outflow from the left ventricular (LV), inadequate cardiac output, heart failure, and even sudden death. Prevalence of stenosis consistently increases with age, 0.2% incidence observes in the 50–59 year cohort of patients and almost 10% in patients from the 80–89 year cohort. We report the case of a 78-year-old man who presented with severe aortic valve stenosis. Since conventional valve replacement was problematic because of a small annular diameter we performed ultrasonic aortic valve decalcification. As a result, the systolic pressure gradient across the aortic valve decreased from 106 mm Hg to 22 mm Hg. Our case demonstrated that even in modern era ultrasonic aortic valve decalcification can be an effective option for restoration of mobility of the aortic valve cusps. We advocate the use of it for elderly patients with small aortic annulus in which surgical or transcatheter valve replacement, valve reconstruction could be extremely difficult or unavailable and consider ultrasonic valve decalcification as an additional tool in cardiac surgeon's hands.

KEY WORDS: ultrasonic decalcification, aortic valve stenosis, valve-sparing operation

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INTRODUCTION

Aortic stenosis (AS) – a constantly progressing disease characterized by thickening and calcification of leaflets of the valve, which leads to obstruction of the blood outflow from the left ventricular (LV), inadequate cardiac output, heart failure, and even sudden death. Prevalence of stenosis consistently increases with age, 0.2% incidence observes in the 50–59 year cohort of patients and almost 10% in patients from the 80–89 year cohort [1,2]. Half of the AS patients die within 2 years after detection of the symptoms of the disease [3]. The gold standard for the treatment of AS traditionally is surgical. Since the 60s of the last century, mechanical decalcification is well known method that allows repair the aortic valve [4]. Due to high risk of cusp perforation and high rate of valve restenosis this method has not been practiced for some time. In 1972, with the development of ultrasound medical equipment the decalcification approach has found its rebirth [5]. Almost 45 years after, we are introducing our own experience of ultrasonic valve decalcification for patient having AS.

CASE REPORT

A 78-years-old man was admitted because of dyspnea, palpitations, periodical chest pain. Echocardiography studies revealed a severe AS with the peak systolic pressure gradient 106 mm Hg (mean 64 mm Hg), fibrosis and severe calcification of the valve cusps 3+ (Fig. 1) with effective aortic valve area 1.0 cm^2.

According to coronary angiography, there were no signs of the coronary arteries lesions. The patient was taken to the operating room where median sternotomy was performed and cardiopulmonary bypass with moderate hypothermia (32°C) and standard heparinization (300 IU/kg IV) was established. After aortic cross clamped, ascending aorta was opened and cardioplegia solution (Custodiol; Koehler Chemi, Alsbach Haenlien, Germany) to volume of one liter was pumped directly into the ostia of the coronary arteries. Then, aortic valve was carefully examined for localization, prevalence and the depth of tissue calcification. It turned out that the inclusion of calcium was located solely on the aortic side the cusps, seized mostly free edges and did not penetrate into the entire thickness of the cusp tissue. Decision was made to perform valve-sparing operation – aortic valve decalcification using an ultrasonic dissector Sonoca 300/MBC 601 UAM (Soring GmbH, Germany). We used mode of maximum power (35 kHz) with supply of sterile saline with a speed 10 ml/min and simultaneous vacuum evacuation of calcium detritus from the wound. We started decalcification procedure from the most affected cusp. Calcium deposits were gently and carefully removed within the border of healthy tissue, preventing rupture or perforation of the cusps (Fig. 2).

To avoid the entering of waste into the ostia of the coronary arteries external suction were additionally used. At the end of the decalcification procedure a test for aortic valve mobility and competence was carried out. Postoperative course was uneventful with no complications. Echocardi-

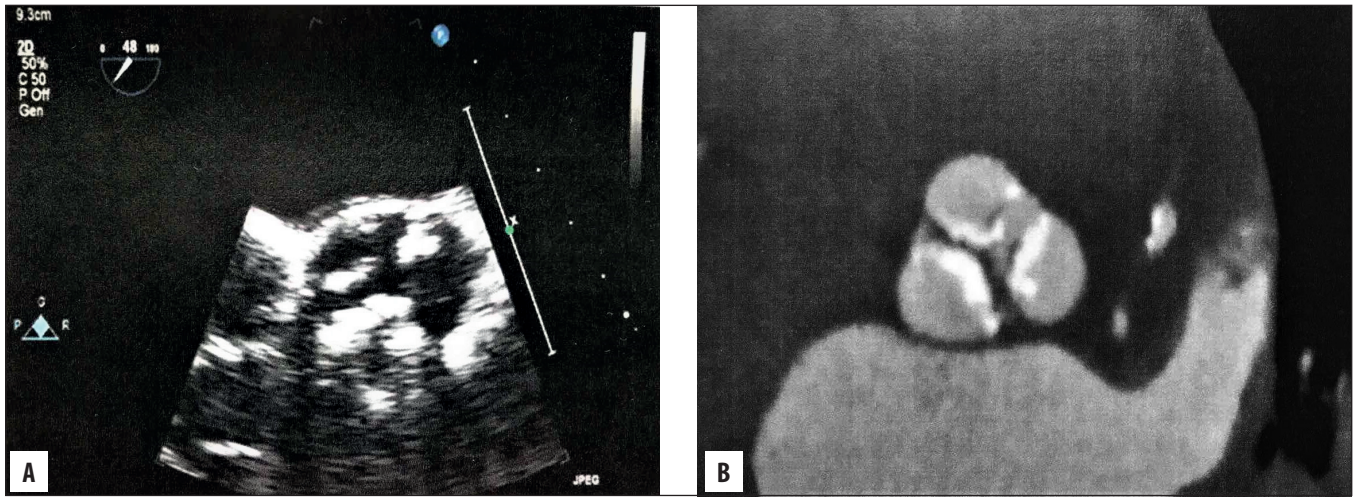

Fig. 1. A). 2D transthoracic echocardiography, shot axis view shows severe calcified aortic valve B). CT scan demonstrates severe calcinosis in aortic valve cusps

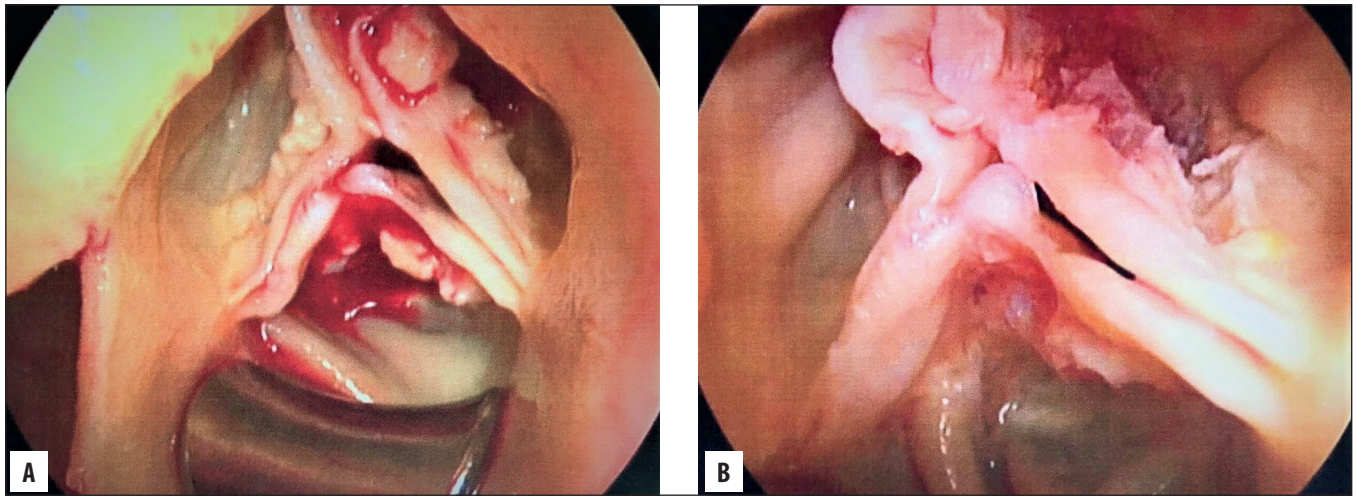

Fig. 2. A). Intraoperative view of stenotic calcified aortic valve and B). aortic valve after decalcification

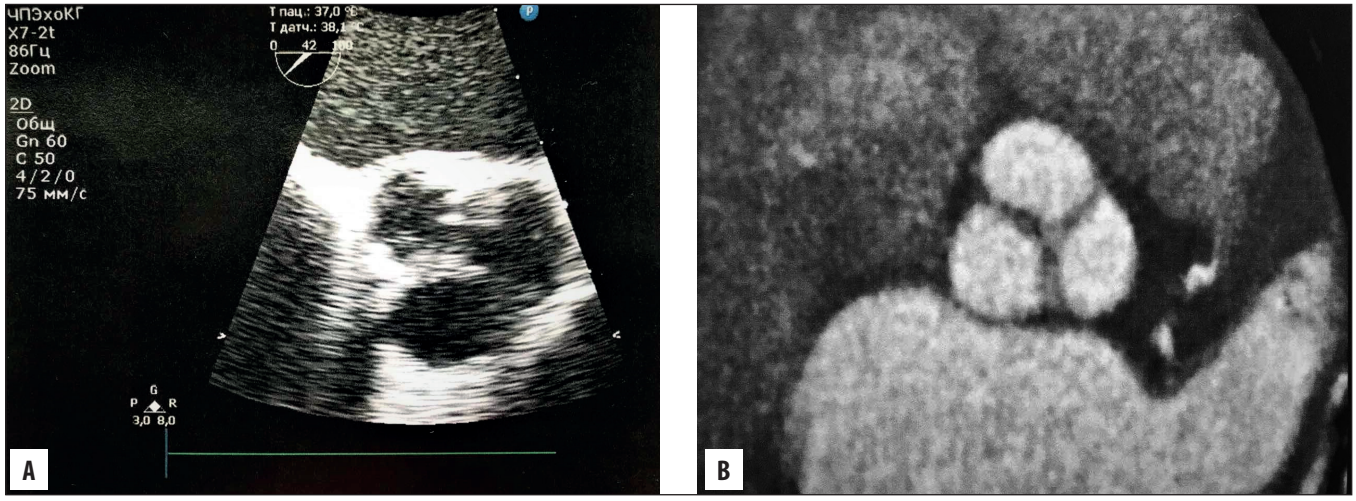

Fig. 3. A). 2D transthoracic echocardiography, shot axis view and B). CT scan demonstrates near to normal, decalcified aortic valve cusps

ography before discharge showed the peak systolic pressure gradient - 22 mm Hg (mean 10 mm Hg), regurgitation - 1/2 +, calcification - 1/2 + (Fig. 3).

On the 7th postoperative day the patient in a stable condition was transferred to the cardiology department for rehabilitation.

The objective of decalcification procedure was to restore mobility of the valve cusps and to slow the progression of stenosis. However, the outcome of mechanical valve-sparing operation was not long lasting, as a high incidence of restenosis and regurgitation was often to go along with the results of the procedure [6,7]. Over time, the surgical equipment, innovations were constantly improving and created the second wave of decalcification in cardiac surgery. It happened due to emergence of a new ultrasound instruments, the principle of which was based on the transformation of electricity into ultrasonic vibrations. Exposure of biological tissue into ultrasound at a frequency of 25-35 kHz leads to appear of cavitation effect. Cavitation is characterized by the formation of micro bubbles in the liquid filled with gas. The rupture of these bubbles into intracellular fluid create a tiny crack destructions of the tissue. Thus, by means of ultrasound the calcium deposits are broken and becomes finely dispersed and expelled from the tissue outwards.

CONCLUSIONS

Our case demonstrated that even in modern era ultrasonic aortic valve decalcification can be an effective option for restoration of mobility of the aortic valve cusps. We advocate the use of it for elderly patients with small aortic annulus in which surgical or transcatheter valve replacement, valve reconstruction could be extremely difficult or unavailable and consider ultrasonic valve decalcification as an additional tool in cardiac surgeon's hands.

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CASE STUDY

BASAL CELL CARCINOMA AND SQUAMOUS CELL CARCINOMA IN A PATIENT TREATED WITH FINGOLIMOD FOR MULTIPLE SCLEROSIS – A CASE REPORT AND LITERATURE REVIEW

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ABSTRACT

The aim: Multiple sclerosis (MS) is a disease of the central nervous system (CNS) characterized by inflammation and demyelination, which leads to chronic progressive disability. Fingolimod is the first registered oral disease-modifying drug (DMD) approved for the treatment of highly active relapsing-remitting multiple sclerosis (RRMS). Fingolimod statistically significantly reduced the number of relapses, clinical and radiological disease activity and disability progression. However, fingolimod can be associated with an increased risk of cancer. This study is aimed to underline how important is regular specialist follow-up during fingolimod therapy.

Materials and methods: The literature review was conducted using the key words: "fingolimod", "multiple sclerosis", "fingolimod and cancer", "relapsing-remitting multiple sclerosis", "fingolimod adverse effects", "basal cell carcinoma fingolimod", "squamous cell carcinoma fingolimod". The study is based on the case report of a 67-year-old male patient with metachronous skin cancer treated with fingolimod. The drug had an influence on the inhibition of clinical and radiological activity of the disease. Despite the control of the underlying disease, skin cancers occurred during treatment. Basal cell carcinoma and squamous cell carcinoma were diagnosed at an early stage when complete resection was possible and negative (R0) margin resection was achieved.

Conclusions: Dermatological examination should be performed at the beginning and during treatment with fingolimod. Patients need to be informed about the risk of malignancy. Patient education are crucial during treatment, which allows achieving a good therapeutic effect, thus minimizing the risk of malignancy and enabling its early detection and cure.

KEY WORDS: multiple sclerosis, basal cell carcinoma, squamous cell carcinoma, fingolimod, metachronous skin cancer, relapsing- remitting multiple sclerosis

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INTRODUCTION

Multiple sclerosis (MS) is a multifactorial chronic disease of the central nervous system (CNS) characterized by inflammation and demyelination, which leads to chronic progressive disability, thus impairing the quality of life.

The disease predominantly affects young adults between 20 and 40 years of age. It is more prevalent in the Caucasian population. Women are more frequently affected [1-4].

The current treatment of MS consists primarily of disease-modifying drugs (DMDs). The therapy includes first-line agents (β-interferons, pegylated interferon beta 1a, glatiramer acetate, dimethyl fumarate) and second-line agents (fingolimod, natalizumab, ocrelizumab, cladribine). The latter group of drugs is indicated in rapidly progressive severe disease or when the first-line treatment is not effective [5].

Fingolimod was approved by the Food and Drug Administration (FDA) for the treatment of MS in 2010 [5-7]. It is an oral immunosuppressive drug which binds to the sphingosine-1-phosphate receptor located on immune cells [7, 8]. By modulating the function of S1P receptors on lymphocytes, it inhibits their migration from lymph nodes to the peripheral blood and the central nervous system [9, 10]. The drug reduces their autoimmune activity in the CNS, which is the pathogenesis of MS lesions.

As with other drugs, the use of fingolimod is also related to the risk of adverse effects. The most commonly reported side effects include headache, flu-like symptoms, diarrhea, nausea, cough, back pain, sinusitis, and rhinitis. In addition, elevated liver enzymes (ALT, GGT, AST) are also reported [6, 7, 10, 11].

Furthermore, the use of fingolimod can be associated with an increased risk of cancer [8]. FDA and the European Medicine Agency (EMA) list lymphomas as a potential adverse effect of fingolimod therapy. Additionally, EMA pays attention to the possibility of developing skin cancer during therapy [9].

Studies reported that patients treated with fingolimod developed basal cell carcinoma, squamous cell carcinoma, cutaneous melanoma, Kaposi's sarcoma, squamous cell carcinoma of the palatine tonsil related to human papillomavirus infection, breast cancer, primary cutaneous CD30+ anaplastic large-cell lymphoma (PCALCL), or Merkel cell skin cancer [4, 6, 7, 11-19].

THE AIM

The purpose of this study is to highlight the need for regular specialist follow-up during fingolimod therapy and to indicate the relationship between treatment with this drug and skin cancer occurrence based on our case report and on the literature review.

MATERIALS AND METHODS

Polish and English-language publications were found according to the given keywords: "fingolimod", "multiple sclerosis", "fingolimod and cancer", "relapsing-remitting multiple sclerosis", "fingolimod adverse effects", "basal cell carcinoma fingolimod", "squamous cell carcinoma fingolimod" in the databases of the PubMed and Google Scholar.

CASE REPORT

A 67-year-old male patient was admitted to the Department of Otorhinolaryngology and Oncological Laryngology in Zabrze in early March 2020 due to a skin tumor of the right nasal vestibule. The patient reported impaired nasal patency on the right side, which worsened over a short period of time. Anterior rhinoscopy revealed a nodular lesion obstructing the right nasal passage.

In July 2019, the patient had undergone resection of a skin tumor of the left nasal wing. Histological evaluation showed basal cell carcinoma. Total resection of the lesion was performed and the patient underwent a periodic follow-up.

The patient's medical history included surgical removal of a skin tumor of the left thigh in 2019 and resection of a neck skin tumor in 2018. Postoperative histological assessment showed keratoacanthoma and basal cell carcinoma, which was resected completely.

The patient started to be under the care of the department of neurology due to relapsing-remitting MS (RRMS) in May 2009. In 2009, the patient developed limb weakness on the right side and right-sided hypoesthesia. Similar symptoms also occurred in 2000. Outpatient brain magnetic resonance imaging (MRI) showed no pathological lesions. However, MRI of the cervical spinal cord showed a single demyelinating lesion at the C6-C7 level. The symptoms resolved spontaneously. Except for urinary incontinence, the patient had no other complaints. In 2009, contrast-enhanced MRI of the brain showed diffuse supratentorial and infratentorial demyelinating lesions with one active lesion in the cervical region without radiological progression. Visual evoked potentials showed bilateral visual pathway damage. After glucocorticoid treatment, the neurological condition of the patient improved. In February 2010, the patient had another relapse in the form of right limb paresis. As a result, interferon-beta-1 b was administered. The patient was given subcutaneous injections of recombinant interferon-beta-1b for 3 years. During the third year of treatment, other relapses of moderate intensity occurred (June and July 2013). The neurological status was assessed according to the Expanded Disability Status Scale (EDSS =4.5). In September 2013, a follow-up MRI of the brain showed progression of demyelinating lesions with the presence of 3 new lesions on T2-weighted sequences. The patient met the inclusion criteria for treatment with fingolimod (two moderate relapses during the first-line therapy and lesions on MRI).

In September 2013, the patient was qualified for treatment with fingolimod after undergoing basic and additional tests (including tests for the presence of antibodies against hepatitis B virus) and cardiac, ophthalmological and dermatological assessment. The treatment was initiated without complications. During therapy, no relapses were observed and the patient's neurological condition improved (EDSS = 2.0). Follow-up brain MRI examinations, which were performed in September 2014 and 2015, showed no radiological progression. In September 2016, a reduction of two demyelinating lesions was found on MRI with no enhancement following contrast administration. In subsequent years, follow-up MRI examinations showed no progression of demyelinating lesions. The patient was on oral fingolimod 0.5 mg for 7 years.

The subject was also diagnosed with peptic ulcer disease and had a gastric perforation which was surgically treated in 2017. As a result, the patient was on pantoprazole (20 mg/day). Other medications taken by the patient included zopiclone (3.75 mg/day), and bisoprolol (2.5 mg/day).

The patient was retired and did not work. He had previously been employed in a gas plant but had very little exposure to ultraviolet radiation.

The patient had a 50-year history of smoking (10-20 cigarettes/day) and his family history was positive for cancer (brothers diagnosed with hepatic cancer and multiple myeloma, while father died of gastric cancer).

In March 2020, the patient was admitted to the Department of Otorhinolaryngology for surgery. An excisional biopsy of the lesion was performed. Histological examination showed keratinizing squamous cell carcinoma (G1) without angioinvasion or neuroinvasion. Cancer infiltration was found in the surgical margins. Therefore, the patient was referred for radical surgery. On April 16, 2020, a lateral rhinotomy was performed under general anesthesia. After injecting the incision site with marcaine and epinephrine, an incision was made around the right nasal wing, the flap was inverted, the skin scar of the nasal vestibule from the previous surgery was located and removed with a wide margin of surrounding tissue. Surgical margins were collected and the specimen was sent for histological examination. Subcutaneous and skin sutures were placed. A seton with the ointment was placed to the right nasal passage. Histological findings showed keratinizing squamous cell carcinoma (G1) (maximum size of 0.6 cm; infiltration depth of 0.2 cm). No vascular or nerve invasion was found. The lesion was completely resected with surgical margins (the transverse margins were 0.3 cm, longitudinal margins were 0.4 cm, and the deep margin was 0.1 cm). The surgical margins which were additionally referred for assessment were free of tumor tissue. R0 resection was achieved in the patient. Due to histological findings, the administration of fingolimod was discontinued.

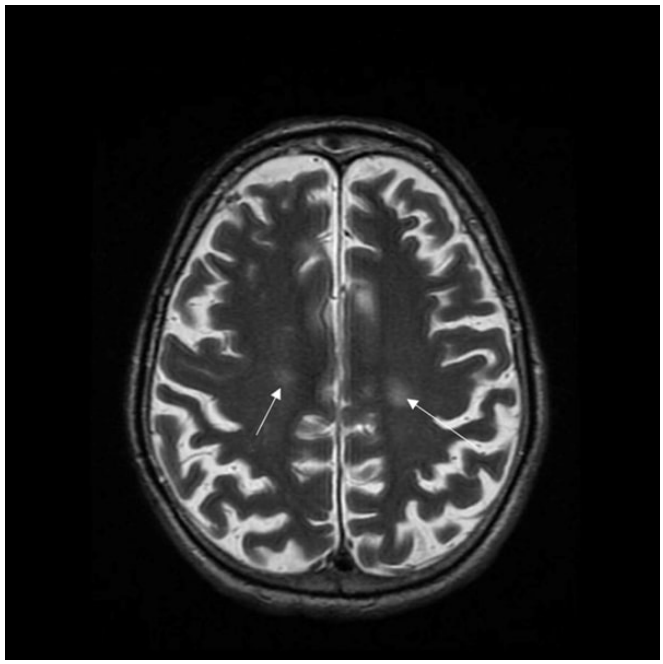

Fig. 1. Magnetic resonance imaging (MRI) after fingolimod treatment shows a reduction of demyelinating lesions. Axial MRI, T2- weighted image showing periventricular lesions and subcortical foci in the centrum semiovale (arrows).

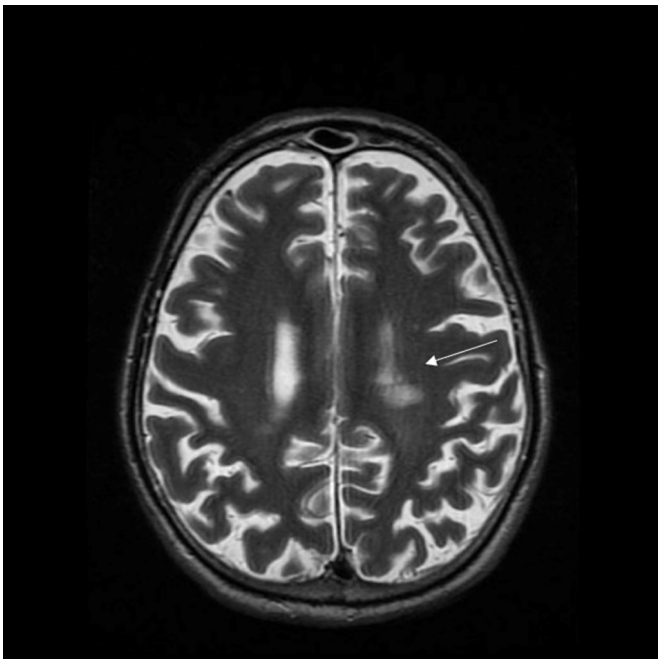

Fig. 2. Magnetic resonance imaging (MRI) after fingolimod treatment shows a reduction of demyelinating lesions. Axial MRI, T2- weighted image showing periventricular lesions and subcortical foci in the centrum semiovale (arrows).

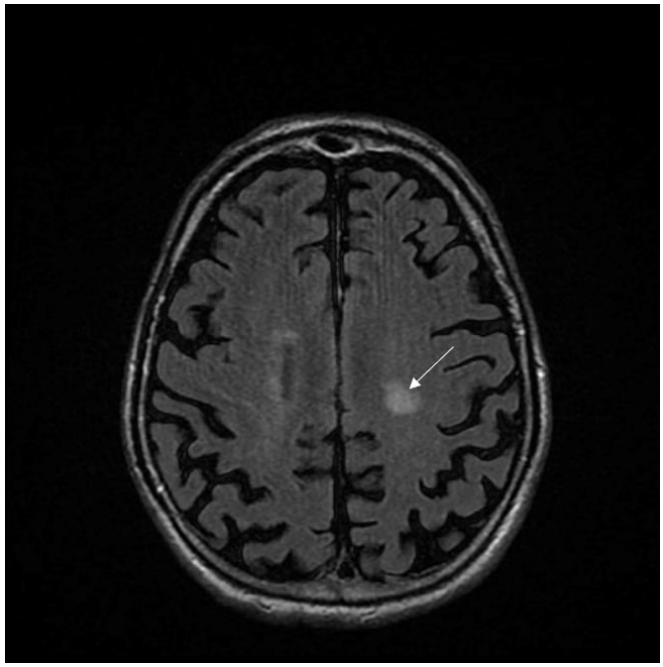

Fig. 3. Magnetic resonance imaging (MRI) after fingolimod treatment shows a reduction of demyelinating lesions. Axial MRI T2/FLAIR images show periventricular demyelinating plaques (arrows).

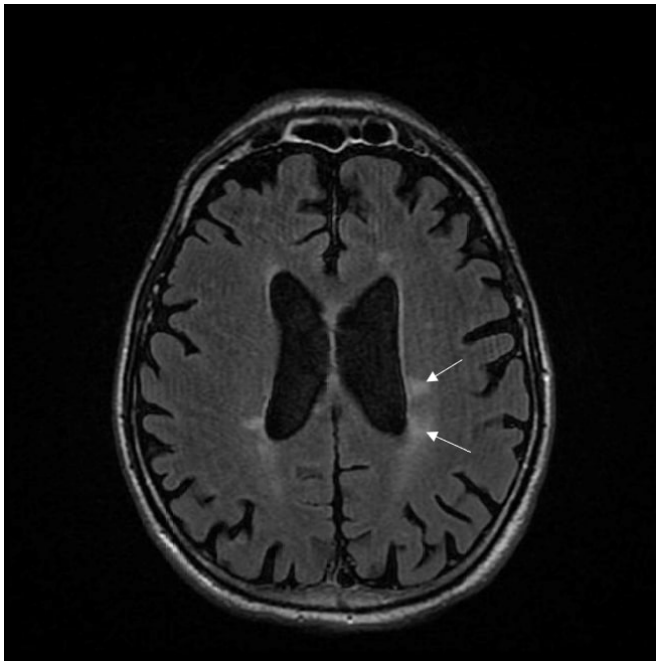

Fig. 4. Magnetic resonance imaging (MRI) after fingolimod treatment shows a reduction of demyelinating lesions. Axial MRI T2/FLAIR images show periventricular demyelinating plaques (arrows).

REVIEW AND DISCUSSION

Baharnoori et al. showed that most epidemiological studies reported a lower incidence of malignancies in patients with MS compared to the general population. However, they stressed that the data had been collected before the introduction of new immunomodulatory drugs into therapy [5].

Bahmanyar et al. emphasized that cancer risk in MS patients was lower compared to the general population, the exception being brain and urinary tract tumors, which are more prevalent in MS patients. According to Bahmanyar et al., a lower prevalence of tumors in MS patients might be due to an increase in systemic autoimmune responses

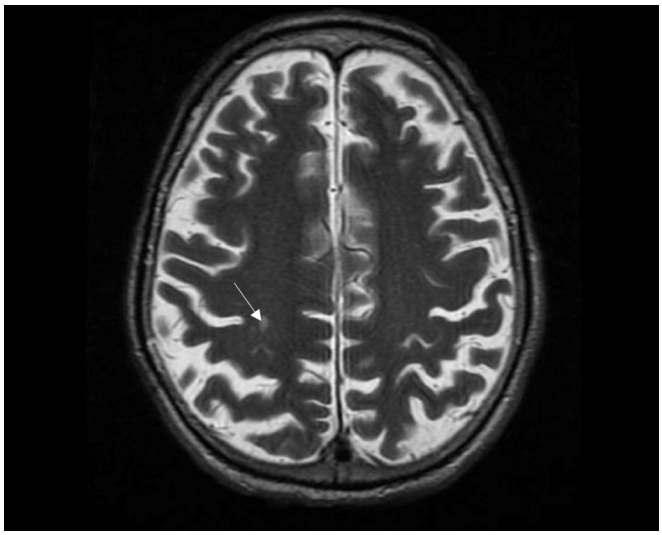

Fig. 5. Magnetic resonance imaging (MRI) after fingolimod treatment shows a reduction of demyelinating lesions. Axial MRI, T2- weighted image showing periventricular lesions and subcortical foci in the centrum semiovale (arrows).

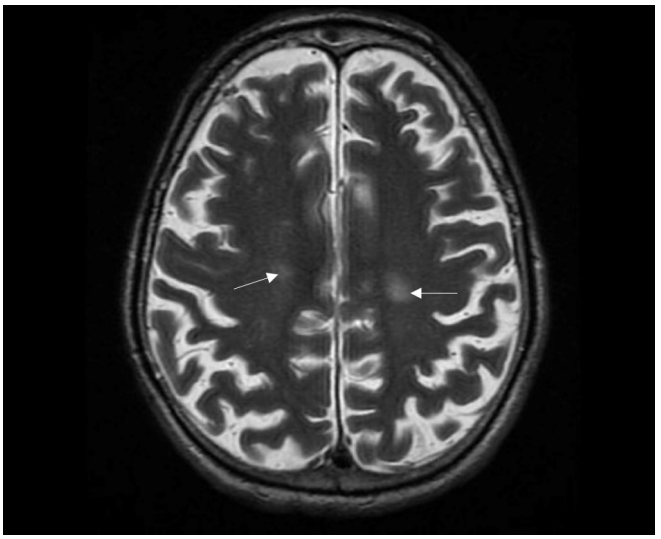

Fig. 6. Magnetic resonance imaging (MRI) after fingolimod treatment shows a reduction of demyelinating lesions. Axial MRI, T2- weighted image showing periventricular lesions and subcortical foci in the centrum semiovale (arrows).

during the course of the disease, which could be a protective mechanism against the formation of some tumors. Additionally, lifestyle changes in patients after diagnosis of MS are listed as cancer protective factors [20].

However, Lebrun and Rocher found that the number of cancer cases in MS patients could be underestimated. According to them, physicians do not systematically report the incidence of cancers in MS patients. It is likely that the incidence of cancer is underestimated as regards previously used DMDs [4].

Fingolimod is the first registered oral DMD approved for the treatment of highly active RRMS. The advantage of fingolimod is related to the way of administration. The recommended dose is one capsule (0.5 mg/day) [6]. The efficacy of fingolimod has been confirmed in multicenter FREEDOMS and TRANSFORMS trials, which demonstrated that the drug statistically significantly reduced the number of relapses and significantly reduced clinical and radiological disease activity [3, 8, 21, 22]. These trials showed its superiority over interferon beta-1a, which is the first-line drug [21]. In addition, the FREEDOMS trial also highlighted that fingolimod reduced disability progression [6, 23].

The drug interferes with the immune system, thus resulting in a reduction in the peripheral blood lymphocyte count to 20%-30% of the baseline value. The low lymphocyte count is maintained during long-term use of the drug [8]. Chronic intake of fingolimod also leads to a reduction in the number of neutrophil granulocytes to about 80% of the baseline value [10]. The influence on the number of circulating lymphocytes is due to their sequestration in lymph nodes and not due to lymphotoxicity, and hence the effect of the drug is reversible [6].

It is known that immunosuppression promotes the development of cancer [6, 12, 24]. Cohen et al. in the LONGTERM study showed that the effect of fingolimod on the immune system may be associated with an increased risk of malignancy, as with other immunomodulatory drugs. The analysis showed that solid organ cancers or hematologic malignancies were rare. However, skin cancers, including basal cell carcinoma and squamous cell carcinoma, were more prevalent.

The INFORMS phase III trial, conducted between 2008 and 2011 in which patients (n= 336) were on fingolimod (0.5 mg/day) and placebo (n=487), showed that basal cell carcinoma and squamous cell carcinoma were more prevalent in the group of patients on fingolimod. [25]

In turn, the FREEDOMS double-blind randomized trial, which lasted 24 months and involved 425 patients on fingolimod 0.5 mg/day, 429 patients on fingolimod 1.25 mg, and 418 patients on placebo, did not show significant differences in the incidence of malignant melanoma in these groups. The FREEDOMS II trial showed that the incidence of skin cancers was similar between the groups on fingolimod and placebo, with the exception of basal cell carcinoma whose incidence was higher in patients on fingolimod [21].

The TRANSFORMS study compared the use of fingolimod with interferon beta in RRMS for 12 months. In this study, Cohen et al. found a higher risk of malignancy in patients on fingolimod 1.25 mg (2 cases of basal cell carcinoma and 2 cases of breast cancer) and fingolimod 0.5 mg (3 cases of basal cell carcinoma, 3 cases of malignant melanoma and 2 cases of breast cancer) compared to interferon beta (2 cases of basal cell carcinoma). [22]

There are also other case reports of cancer in patients treated with fingolimod, including lung, brain, hematopoietic, and lymphatic cancers. However, many cases are related to various types of skin cancer [14, 24].

Manouchehri et al. described a female patient with MS

who had been treated with fingolimod for 2 years and developed cutaneous anaplastic lymphoma during treatment. After discontinuation of the drug, the symptoms of lymphoma resolved, which could indicate a direct link between the development of this malignancy and the drug intake.

A similar case was reported by Papathemeli et al. Cutaneous anaplastic lymphoma was diagnosed in a patient treated with fingolimod and resolved shortly after discontinuation of treatment. The authors highlighted that primary cutaneous lymphomas should be considered a potential adverse effect in patients on fingolimod and should be taken into account during patient examination [11, 18].

Baharnoori et al. described a female patient on fingolimod who developed lymphoplasmacytic lymphoma located in the left frontal lobe. After discontinuation of the drug, the size of the tumor decreased, despite the absence of additional treatment, which may suggest that fingolimod may contribute to lymphoproliferative diseases [5].

In the summary of product characteristics of fingolimod, basal cell carcinoma is listed as a common adverse effect. Malignant melanoma is uncommon (1:100), squamous cell carcinoma is rare (1:1000), whereas Kaposi's sarcoma is very rare (1:10,000).

In the general population, basal cell carcinoma accounts for approximately 80% of all non-melanoma skin cancers. It is also the most prevalent skin cancer among Caucasians [26]. The incidence of this cancer is related to the latitude. The highest incidence is found among men over 60 years of age. It is characterized by slow growth and a low propensity to metastasize. [27]. High exposure to ultraviolet radiation is reported as a major risk factor for the disease. Additionally, immunosuppression is also a predisposing factor [28]. Surgical removal of the lesion with margins of healthy tissue is the treatment of choice, which provides the highest cure rate [27].

Squamous cell carcinoma is the second most prevalent skin cancer and accounts for approximately 20% of all cases, with the head and neck region being the most common location. It is characterized by faster tumor growth and a greater propensity to metastasize to lymph nodes compared to basal cell carcinoma [28]. It often arises from precancerous lesions. However, it can also arise from normal skin, and the lesions are often localized at the border of skin and mucosa [29]. The major risk factors for its occurrence include significant exposure to ultraviolet radiation, exposure to chemicals (such as arsenic, coal tar, soot, nitrogen mustards, aromatic polycyclic compounds - biphenyl derivatives, psoralen), HPV infections, smoking, and genetic factors. The prevalence of this cancer increases with age.

Immunosuppression significantly increases the risk of its occurrence. The course of the disease is more aggressive. Lesions are often multifocal and involvement of lymph nodes is more frequent. Surgical resection is the mainstay of treatment for squamous cell carcinoma. Adjuvant radiotherapy may be indicated in patients with the aggressive course of the disease [28]. It is applied to treat clinically advanced lesions, including cases with lymph node and/or CNS involvement.

A surgical margin of no less than 6 mm is recommended in the cases of SCC. When it is difficult to obtain such a margin due to the location of the lesion and the subsequent cosmetic effect, achieving a margin negative resection (R0) is considered satisfactory. Systemic therapy is warranted when the disease is advanced, recurrent and multifocal. However, there are no conclusive data which confirm the efficacy of chemotherapy in the treatment of squamous cell carcinoma [28, 29].

Considering the above case and the presented data, it seems reasonable to recommend careful monitoring of patients treated with fingolimod. Dermatological examination should be performed at the beginning and during treatment [4, 7, 15, 16].

Prior to disease-modifying therapy (DMT), next to dermatological assessment, gynecological consultation should be performed. Additionally, X-ray of the lungs should be performed in smoking patients. Fecal occult blood testing should be done in patients over 50 years of age [4].

Patients need to be informed about the risk of malignancy. They should also be provided with the detailed information on alarming symptoms and on skin lesions that require medical attention, which can allow early detection of lesions at an earlier stage, thus increasing the chances of cure. Screening and specialist consultations are also crucial [3, 4, 7, 17]. Most DMDs are contraindicated in patients with a history of malignancy. However, this does not apply to patients with a history of basal cell carcinoma [4].

In our patient, fingolimod resulted in very good control of the underlying disease and even led to the resolution of lesions on MRI. Considering the prevalence of basal cell carcinoma in the general population, especially in individuals over 60 years of age and usually very good surgical results and good prognosis, the benefits of treatment with fingolimod seemed to outweigh the risks in our patient.

Importantly, the patient had also other risk factors for malignancy (i.e., long-term smoking, a positive family history of cancer, age). Of note, the patient developed two different nasal skin malignancies within a short period of time. Additionally, the patient underwent surgical excision of basal cell carcinoma of the neck in 2018 and keratoacanthoma of the left thigh in 2019. Interestingly, the incidence of cancer increased significantly after a 5-year treatment with fingolimod, which may be related to the cumulative drug dose. This observation seems to support the observation of Lebrun and Rocher who found that the possibility of cancer during MS treatment was more connected with duration and the cumulative dose of the DMD than with a specific immunosuppressive drug [4]. However, in the case of fingolimod, the LONGTERMS trial showed that such a relationship was not found [8].

Due to its more aggressive course and a greater propensity to metastasize, the diagnosis of squamous cell carcinoma, particularly in immunocompromised patients, was the indication to discontinue the administration of fingolimod.

Good effect of MS treatment with fingolimod should be considered when the safety of the drug is taken into consideration. Patient-doctor compliance, careful assessment

of patient condition, and patient awareness of drug-related risks are of paramount importance. ENT assessment of patients with MS also remains to be considered.

CONCLUSIONS

Fingolimod is a highly effective drug for the treatment of RRMS. In our case, the drug had an influence on the inhibition of clinical and radiological activity of the disease. Despite the control of the underlying disease, skin cancers occurred during treatment, which may be strongly related to the use of the drug, as shown by many studies. Basal cell carcinoma and squamous cell carcinoma were diagnosed at an early stage when complete resection was possible and negative (R0) margin resection was achieved.

Therefore, it is crucial to highlight the recommendations for regular specialist follow-up during fingolimod therapy. ENT assessment of patients with MS seems to be warranted. Patient compliance and patient education are crucial during treatment, which allows achieving a good therapeutic effect, thus minimizing the risk of malignancy and enabling its early detection and cure.

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CASE STUDY

PLACENTAL MOSAICISM: COMPLETE DISCORDANCE BETWEEN THE PLACENTA AND THE FETUS. CLINICAL CASE RECORD

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ABSTRACT

NIPT with the analysis of all chromosomes for aneuploidy screening. Chromosomal microarray 750K, routine karyotyping and Whole-exome sequencing and Sanger sequencing were used for the analysis of the clinical situation

Sonographic fetal abnormalities were accompanied by the placental mosaicism (trisomy 16), fetal partial uniparental disomy of the short arm of chromosome 16.

NIPT with the analysis of all chromosomes is a powerful tool to identify placental mosaicism, which in turn can manifest itself as nonspecific abnormalities in biochemical markers, placental dysfunction, growth retardation, fetal malformations, preterm birth, etc. If placental mosaicism is suspected, the optimal clinical strategy is to perform amniocentesis and placentocentesis simultaneously with a complete genetic examination of the obtained material

KEY WORDS: Placental mosaicism, congenital malformations, NIPT, karyotyping, chromosomal microarray analysis, pregnancy planning

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INTRODUCTION

Chromosomal mosaicism is the presence of two or more cell lines with different karyotypes in an organism developing from a single zygote. It can involve all tissues of the body (true fetal mosaicism) or be limited to only some of them (tissue-limited mosaicism). When the karyotype of the embryo itself is normal and chromosomal abnormalities are found only in the provisional tissues of the embryo (chorion, placenta), limited placental mosaicism is implied. Chromosomal abnormalities of the placenta can affect embryogenesis in different ways: from complete lack of influence to intrauterine growth retardation and fetal death, which depends on the type of chromosomal abnormality, involvement of extraembryonic tissues and quantitative ratio of normal and abnormal clones associated with epigenetic effects [1-3].

The most common phenomenon is the limited fetal mosaicism (Table I), with a rate of 87.3% among all pathologies. At the same time, the marker chromosomes are most often involved in mosaicism (31.6%); most rare are chromosome anomalies, excluding vital and sexual (2.8%) (Table II) [4].

It is known that Type III mosaicism has the most significant effects on the course and outcomes of pregnancy, resulting in "false-positive" nonspecific results of screening studies with individual risk calculation, reduced PAPP-A protein levels, placental dysfunction, premature birth, fetal growth retardation, stillborn pregnancy, fetal malformations and other adverse effects in pregnancy [5, 6].

Diagnosis of placental mosaicism is a complex practical and methodological problem that requires a clear understanding of both the possibilities of each analytical method and the peculiarities of histogenesis at the early stages of embryonic development, as there is no universal method of diagnosis, including placental mosaicism (Fig. 1 [4]).

CASE REPORT

Patient K., 31 years old, 16th week of pregnancy, on her own initiative, presented at the Nadiya Clinic of Reproductive Medicine for non-invasive prenatal genetic DNA testing (NIPT) for chromosomal abnormalities of the fetus, NIPT Verify, Illumina (for all chromosomes).

The obtained result: trisomy of chromosome 16. Medical and genetic consultation is recommended.

History:

Suffered from primary infertility for 3 years.

Diagnosis: First pregnancy, primary infertility managed using ART (intrauterine insemination with controlled ovarian stimulation). Placenta previa.

Family history not compromised. Occupational hazards not determined. Exacerbation of HSV and acute pharyngitis at week 9, ARVI at week 13-14 (without serological diagnosis).

Screening tests in the first trimester of pregnancy: week 12+4 days: CRL 61.6 mm, nuchal translucency thickness 1.4 mm, beta-HCG 1.017 IU, PAPP-A 0.177 MoM, PIGF 0.329 MoM.

Individual estimated combined risk:

- Trisomy 21: 1/50;

Table I. Proportions of the types of mosaicism in its general structure (according to [4])

Mosaicism type	Group	Karyotype			Specific weight
		Trophoblast	Mesenchyme	Amniocytes	
I	CPM	Abnormal	Normal	Normal	34.8%
II	CPM	Normal	Abnormal	Normal	42.3%
III	CPM	Abnormal	Abnormal	Normal	10.2%
IV	TFM	Abnormal	Normal	Abnormal	1.6%
V	TFM	Normal	Abnormal	Abnormal	5.8%
VI	TFM	Abnormal	Abnormal	Abnormal	5.4%

CPM - Confined Placental Mosaicism
TFM - True Fetal Mosaicism

Table II. Proportions of mosaic anomalies in the general structure of mosaicism (according to [4])

Aberration	Specific weight
47,+mar	31.6%
Sex chromosome aneuploidies	26.00%
Frequent (vital) trisomies(13, 18, 21)	20.00%
Structural displacement	9.9%
Polyploidy	3.3%
Rare autosomal trisomies	2.8%

- Trisomy 18: 1/1022;
- Trisomy 13: 1/1220;
- Fetal growth retardation: 1/8.

The patient was informed that the result was more likely due to the tissue-limited placental mosaicism, there was a high risk of intrauterine growth retardation or fetal death.

Recommended:

- Ultrasound examination of the fetus;
- Invasive genetic diagnosis (placentocentesis and amniocentesis with karyotyping);
- Free verification of NIPT Verify results using the NIPT SAGE-Nadiya.

The results at week 18-19:

NIPT SAGE-Nadiya: trisomy 16 (z-score 13.75 (N -6… + 6))

Placentocentesis: 47,XX,+16.nuc ish(D16Z3x3) [50]. Female karyotype with regular trisomy of chromosome 16 (verified by FISH: 3 signals corresponding to chromosome 16, 100%).

Amniocentesis: 46,XX.nuc ish(D16Z3x2) [50]. Normal female karyotype. FISH verified (2 signals corresponding to chromosome 16, 100%).

Ultrasound:

- unilateral aplasia of the radial bone (HP:0011908)
- unilateral agenesis of the kidney (HP:0000122)
- fetal growth retardation (HP:0001511).

The pregnancy was terminated due to medical reasons.

Results of fetal fibroblast karyotyping: 46,XX.nuc ish(D16Z3x2) [50]. Normal female karyotype.

In order to exclude genetic factors of fetal pathology, as well as to address the strategy for pregnancy planning and calculation of a posteriori genetic risks, a sample of fetal fibroblasts was sent for chromosomal microarray analysis (comparative genomic hybridization) and full exome sequencing.

Chromosomal microarray analysis showed a partial uniparental disomy of the short arm of chromosome 16: arr[hg19]16p13.3p13.11(4,781,662-15,965,258) x2 hmz (11.18 MB) (Fig. 2).

The region of uniparental disomy includes 103 OMIM genes, of which the phenomenon of haploinsufficiency leads to confirmed pathogenicity: 13, including ABAT, ALG1, CIITA, EMP2, ERCC4, GRIN2A, LITAF, MYH11, NDE1, PARN, PMM2, ROGDI and SET.

In addition, 2 microstructural syndromes of predisposition to neurocognitive disorders have been described for this region: 16p13.11 reversed microdeletion syndrome and 16p13.11 reversed microduplication syndrome. These syndromes are formed in the germ cells reciprocally, i.e., duplication in one will be accompanied by deletion in the other and vice versa, due to the presence of highly homologous repeated DNA fragments (LCR16's), and will be manifested in delayed psychocognitive development and certain birth defects. That is, the molecular structure of the 16p13 locus provides a basis for genetic instability and increased risks of clinically significant microstructural disorders.

Schulze et al. [7] confirm the presence of 16 loci with differential methylation in the chromosome, where map abnormalities result in the development of congenital genetic pathologies, as evidenced by cases of fetal growth retardation and/or congenital genetic conditions associated with partial uniparental disomy and abnormalities in the DNA methylation map of different loci of chromosome 16 in the normal karyotype of the fetus/child [8].

Whole exome sequencing.

The results showed a mutation in heterozygous status with unknown clinical significance, which is likely to have a clinical outcome in the form of the above clinical picture. Gene: NIPBL. Mutation: c.4332A>C (p.Arg1444Ser). The mutation is found in the gene associated with Cornelia de Lange syndrome, type 1 (autosomal dominant inheritance). Most cases of the disease are sporadic, occurring de novo. Cornelia de Lange syndrome, type 1 is characterized by severe prenatal hypoplasia, significant retardation in physical and intellectual development and significant malformations.

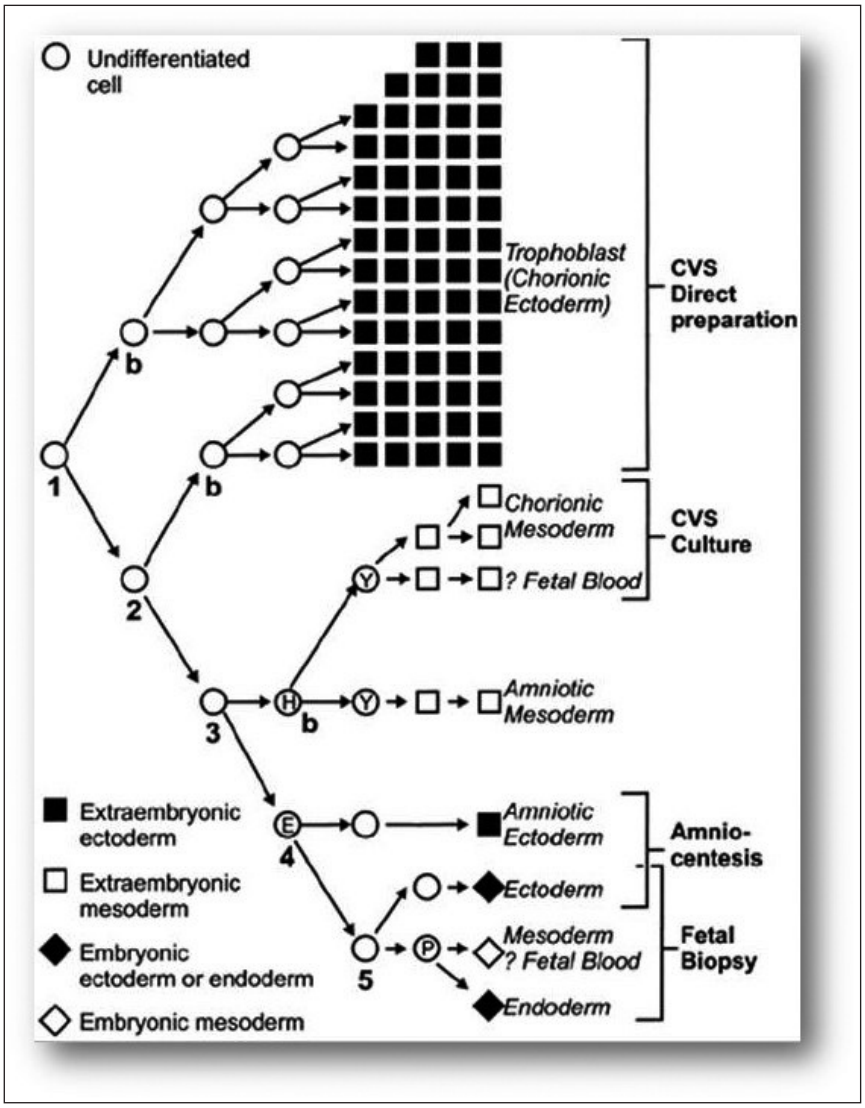

Fig. 1. Diagram of cell lines that differentiate at the early stages of embryonic development [4].

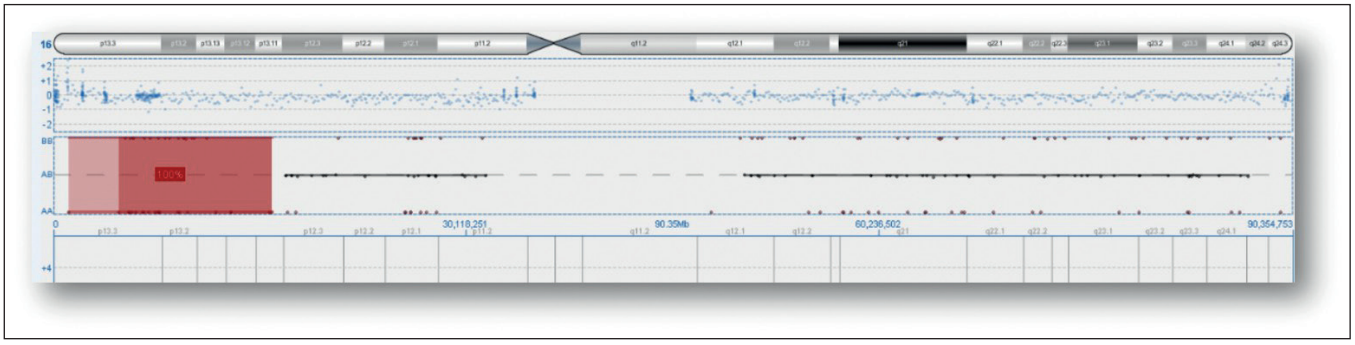

Fig. 2. Partial uniparental disomy of the short arm of chromosome 16 (bands 16p13.3-16p13.11)

Given that the clinical significance of the mutation has not been identified, genetic study of the parents is recommended for NIPBL mutation c.4332A>C to determine its causality.

Random findings of the whole exome sequencing included: heterozygous carrier of mutations of autosomal recessive pathologies:

- gene of spastic paraplegia, type 47 AP4B1, mutation c.1160_1161del (p.Thr387Argfs*30) (pathogenic);

- BTD biotinidase deficiency gene, mutation c.1336G>C (p.Asp446His) (pathogenic) and autosomal-dominant pathology

- Charcot-Marie-Tooth gene, type 2Q DHTKD1, mutation of the splicing site c.1897-1G>A (probably pathogenic).

Based on the above, the couple should be examined for a hidden carrier of recessive pathology (Carrier Screening) in order to minimize the risks of having a child with recessive genetic pathology.

Additional examination of the couple for the carrier of the NIPBL mutation c.4332A>C will probably clarify the origin of the described pathology. If a mutation is found in one of the genetic parents, it will allow the exclusion of the causative nature of the mutation and the conclusion that the mutation is nonpathogenic, and therefore the only factor of fetal malformations may be fragmentary uniparental disomy of the short arm of the chromosome 16. The latter probably occurred after meiotic chromosome nondisjunction in the gametogenesis of one of the parents with subsequent self-correction of the embryo, during which a cascade of chromosomal "breaking-assembly" (chromothripsis) occurred and a fragmentary uniparental disomy was formed. A priori recurrent genetic risk for the couple is low for uniparental disomy and increased for possible chromosomal abnormalities (total 8%, chromosome trisomy 16 - 2%). In this case, the couple is advised to perform pre-implantation genetic testing for further ART to exclude the transfer of the aneuploid embryo. Chromosomal microarray analysis is recommended at the stage of prenatal examination to exclude uniparental disomy.

If the mutation is not identified in the couple, this will give the grounds to recognize possible causality of the mutation. Most cases of Cornelia de Lange syndrome occur de novo. Given the possibility of gonadal mosaicism in the parents (germline mosaicism in the parents), the recurrence risk is 1.5%. There is still a recurrence risk for chromosomal abnormalities, as described above. In this case, it would be appropriate to include pre-implantation genetic testing of embryos for NIPBL mutation c.4332A>C in the diagnostic program.

The preconception genetic testing showed that the husband is a carrier of the NIPBL mutation c.4332A>C. No additional genetic risks were identified after the couple had been tested for hidden carrier of recessive pathology (Carrier Screening). The couple is planning a pregnancy using ART with pre-implantation genetic testing.

It should be noted that the described clinical case highlighted another urgent problem: improving the quality assurance system in obstetrics and gynecology, given its importance in the prevention of maternal and infant losses and in population health in general. It is known that financial availability of expert genetic testing for more than half of patients is significantly limited [9]. Under conditions of out-of-date regulations of medical genetics (Order No. 641/84 of the Ministry of Health of Ukraine of 31.12.2003), absence of unified clinical protocols, including expert methods of genetic testing, unavailability of clinical protocols from international sources (due to financial factors), it is impossible to unify approaches in patient management, ensure that patients have access to the necessary test methods and, as a result, to provide timely and high quality medical care. Therefore, organizational measures to improve the quality of obstetric and gynecological, as well as genetic services, aimed at preserving the life and health of a newborn and improving the quality of medical care for pregnant women and mothers, are extremely important. It is a necessary condition for ensuring the citizens' right to health, successful development of the national health care system and improvement of the demographic situation in Ukraine.

CONCLUSIONS

NIPT with the analysis of all chromosomes is a powerful tool to identify placental mosaicism, which in turn can manifest itself as nonspecific abnormalities in biochemical markers, placental dysfunction, growth retardation, fetal malformations, preterm birth, etc. If placental mosaicism is suspected, the most optimal clinical strategy is to perform amniocentesis and placentocentesis simultaneously with a complete genetic examination of the obtained material. Close collaboration between geneticists and patients at the screening phase is the key to accurate genetic diagnosis and the development of a pregnancy planning program to minimize genetic risks. Organizational measures to improve the quality of obstetric and gynecological, as well as genetic services, aimed at preserving the life and health of a newborn and improving the quality of medical care for pregnant women and mothers, are extremely important.

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