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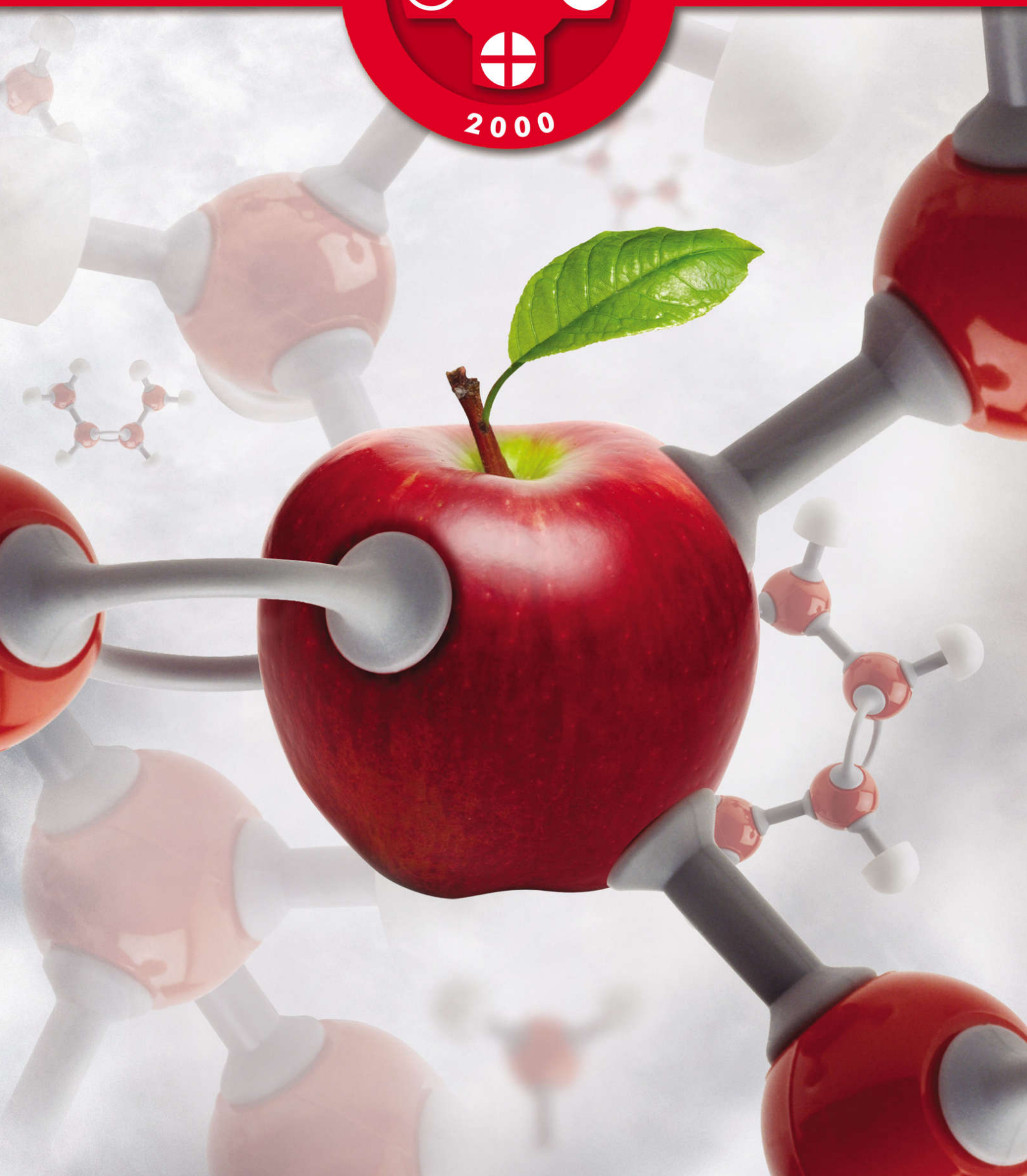

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PUBLIC HEALTH AND THE NEW LAW OF PUBLIC HEALTH IN REPUBLIC OF SERBIA

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JAVNO (NARODNO) ZDRAVLJE I NOV ZAKON O JAVNOM (NARODNOM) ZDRAVLJU U REPUBLICI SRBIJI

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ABSTRACT

The new Law on Public Health was published in the Official Gazette of the Republic of Serbia No. 15 dated 25 February 2016. Comparing to the previous Law, the biggest changes have been made in the domain of monitoring the indicators of the environment and population health. The responsibility of controlling the quality and safety of food was given back to the Ministry of Health. This paper presents the principal regulations regarding the main functions, principals and organizational features of the public health system in Serbia.

Keywords: *Public Health, New Legislation, Health System, Serbia, Health Policy*

SAŽETAK

Novi Zakon o javnom zdravlju objavljen je u Službenom glasniku Republike Srbije br. 15 od 25.02.2016.godine. Osnovne promene u odnosu na stari zakon se odnose na praćenje indikatora životne sredine i njihovom uticaju na zdravlje stanovništva. Odgovornost za kontrolu kvaliteta i bezbednosti hrane je ovim zakonom vraćena u domen rada Ministarstva zdravlja. Ovaj Zakon trebalo bi da unapredi nivo zdravstvene zaštite građana kao i da aktivno radi na očuvanju i unapređenju zdravlja stanovništva. U ovom radu su prikazane osnovne regulativne norme koje se tiču glavnih funkcija, principa i organizacionih osobina sistema javnog zdravlja u Srbiji.

Ključne reči: *zakon, javno (narodno) zdravlje*

INRODUCTION

A law on public health is one of the most important legal acts in the health sector. Health is the main resource of every individual and society as a whole. Along with developing curative methods and techniques, modern medicine also developes methods of prevention. A large number of developed countries allocate significant resources to the development of public health (1-5).

The new Law on Public Health was published in the Official Gazette of the Republic of Serbia No. 15 dated 25 February 2016 (6). As in the previous law, it has been established that all programs of public health should be implemented and actively participated by the health service networks - primarily the Institutes (led by the National Institute of Public Health of Serbia), health and social insurance, and the broader community (including state authorities and ministries, educational and other institutions, public companies, the Red Cross of Serbia, churches and religious communities, citizens, local authorities etc.) (7). Based on the results of studies on the health status of the relevant population, Institutes actively participate not only in the preservation and improvement of human health, but also in establishing the current health policy (8). This Act should enhance the level of health protection of citizens and preserve and improve their health condition (6, 7).

The Law has stipulated that social care should include the policy and the strategy of public health, monitoring the health status of the population, identification of health problems, determination of the main tasks, the adoption of special programs in the field of public health, implementation of tax measures and economic policies that encourage healthy lifestyles, providing conditions for health care and education of the population and the conditions for rapid

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and adequate response in emergency situations. The main changes have been made in the domain of monitoring the indicators of the environment and population health. Public health departments and institutes have taken the responsibility of controlling the state of the environment, the quality and safety of drinking water, food and objects of general use. They are also in charge of monitoring the hygiene standards in health care facilities, schools and kindergartens, sport facilities, facilities where food is produced, processed, distributed and sold etc. The health care institutions should estimate the health risks to the population, based on the register of polluters, and they should monitor and analyse the population health based on the state of the environment. The assessment of the epidemiological situation is also under the competency of these institutions. The important thing is that monitoring of food quality has been brought back under the jurisdiction of the Ministry of Health (6).

A DEFINITION OF PUBLIC HEALTH

Concepts and definitions of public health are quite voluminous and complex in all countries with rather developed or intensively developing public health. Most of these definitions are based on the concepts of preserving and improving health of population (9). According to the World Health Organization, "Public health is a social and political action that aims to improve health, extend life and improve its quality in the entire population through health promotion, disease prevention and other forms of medical intervention" (1).

THE HISTORY

Social care for health was developing along with the first medical skills, so that some of its roots could be found even in the ancient Egyptian and Greek medicine. During the historical development, the concepts of public health have undergone significant changes. Historically, the development of public health can be divided into four periods:

1. the period of hygienic sanitation;
2. the period of individual health care;
3. the period of therapeutic procedures;
4. the new public health period.

The development of public health and organized health care in our country began in 1839, with the adoption of the first Constitution that enabled foundation of a quarantine ward and an ambulance with the task "to do all that can serve to protect the life and health of people". At the beginning of the twentieth century and with the opening of the first medical school in Belgrade, health institutions were established and they performed public health services (The Central Hygiene Institute in Belgrade) (10,11).

THE MAIN PRINCIPLES OF PUBLIC HEALTH

The main principles of public health are: equality and solidarity, sustainability, participation, efficiency, justice and peace. The main principle of the new public health is to connect the traditional goals of public health with the work of institutions involved in the individual health protection and social activities (12, 13).

Modern human activities and lifestyles have brought new challenges in the field of public health, including new techniques and weapons of mass destruction (terrorism, particularly bioterrorism) (14), emergency situations (especially natural disasters like earthquakes, floods, volcanic eruptions, hurricanes, tsunami etc.) (15), some new infectious diseases (SARS, "bird flu", "swine flu") (16) and old diseases with a large number of newly infected people (such as tuberculosis), increasing resistance of microorganisms to antibiotics and disinfectants (17), expansion of chronic diseases on a global scale (depression, obesity etc.) (18), diseases associated with unhealthy habits (e.g. smoking) (19), and many others (20). The conditions of modern life induce new threats to population health, such as globalization, the collapse of public health infrastructure, poverty and hunger in the world, wars, mass migrations, etc (21). Modern medicine can not be imagined without the public health achievements such as vaccination programs, the safety of drinking water, food and objects of general use, the safety at workplaces, health risk assessment, control and combating infectious diseases, reducing mortality from chronic noncommunicable diseases, family planning, improving the health of mothers and babies, recognition of tobacco use as a health hazard, improving the health care system, etc (22).

FUNCTIONS OF PUBLIC HEALTH

Functions of public health are numerous and quite demanding. They include:

1. Monitoring, evaluation and analysis of population health;
2. The public health surveillance, investigation and control of risks and threats to health;
3. Improving health of the population;
4. Social participation in all aspects of health care;
5. Development of policies and institutional capacities for planning and managing in the public health sector;
6. Improving the institutional capacities for planning and managing in the public health sector;
7. Evaluation and promotion of equitable access to necessary health services;
8. Human resource development and training in the area of public health;
9. Ensuring the quality of health care and the orientation towards an individual and population;
10. Public health survey;
11. Reducing emergencies and disasters by prevention, migration, readiness for response and rehabilitation;
12. The assessment of public health risks (1, 6, 12).

THE ORGANIZATION OF PUBLIC HEALTH IN THE REPUBLIC OF SERBIA

Public health care in Serbia is organized at several levels. The first level includes:

1. The Ministry of Health;
2. Other Ministries: the Ministry of labour, employment, veteran and social affairs; the Ministry of education, science and technological development, the Ministry of agriculture and environmental protection etc.
3. Inspection bodies (health and sanitary inspection bodies, market and veterinary inspection bodies, etc.);
4. The Institute of Public Health of Serbia "Dr Milan Jovanovic Batut" with the network of other institutes;
5. Community Health Centres;

The second (local) level of the organization includes:

6. Educational institutions - universities, higher and secondary medical schools, primary schools and preschool institutions;
7. Institutions of Social Protection;
8. Municipalities and local authorities;

The third level includes non-governmental sector:

9. The non-governmental organizations such as the Red Cross (1).

THE LEGAL REGULATIONS

The Republic of Serbia began with the adoption and implementation of the first legal document related to public health at the beginning of 21th century. The first document entitled "The Health Policy of the Republic of Serbia" was adopted in 2002, based on the premise that health of people was a question of general public interest and the most important resource for development. Thereafter, the health care started to develope in accordance with the European Union strategy in this field (23).

The Ministry of Health is responsible for the organization of the public health system in the Republic of Serbia. A part of the responsibility is attributed to the other ministries, including those responsible for education, environment, social affairs, science, sports, agriculture, economy etc. The Regulation on Health Institutions' Network Plan ("Official Gazette of RS", no. 42/06, 119/07 and 84/08) has defined the institutions that make up the public health system. Twenty three institutes were established in the domain of public health. Their task is to coordinate the entire area of public health, and to directly participate in health promotion, disease prevention and environmental protection. Community health centers also have an important role in the public health system in the corresponding teritorries. The community health centers' network in Serbia consists of 160 institutions. The public health system also includes the inspection services (related to health care, sanitary surveillance, utilities, market and veterinary care) as well as the institutions in the field of education and social welfare (6-8).

Public health promotion can be performed by the organizations whose activities must be coordinated with the official policy of public health and regulated by the current legislation (13). Public health is regulated by a number of laws and regulations (the Act on Public Health, Public Health Strategy, Youth Strategy, etc.) (20, 22), and is funded by different sources: the funds of the Republic Institute for Health Insurance, Republic of Serbia budget and the budgets of local authorities (24, 25).

PUBLIC HEALTH IN EUROPE

The European Union public health policy is a part of the jurisdiction of the Health Council of the European Union. The new public health program was established in June 2002 and it has been evaluated and modernized in every 5 years (26). The program activities are under the competence of the Commission of the Health Council of the European Union (27). The program that was implemented during the period of 2003-2008 reffered to the main objectives of the new public health:

1. increasing the information and knowledge in the field of public health;
2. enabling rapid reaction and response to all health hazards;
3. determination of the major determinants of health in order to reduce mortality and prolong the life expectancy of the population.

The next program was defined for the period of 2007-2013. Besides the main objectives, the public health priorities of the Commission of the EU Health Council were also:

1. increasing the efficiency and responsibility in the provision of health services;
2. helping the countries in preventing diseases in order to extend the life expectancy of the active-aged population;
3. the promotion of the cooperation among the health systems of the member states and the dissemination of knowledge in the field of public health (28).

European countries have different models of public health systems (29). However, a common feature are the national public health institutes that have particularly important roles, tasks and responsibilities (30).

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MANUFACTURING OF BIODEGRADABLE SCAFFOLDS TO ENGINEER ARTIFICIAL BLOOD VESSEL

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IZRADA BIORAZGRADIVIH MATRICA ZA VEŠTAČKI KRVNI SUD

Maja Milošević[1], Nikola Mijailović[2,3], Dalibor Nikolić[2,3], Nenad Filipović[2,3], Aleksandar Peulić[2,3], Mirko Rosić[1] i Suzana Pantović[1]
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ABSTRACT

Blood vessels diseases such as cardiac infarction with coronary artery occlusion, peripheral arterial disorders, or stroke of carotid or cerebral arteries, are the leading causes of death in the world. One of medical procedures for clinical treatment of vascular diseases is the blood vessels grafting. As the autologous blood vessels, which are the "golden standard" for coronary grafting, are not always suitable for blood vessels grafting, there is a need to develop artificial blood vessels as a vascular prostheses, either from natural and synthetic materials, permanent synthetic or biodegradable scaffolds which would be suitable for vascular grafts. Considering this to be our study goal we made bilayered biodegradable polycaprolactone scaffolds with different properties and evaluated their morphological and biomechanical characteristics.

Keywords: *blood vessel, vascular grafts, biodegradable scaffolds, electrospinning*

SAŽETAK

Oboljenja krvnih sudova, kao što su infarkt miokarda sa okluzijom koronarne arterije, poremećaji perifernih arterija, ili infarkt karotidnih ili cerebralnih arterija su vodeći uzrok smrti u svetu. Jedna od medicinskih procedura za klinički tretman vaskularnih oboljenja je primena graftova krvnih sudova. Kako autologi krvni sudovi, koji predstavljaju „zlatni standard" u primeni koronarnih graftova, nisu uvek pogodni, postoji potreba za razvojem veštačkih krvnih sudova kao vaskularnih proteza, od prirodnih ili sintetičkih materijala, permanentnih ili biorazgradivih skafolda koji bi bili pogodni da se primene kao graftovi. Imajući ovo u vidu, cilj naše studije je bio da se naprave dvoslojni biorazgradivi skafoldi od polikaprolaktona sa različitim svojstvima i da se zatim procene njihove morfološke i biomehaničke karakteristike.

Ključne reči: *krvni sud, vaskularni graft, biorazgradiva matrica, elektrospining*

ABBREVIATIONS

DMF - N,N – dimethylformamide
ECs – endothelial cells
ECM – extracellular matrix
PCL – polycaprolactone
PEG – polyethyleneglycol
SEM – scanning electron microscopy
SMCs – smooth muscle cells

INTRODUCTION

Blood vessels diseases such as cardiac infarction with coronary artery occlusion, peripheral arterial disorders, or stroke of carotid or cerebral arteries are the leading causes of death worldwide (1). Clinical treatments of vascular diseases include various drugs and medical procedures with different success of treating. One of medical procedures for clinical treatment of vascular diseases is the blood vessels grafting. As the autologous blood vessels, which are the "golden standard" for coronary grafting, are not always suitable for blood vessels grafting, there is a need to develop artificial blood vessels as a vascular prostheses. In recent years, a significant progress has been made in the development of prosthetic grafts from natural (2, 3) and synthetic materials (4, 5). Tissue engineering offers an attractive option to vascular grafting, particularly for creating small diameter vessels using biodegradable scaffolds technologies for vascular grafts in which layers of endothelial cells (ECs), smooth muscle cells (SMCs), or fibroblasts are grown to resemble the blood vessel structure. These scaffolds are designed to degrade over time as vas-

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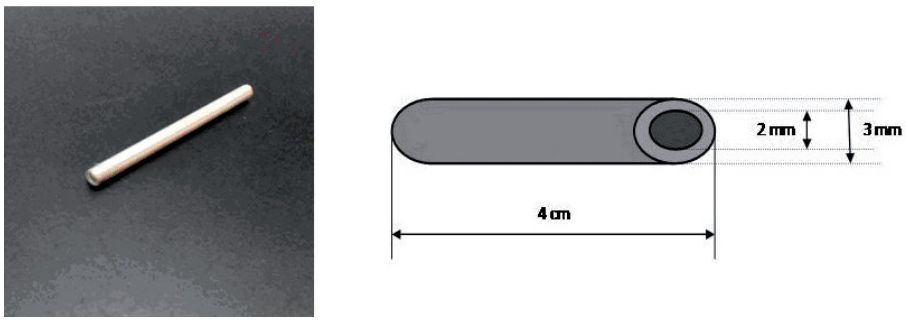


Figure 1. Metal tube collector (mandrel) presented as photo images (left) and as schematic diagram (right).

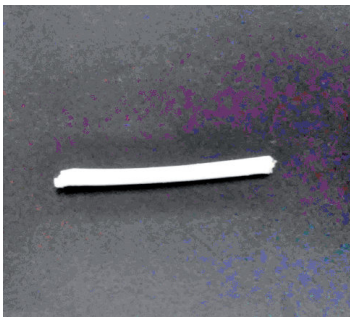


Figure 2. Tubular scaffold (one representative specimen).

cular cells produce extracellular matrix (ECM) and form functional vessels through a tissue remodeling process in vivo (6, 7). The vascular scaffold should be composed of a durable biomaterial capable of withstanding physiological hemodynamic forces while maintaining structural integrity until mature tissue forms in vivo (6). Electrospinning technology has been widely used for this purpose because this technique permits fabrication of nano- to microscale fibrous matrices and allows for control of the composition, structure, and biomechanical properties of scaffolds (8-10).

The aim of this study was to prepare scaffolds by means of electrospinning as well as to test morphological and biomechanical properties of the scaffolds aiming to create small diameter bilayered blood vessel grafts.

MATERIAL AND METOD

Scaffold fabrication

In order to prepare bioengineered circumferential bilayered scaffolds, biodegradable materials were used. For the first (inner) layer polycaprolactone (PCL, Sigma-Aldrich, U.K., Mn 80000, pellets (~3 mm)) and polyethyleneglycol (PEG, Sigma-Aldrich, Germany, Mn 4000, platelets) were dissolved in chloroform solution (Chloroform, Sigma-Aldrich, France, ACS, reagent, ≥ 99.8%) in the period of 24 hours. Different ratios of PCL and PEG were used (1:1.1 ratio and 1:1.25 ratio) dissolved in chloroform solution as 16.5% (w/v), 22% (w/v), and 30% (w/v), in order to obtain proper concentrations which would in the end have gratifying amount of pores (important for coating with cells). A metal tube collector with the diameter of 3-5 mm (mandrel) was used for manufacturing the inner layer of circumferential scaffolds (Figure 1). After 24 h drying scaffolds were immersed into the water for another 24h (in order to dissolve some amount of PEG to obtain pores). After drying, scaffolds were ready for creating outer layer (Figure 2).

Electrospinning method

Outer layer was obtained using electrospinning method and apparatus. The electrospinning system consists of syringe pump, high voltage supply and rotating rod (outer diameter, 2.1 mm). The Acopian Power Supply Model P030HP1 was used (in collaboration with Faculty of Engineering and BioIRC, University of Kragujevac). The positive electrode was connected to the cusp of the syringe needle and negative electrode was attached to the rotating rod. One-layered scaffolds were put onto the rod collector. A polymeric solution for electrospinning was prepared as 20% (w/v) PCL using an organic solvent mixture composed of chloroform and N,N-dimethylformamide (DMF, Sigma-Aldrich, USA, anhidrous, 99.8%) (7:3 ratio) (11). For the electrospinning process the polymeric solution was placed in a 10 ml plastic syringe fitted with a metal needle with a tip diameter of 0.8 mm. The syringe pump provided constant flow rate of polymer of 1 ml/h through syringe needle. The polymer solution was converted to nanofiber in presence of electric field. The voltage supply between 15 kV and 18 kV was applied to polymer solution. These nanofibers were collected to the already prepared one-layered scaffold which was put onto 436 stainless steel rod with outer diameter 2.7 mm. Different numbers of rotations were applied (50 revolutions per minute and 60 revolutions per minute) for solvent cast PCL layer. Electrospinning was performed in 1 and 2 min. The distance between the syringe tip and the collection rod was 20 cm that allows fibers to be wider and better spread on collector in order to achieve better ar-

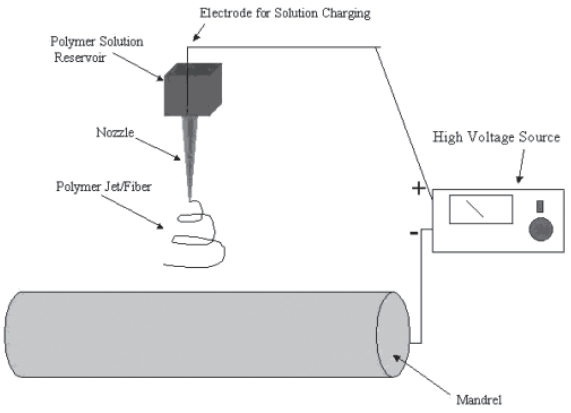
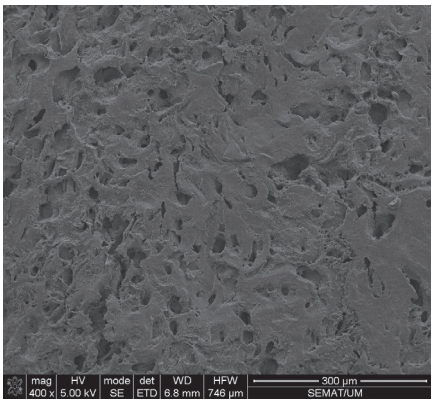


Figure 3. Electrospinning apparatus setting



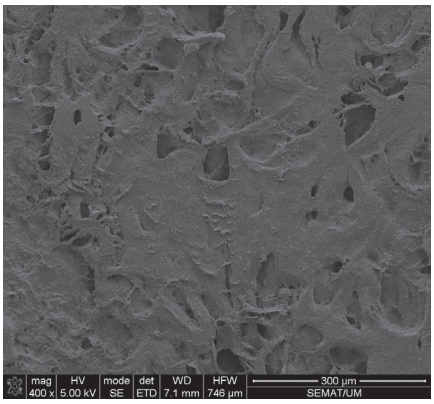


Figure 4. Photomicrographs obtained by SEM represent: a) inner layer of scaffold 16.5% (w/v) chloroform solution, PCL:PEG=1:1.1, b) inner layer of scaffold 16.5% (w/v) chloroform solution, PCL:PEG=1:1.25.

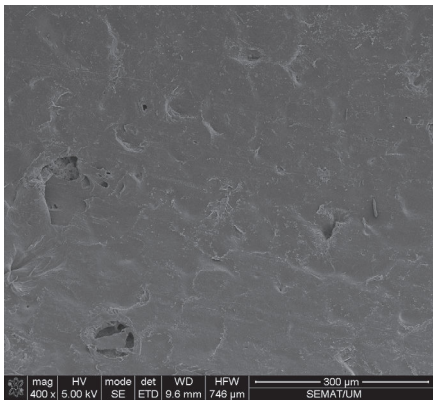
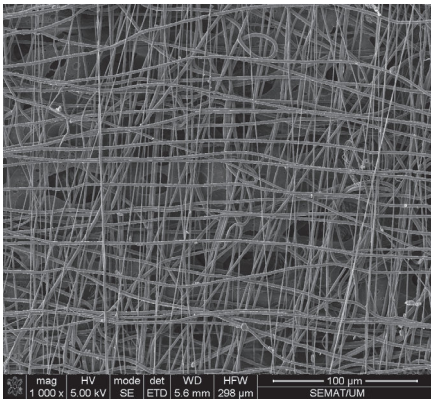


Figure 5. Photomicrograph obtained by SEM represents an inner layer of scaffold which consists chloroform solution 30% (w/v) and PCL:PEG= 1:1.1.



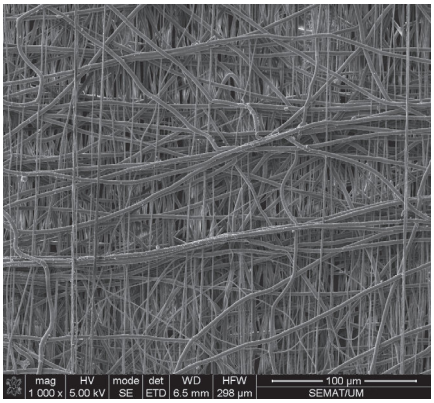


Figure 6. Photomicrographs obtained by SEM represent: a) outer layer of scaffold 60 rpm, 1 min, b) outer layer of scaffold 60 rpm, 2 min.

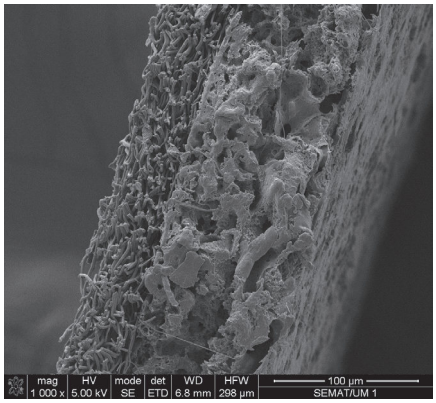


Figure 7. Photomicrograph of bilayered scaffold (cut at the edge, one representative specimen).

rangement of fibers. Solvent evaporation was performed at room temperature for 2 days. Many conditions were changed in order to obtain gratifying scaffold that will be used for cell seeding (Figure 3).

Mechanical characterization of tubular scaffolds

Electrospun bilayered tubular specimens were used for mechanical testing. Different ratios of PCL and PEG, as well as the percentage of chloroform solutions (w/v), of scaffolds' were used in order to obtain optimal biomechanical characteristics of specimens (Young's tensile modulus, Max stress and Strain at break). Tensile properties were measured with load test machine (Bose ElectroForce® 3200, TA Instruments®, USA) (in collaboration with Faculty of Engineering and BioIRC, University of Kragujevac) equipped with a maximum 225 N load cell.

Morphological characterization of tubular scaffolds

Bilayered parts of membranes were observed using scanning electron microscopy (SEM) (Pegasus X4M). The membranes were cut into square specimens (1 cm^2), glued with carbon tape to copper supports and sputter coated with gold to a thickness between 10 to 15 nm. Images were acquired using SEM operating at accelerating voltage of 5 kV.

Statistical analysis

Statistical analyses of data obtained after mechanical tests were performed using independent t-test with significance threshold $p<0.05$. All statistical calculations were done with the computer program SPSS, version 19.0. Data are presented as mean ± standard deviation (SD).

RESULTS

In order to adjust experimental conditions for preparing bioengineered scaffolds that would have desired properties for creating vascular grafts, several adjustments were made. Scaffolds were made using different values and combinations of materials and conditions, as we already described in the Material and Methods section and presented in the Table 1.

All specimens then subjected to scaffolds' analysis consisted of:

1. Morphological characterization (Figures 4-7),
2. Biomechanical characterization (Young's tensile modulus, Max stress and Strain at break) (Table 2, Figures 8-11)

Table 1. The values and combinations of used materials and conditions in order to create bioengineered scaffolds.

The inner layer of scaffold		The outer layer of scaffold		
Ratio PCL:PEG	w/v %	PCL	Rotations	Time of rotations (electrospinning)
1:1.25	16.5	7:3	50	1 min and 2 min
	22			
	30		60	1 min and 2 min
1:1.1	16.5	7:3	50	1 min and 2 min
	22		60	1 min and 2 min
	30			

PCL is polycaprolactone, PEG is polyethyleneglycol, PCL is dissolved in chloroform and N,N-dimethylformamide (ratio 7:3)

Table 2. Results of biomechanical characterization of scaffolds (PCL:PEG=1:1.1 and PCL:PEG=1:1.25, made under 60 rpm, duration 1 minute), performed by estimation of Young's tensile modulus, Max stress and Strain at break.

Scaffold ratio PCL : PEG	Young's tensile modulus (Mpa)[1]	Max stress (Mpa)	Strain at break (%)
1 : 1.1	50.2 ± 5.2	5.3 ± 0.6	320 ± 128.8 **
1 : 1.25	49.2 ± 14.5	4.3 ± 0.9	179 ± 39.2 **

* results are presented as Mean ± SD
** stat. significant difference, p<0.05
[1]- Tensile modulus calculated as difference between two points in linear part of graph
[2]- Tensile modulus calculated using function LINEXP, and all the spots between two chosen in linear part of the graph

1. Morphological characterization - by scanning electron microscopy (SEM)

All created scaffolds were subjected to analysis by scanning electron microscopy (SEM). Analysis of the inner layer of the specimens showed that scaffolds made as 16.5% chloroform solution with both ratios PCL:PEG=1:1.1 and PCL:PEG=1:1.25 had the most appropriate characteristics in respect to desirable size and number of pores (Figures 4a) and 4b)). As an example, we presented in the fig. 5, how one of the scaffolds looks like (30% chloroform solution, PCL:PEG=1:1.1), which showed no suitable morphological characteristics.

Analysis of the outer layer of the specimens (by SEM) showed that scaffolds made using wire collector under 60 revolutions per minute (with the duration of electrospinning of 1 minute) had the most appropriate characteristics in respect to arrangement and amount of fibers and pores (Figure 6a), according to Nam and coauthors (12). As an example, we presented in the Figure 6b, how one of the scaffolds looks like (30% chloroform solution, PCL:PEG=1:1.1), which showed no suitable morphological characteristics.

In the Figure 7, we presented photomicrograph of one representative specimen of bilayered scaffold (cut at the edge).

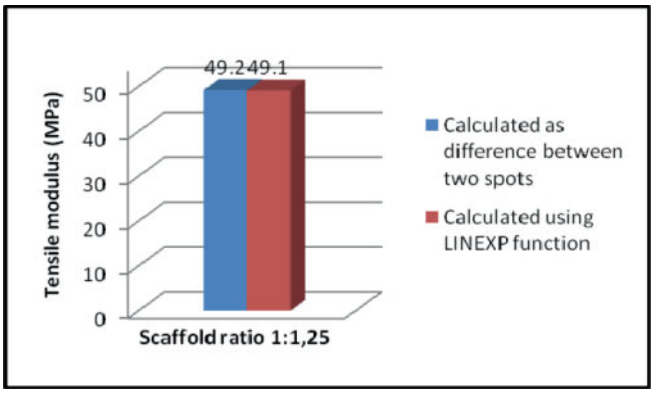


Figure 8. Calculated Tensile modulus; scaffold ratio (PCL:PEG) 1:1.25.

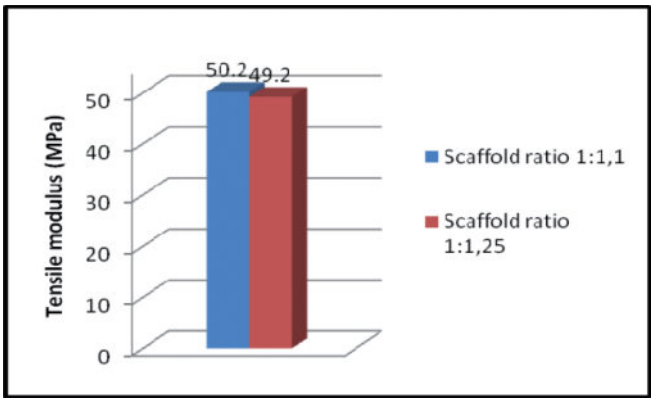


Figure 9. Comparison of Tensile modulus between scaffold ratios (PCL:PEG), calculated as difference between two spots.

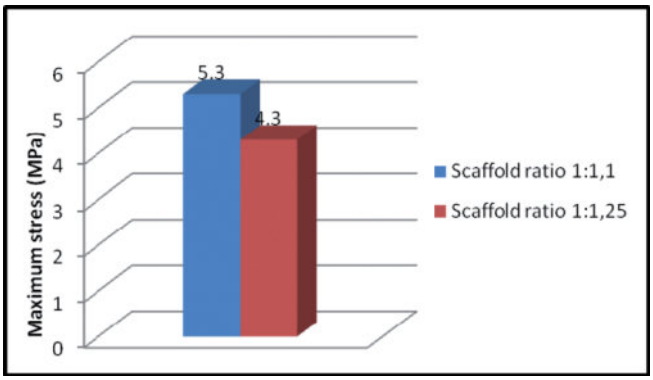


Figure 10. Maximum stress; scaffold ratios PCL:PEG.

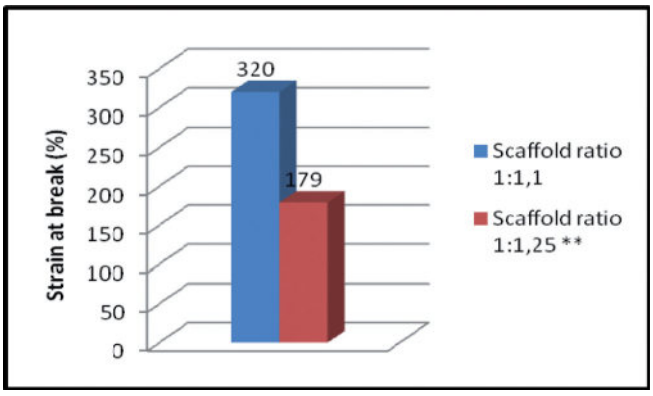


Figure 11. Strain at break; scaffold ratios PCL:PEG. (** stat. significant difference, p<0.05)

2. Biomechanical characterization

Electrospun bilayered tubular scaffolds which showed the most suitable morphological characteristics (PCL:PEG=1:1.1 and PCL:PEG=1:1.25, made under 60 revolutions per minute (rpm), 1 minute duration) were used for mechanical testing in order to evaluate biomechanical characteristics of specimens. Biomechanical characterization was performed by estimation of Young's tensile modulus, Max stress and Strain at break. Obtained results are presented in Table 2 and Figures 8-11.

DISCUSSION

Having in mind that each year over a million patients worldwide need arterial prostheses which cost more than US$25 billion (13), the need for vascular grafts replacement is growing. It is also known that pathologies (mainly caused by atherosclerosis) affecting small and medium-sized blood vessels are the primary cause of death (14, 15). In these situations cardiac and peripheral bypass surgeries are necessary, requiring the replacement of a segment of blood vessels. The currently available options for these transplants are autologous grafts, allografts, xenografts and synthetic vascular grafts (16). The use of autografts and allografts is limited due to the lack of tissue donors, previous harvesting or anatomical variability (17). Xenografts suffer from their relatively shorter life span (18). Synthetic prosthetic grafts become rejected by the immune system of the body if the diameter of the vessel is smaller than 6 mm (reocclusion, thrombosis and aneurysm) due to mismatch of compliance (17-20). So far it has been shown that tissue engineering could be an alternative approach for creating new vascular grafts. However, synthetic vascular grafts have rarely been proved successful in small blood vessel replacements (inner diameter < 6 mm) (21). The principle of designing tissue engineered scaffolds is simple: the scaffold should mimic the structure and biological function of native extracellular matrix (ECM) as much as possible, and in addition not to be toxic for tissues. In tissue engineering, the basic idea is that cells seeded onto scaffold produce ECM while the polymer is degraded, gradually creating the intended tissue. Moreover, vascular grafts should have further characteristics: enough strength to resist rupture or excessive dilation when subjected to pulsatile pressure *in vivo*; also to have stable mechanical properties during their expected lifetime. Many previous studies have used PCL as biodegradable polymer for vascular tissue engineering (22) or to make *in vitro*, scaffolds coated with neuronal cells (23) which show its nontoxic effects. That was one of the reasons why it was used for this research.

Poly(ε)-caprolactone (PCL) is wide used biopolymer in many studies (24). PCL is semicrystalline, aliphatic polyester synthesized by the ring-opening polymerization of ε-caprolactone (25-27). It shows good mechanical properties, specifically high elongation and strength, and good biocompatibility (27, 28). Furthermore, PCL degrades very slowly *in vivo* by enzymatic action and by hydrolysis to caproic acid and its oligomers (25, 27). It takes more than one year to completely degrade in vivo (27, 28). PCL is a FDA approved polymer (25). The degradation products are ultimately removed by giant macrophage cells (29, 30). PCL elasticity closely matches native values (31) and has a high extension rate before breakage, but tensile strength of PCL is less appreciable (31). PCL based small diameter vascular constructs have been reported to have good suture retention value and compliance to withstand physiological conditions of blood vessels (32). Electrospun PCL with differential porosity in two layers (inner low porosity than outer), when implanted in a rat showed complete endothelization and perfect patency with no thrombosis (33, 34). In another study, an electrospun PCL tubular scaffold implanted into rat proximal native artery showed excellent structural integrity throughout the study, with no aneurysmal dilation, and perfect patency with no thrombosis and limited intimal hyperplasia (34).

In the first phase of our research we wanted to create scaffolds with the most suitable characteristics, which could be used for seeding cells, in the second phase of our research.

Regarding the morphological characteristics, all the adjustments performed in this research (Figures 4-7) were made in order to obtain effective pore diameters for cell ingrowths. Pores in a tissue-engineered scaffold make up the space in which cells reside. In this study, the majority of pore diameters are limited to the range of 25 to 100 μm (Figures 4-7) According to literature data of vascular grafts, the effective pore diameters for cell ingrowths are between 20 and 60 μm while for bone ingrowths the pore-diameters between 75 and 150 μm are required (35). The pore size that would permit adequate cellular infiltration has been suggested to be greater than 10 μm (9). For the engineering of blood vessels, small pore size does not present a problem with respect to coating of the lumen using endothelial cells (EC). However, this pore size would limit the ability of smooth muscle cells (SMC) to colonize the outer portion of neo-vessel and facilitate remodeling of ECM (12). As already described in the Material and Methods section, all manufactured specimens were subjected to evaluation of morphological properties by scanning electron microscopy (SEM). Results of these evaluations showed that the most appropriate properties in respect to desirable size and number of pores for the inner layer showed scaffolds made as 16.5 % chloroform solution with both ratios PCL:PEG = 1:1.1 and PCL:PEG = 1:1.25 (Figures 4a) and 4b)). Regarding to outer layer using electrospinning method the most gratifying properties showed scaffolds made using thicker wire collector under 60 revolutions per minute (duration of electrospinning was also one minute) (Figure 6a) because arrangement and amount of fibers and pores would be satisfactory for smooth muscle cells' growth (12).

As far as biomechanical tests are concerned (Figures 8-11), our results showed that no statistical significance were obtained between scaffolds' Young's modulus values.

Young's modulus defines the relationship between stress (force per unit area) and strain (proportional deformation) in a material, it predicts how much a material sample extends under tension or shortens under compression. This value of the Young's modulus provides strong and compact tissue formation. More flexible tissue formation can be achieved using thinner layer of scaffold material (36). Maximal stress was no significantly different regarding the scaffold's ratio, which is desirable from mechanical point of view. However, the material with scaffold PCL:PEG ratio of 1:1.1 has approximately 20% greater maximal stress value and due to this fact can be a better choice in elastic tissue formation (Figure 10). Maximal stress value is in fact maximal force value per area of material for which material reserves elastic behavior what is crucial at blood vessels application due to presence of the cyclic mechanical stress. Further, statistically significant difference was obtained for value of Strain at break (%) ($p=0.021$, group of scaffolds made as ratio PCL:PEG=1:1.1 shows higher level for Strain at break). Strain at break represents ratio between changed length and initial length after breakage of the test specimen. It expresses the capability of a material to resist changes of shape without crack formation. Before break material loses the elastic behavior. The mixture of the scaffold ratio PCL:PEG=1:1.1 has approximately twice larger Strain at break value than mixture with ratio of 1:1.25. In the normal condition this value has never been achieved (37) and both materials are satisfied toward potential crack in application for tissue growth.

CONCLUSIONS

The morphological analysis of results in this study has showed that scaffolds with inner layer made as 16.5 % (w/v) PCL:PEG ratio 1:1.1 and PCL:PEG ratio 1:1.25 have more desirable size and number of pores than scaffolds made as 22% and 30%. Further, biomechanical characterization showed that both specimens with PCL:PEG ratios 1:1.1 and 1:1.25, respectively, have very similar elasticity characteristic. However, scaffolds with PCL:PEG ratio 1:1.1 are stronger and more resistive to the straining and mechanical stress. This fact point, that considered scaffold material, represents a better choice in application for tissue growth. In the same time this material has slightly better elastic characteristics and in the same time can be used for more different types of tissues which is an additional reason for its application.

We believe that described experimental procedures and obtained results extend our knowledge in the technologies for vascular grafts, and represent a good basis for further research that would include and the cells seeded onto scaffold in order to develop artificial blood vessels as vascular prostheses.

Disclosures

Conflict of Interest: None.

Acknowledgements

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INSTRUMENTAL ASSESSMENT OF THE FACE SKIN AGING IN WOMEN

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INSTRUMENTALNE METODE PROCENE STARENJA KOŽE KOD ŽENA

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ABSTRACT

The aim of this study is to conduct several non-invasive methods for assessing the level of circulatory disturbance, elasticity and aging of skin in patients of different age groups in order to expand the diagnostic capabilities and evaluate the effectiveness of current research in aesthetic medicine. Clinical and instrumental exploration of 160 women aged 17 to 75 years with varying degrees of involutional skin changes was carried out. To objectify the assessment of skin condition, in all group of patients modern instrumental methods were used, such as: elastometry, ultrasound examination of the skin, laser Doppler flowmetry, transcutaneous oxygen tension. Concurrent implementation of several non-invasive methods for assessing the level of circulatory disturbance, elasticity and aging of the skin, allowed us to find new possibilities for studying the functional state of the skin. These methods extend the possibilities of ultrasonic research methods used today in aesthetic cosmetology. The obtained comparative data of elastometry, ultrasonography, laser Doppler flowmetry and transcutaneous oximetry in patients of different age groups showed the presence of elasticity and structure defect, skin thickness and subcutaneous fat, as well as microcirculation changes since 25 years and marked changes after 40 years.

Keywords: *facial skin, instrumental diagnostics, aging, elastometry, ultrasound, laser Doppler flowmetry, oximetry.*

SAŽETAK

Cilj ove studije je da prikaz nekoliko neinvazivnih metoda za procenu nivoa poremećaja cirkulacije, elastičnosti i starenja kože kod pacijenata različite starosti sa ciljem predstavljanja drugih dijagnostickih metoda i procene efikasnosti trenutnih metoda u estetskoj medicini. Sprovedeno je kliničko istraživanje koje je obuhvatilo 160 žena starosne dobi od 17 do 75 godina sa različitim stepenom incidualnih promena kože. Da bi se objektivizovala procena stanja kože, u svim grupama pacijenata su korišćene moderne instrumentalne metode, kao što su: elastometrija, ultrazvučni pregled kože, laser i dopler metoda merenja protoka, transkutana metoda merenja zasićenošću kiseonikom. Istovremena primena nekoliko neinvazivnih metoda za procenu nivoa cirkulacijskog poremećaja, elastičnosti i starenja kože, omogućilo nam je da pronađemo nove mogućnosti za proučavanje funkcionalnog stanja kože. Ove metode proširuju mogućnosti ultrazvučnih istraživačkih metoda koje se danas koriste u estetskoj kozmetologiji. Dobijeni uporedni podaci elastometrije, ultrasonografije, laserske i dopler tehnike i transkutane oksimetrije kod pacijenata različitih starosnih grupa pokazali su prisustvo elastičnosti i defekt strukture, debljine kože i potkožne masti, kao i promene mikrocirkulacije od 25 godina i značajne promene nakon 40 godina života.

Ključne reči: *koža lica, instrumentalna dijagnostika, starenje, elastometrija, ultrazvuk, laser i Dopler metoda, oksimetrija.*

INTRODUCTION

An increase in the life expectancy of the world's population is accompanied by an elevation in the proportion of people older than 60 years (1, 2), whose number has doubled in the past half century. If in 1991 13.9% of the total population were persons over 65, in 2014 the share of pensioners reached almost 20% (3). The significant aging of the population of economically developed countries raises a legitimate interest in various aspects of gerontology. The first thing is to study the mechanisms of aging. Aging is a complex multicomponent general biological process characterized by metabolic, structural and functional changes in cells and tissues. These processes are

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formed in connection with the depletion of biological resources of the organism. Free radical processes played an important role in this process. Oxidative stress is recognized as the leading mechanism of aging, including the evolving processes of the skin (4-6). These new realities of the modern world are conditioned by new tasks of medicine. One of the tasks is to improve the quality of peoples` life of mature and advanced age, including the preservation and restoration of aesthetic health of the person, in which the condition of the skin system is considered as one of the main components.

Skin is the most largest and visible organ, which is primarily influenced not only by internal, but also by external aggressive factors. Therefore, the skin is the first visual aid of a person's age.

The availability of medicine and the priorities of a healthy lifestyle allow people to remain more active and fulfill social and physiological functions. However meeting with a mirror every year becomes more and more tragic. In this contex, the market of cosmetology services and plastic surgeries is growing exponentially in different segments every year by 2-10% (7, 8). Despite the financial recession of the last decade, the number of people using plastic facial surgery has increased. This trend can also be associated with the spread of today's most popular minimally invasive procedures (for example, injections of botox, collagen, etc.) (9). So, only in the USA in 2016 more than 16 million cosmetic procedures were performed, including minimally invasive procedures, the cost of which exceeded $ 13 billion (10, 11).

The growth of the aesthetic services market is accompanied by a simultaneous increase in equipment for carrying out cosmetic or surgical procedures. However, there are no large studies that allow you to objectively choose methods of treatment are based on the state of the skin system according to diagnostic hardware systems (11-15). Perhaps this is due to the lack of objective criteria for assessing the state of the skin system.

In this regard, the interest of physicians to new modern functional methods of treatment and research is increasing, allowing timely and reliable assessment of the skin condition, as well as the effectiveness of the therapy used. The authors made an attempt to apply diagnostic systems known in general clinical practice to assess the age-related changes in the skin system.

The purpose of the study is to assess by non-invasive methods the degree of skin aging on the parameters of blood flow and skin elasticity in patients of different age groups.

MATERIAL AND METHODS

Study population

Clinical and instrumental examination of 160 women aged 17 to 75 years, divided into 4 age groups of 40 people each (1st group - up to 25 years inclusive, 2 group - 26-35 years, 3rd group - 36-50 years, the 4th group is over 50 years old).

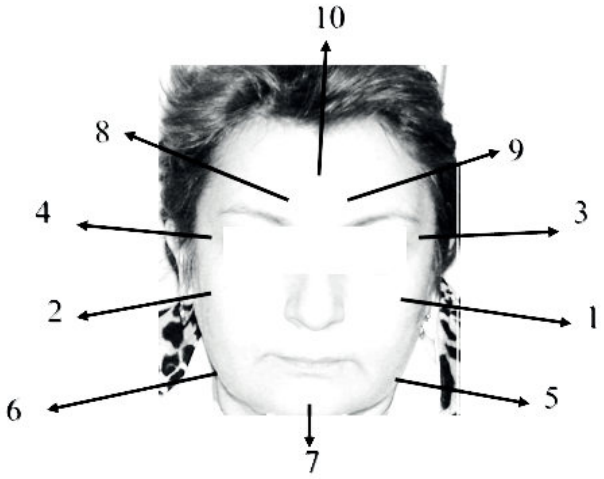

Figure 1. Points on the face for instrumental evaluation of the skin

Methods

To assess the skin condition of all the patients objectively, modern instrumental methods of investigation were used, such as: elastometry, ultrasound examination of the skin of the face, laser doppler flowmetry, transcutaneous oxygen tension.

Measurement of skin elasticity

Measurement of skin elasticity (elasticity) was carried out using the ElastometrR EM 25 device from Courage + Khazaka electronic GmbH (Germany). The degree of elasticity of the skin is determined optically during its suction and is displayed in percentages on the instrument screen and a diode indicator. This measurement was performed around the face at 10 points: cubic (1, 2), orbital (3, 4), mandibular (5, 6), chin (7), and frontal (8-10), as shown in Fig. 1.

To study the structural features of involutional skin changes, ultrasound investigation of the dermis and epidermis was carried out by the method of two-dimensional ultrasound scanning of the skin. Ultrasound investigation (ultrasonography) was performed in 10 standard points of the patient`s face using the LOGIC 1200 apparatus in the "small pats" mode, which allows to reliably estimate the skin type of the patient and identify the most problem areas for more intensive medication.

Measurements of skin microcirculation

Measurements of skin microcirculation by laser doppler flowmetry was performed with using a single-channel BLF-21 laser Doppler flowmeter from Transonic System Inc. (USA) with a surface sensor type "R". Laser doppler flowmetry is based on laser tissue sounding and subsequent recording of radiation reflected from mobile and immobile tissue components. The signal registered with LDF characterizes the blood flow in microvessels in a volume of 1-1.5 cm^3 tissue. The studies were carried out for 15-30 seconds at each of 10 points, after stabilization of the perfusion blood flow parameters. During the study, perfusion

indices of the skin (PS, in conditional perfusion units) and the mean square deviation of the amplitude of the blood flow fluctuations from the arithmetic mean value were calculated.

To directly assess the state of skin metabolic processes, the examination of the state of the tissue (intracutaneous) oxygen tension was made. It was studied by a non-invasive method of transcutaneous oximetry (TO_2) based on the principle of polarographic detection of oxygen in biological objects using the TCM 400 Radiometer device (Denmark).

Statistical analyses

All data are presented by descriptive statistic methods, as mean, median, standard deviations, quartiles, minimum and maximum, as well as frequency of variable in percent (%).

RESULTS

According to the data of elastometry, the heterogeneity of tissues in various areas of the face was revealed. Comparative analysis of elderly patients` skin elasticity in 6 points (3, 4, 7, 8, 9, 10) showed a significant decrease in elasticity indices by 15-25% ($p < 0,05$) (Tab.1).

The best indices of elasticity were registered in group 1 (young patients), especially in the paraorbital region (points 3-4) and in the chin area (point 8). In the second group (26-35 years), the indices decreased reliably: by 13% in the paraorbital region (points 3, 4) and by 10% in the forehead area (points 8, 9, 10). This suggests that these areas are the first problem areas for the wrinkles appearance and need careful attention and care since 25 years. After 35 years (groups 3 and 4), a significant decrease in elasticity was observed at all points. Particularly at this age a deformation type of age-related changes begins to appear. It is ptosis of soft tissues, whose marker is the decrease in elasticity at points 5, 6, in the face oval area. The maximum degree of decrease in elasticity, diagnosed at almost all points, was found in patients over the age of 50 years. By this period, the majority of patients had already formed a deformational type of involutional changes in the skin of the face of the second degree, and in some cases also of the third degree. The results obtained reflect the redistribution of mechanical stress in the examined areas of the facial skin surface.

Consequently, measuring the elasticity of the skin can serve as an objective criterion in the exteriorization of the degree and nature of involuntary changes in the facial skin. In addition, carrying out these survey methods can help in choosing the method of surgical correction in older patients. That is because atrophic skin processes leading to a marked decrease in its elasticity can affect the results of surgical correction, making them less stable in the duration of the effect than in patients of younger age groups.

Analysis of involutional changes in the state of the skin system according to ultrasound parameters revealed that the thickness of the skin and subcutaneous fat depends on the type of skin and body mass index of the patient, directly correlating with the latter. The smallest thickness of the skin, from 0.06 to 0.07 mm, is noted in the paraorbital region, and the largest - in the forehead region, where it is from 0.08 to 0.1 mm.

Table 1. Elasticity of patients` skin in different age groups.

Measurement points	Group 1 (≤25 years)	Group 2 (26-35 years)	Group 3 (36-50 years)	Group 4 (>50 years)
1	34 29 / 39	42 35 / 58	64 55 / 69	68 63 / 78
2	34 28 / 40	38 34 / 50	66 56 / 73	72 62 / 79
3	39 31 / 45	43 35 / 49	66 58 / 70	79 70 / 87
4	38 30/43	39 32 / 46	65 56 / 70	78 71 / 85
5	33 29 / 39	34 30 / 41	67 60 / 75	74 68 / 79
6	34 30 / 40	35 31 / 43	70 62 / 79	73 65 / 81
7	44 34 / 50	48 43 / 57	78 64 / 85	82 78 / 89
8	42 33 / 48	42 37 / 50	68 62 / 77	76 70 / 82
9	42 34 / 45	44 39 / 50	68 60 / 76	74 66 / 79
10	43 35 / 46	46 38 / 53	65 58 / 70	75 68 / 80
The results are presented as Medians (first line) and lower 25% / top 75% quartiles (second line)				

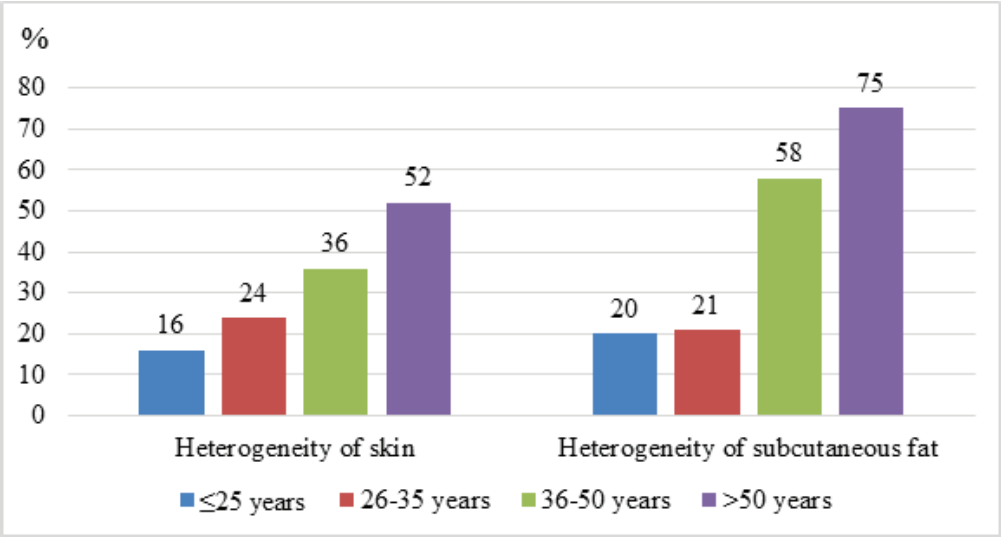

Figure 2. Indicators of the structural organization of the skin (% homogeneity and echogenicity (heterogeneity) of subcutaneous fat in patients of different age (p<0,05).

In the course of the study, a significant decrease in skin thickness was recorded, It had a reverse correlation with age in patients in groups 3 and 4, whose thickness of skin and subcutaneous fat was on average 0.01-0.02 mm less than in more young patients, respectively, the 2nd and 1st group ($p < 0.05$). In addition, there was a progressive thinning of the skin with age and a significant increase in the heterogeneity of the structure of the skin and subcutaneous fat, especially noted in patients after 50 years (Fig. 2).

The significant growth of structural heterogeneity occurring simultaneously with atrophy is caused by different degrees of tissue dismetabolism in different areas of the facial skin. These processes are secondary to different anatomical structures, and thus having different degrees of microcirculatory disorders and different levels of functioning of fibroblasts producing collagen.

According to laser Doppler flowmetry (LDF), it was established that the parameters of skin microcirculation changed significantly with age. However, while comparing border groups, this reliability was not obtained, which later served as the reason for refusing to adopt a generally accepted division into narrow age groups. Thus, the average total LDF (including the total quantitative representation of blood flow in all measured points of the face) in patients under the age of 25 years was 8.0 ± 1.63 ml/min, while in women older than 50 years the value of LDF was 6.5 ± 1.4 ml/min ($p < 0.05$). It was established that the LDF indicators varied in different areas of the face. The highest values of indices of blood supply were noted in the frontal and mandibular regions (points 5, 6, 8-10). This confirms the direct correlation between age and levels of tissue (intradermal) microcirculation.

Quantitative LDF results, which characterize the decrease of microcirculation parameters with aging, correlated with the results of the study of microcirculation parameters for transcutaneous TO_2 oximetry, characterizing the age-induced involution of tissue oxygenation (Tab. 2).

The obtained data confirm the hypothesis about the predominant influence of the microvasculature (blood flow level and oxygen level) on the metabolic processes in the skin. Therefore, the improvement of metabolic processes in cells should be an integral component in the pathophysiologically directed treatment of involutional changes in the skin system. That is because, any tissue ischemia and hypoxemia, including in involutional changes in the skin system, revealed in our studies, lead to the actual mechanisms of skin aging.

DISCUSSION

The purpose of the study is to assess by non-invasive methods the degree of skin aging on the parameters of blood flow and skin elasticity in patients of different age groups. Skin is a multifunctional organ but, alongside every other organ system, is subject to both intrinsic (chronological) and extrinsic (environmental) aging, resulting in a loss of functional capacity. Cutaneous aging manifests as an observable change in the external appearance of the skin, the major accelerator of the aging process being our interactions with our environment, such as chronic exposure to solar irradiation (UV, IR or visible wavelengths of light) (11-14).

Groups	LDF ml / min	TO2 (mm Hg)
I, 18-25 age	8.0 ± 1.63	54 ± 6.5
II-III, 26-50 age	7.4 ± 2.0	45 ± 3.6
IV, over 50 age	6.5± 1.4	37 ± 4.7

Table 2. Indices of tissue oximetry and microcirculation of the facial skin in patients of different age. Results are presented as mean ±standard deviations

Topics of involutional changes in the skin system are basic for cosmetologists and, to a large extent, plastic surgeons. Specialists in these areas, using the concept of age-related skin changes, use mainly subjective scales for assessing skin types and aging. A large number of these scales, actively applied in practice, once again prove the dissatisfaction of specialists with these scales and their subjective assessment. All this does not allow to realize a fully personalized approach while choosing conservative or surgical treatment, and also to objectify the effectiveness of the treatment.

The development of medical technology makes it possible, on the basis of data from instrumental survey methods, to obtain digital indicators for many characteristics of the skin system. The most important of these are the elasticity and density of the skin, as well as its blood supply. In the last few years, there has been increasing demand for aesthetic procedures to improve the effects of skin aging. Previous authors evaluated the anti-aging efficacy, tolerability and skin changes induced by the topical products containing hyaluronic acid, N-acetyl glucosamine and gamma-amino butyric acid through instrumental techniques, clinical and subjective evaluation (12). A clinical assessment of smoothness, expression wrinkles, fine wrinkles and measurements of the parameters using Reveal Imager, X-Rite, Corneometer, Dermalab, Moisture Meter EpiD were taken at day 0, 15, 30 and 60 of study period. A final assessment questionnaire was submitted, and they conclude that the efficacy of the topical products is confirmed by subjective, clinical and instrumental assessment which should be a routine approach in dermatologic practice (12).

The parallel study of several non-invasive methods for assessing the degree of disturbance of blood flow, elasticity and aging of the skin allowed us to find new possibilities for an objective evaluation of the functional state of the skin. The obtained comparative data of elastometry, ultrasound, laser Doppler flowmetry and transcutaneous oximetry in patients of different age groups show age-related disorders of elasticity, structure, and thickness of the skin (7, 8). Iyengar and coauthors investigated the ultrasound as an investigating method, which is relatively deeply penetrating and can be used to evaluate deep dermal and subcutaneous structures (13). They concluded that ultrasound is an inexpensive, noninvasive, and convenient means to monitor dermatologic conditions and guide their treatment (13).

On the other hand, with age, there is a decrease in elasticity, the heterogeneity of the skin and subcutaneous fat is progressing, by means of reducing the collagen content in it, and also reducing the blood supply to the skin system. The first involutional changes are recorded in the areas of the thinnest skin (paraorbital regions). On such areas it is already necessary to pay attention from the age of 25 with the conduct of adequate cosmetic procedures.

After 40-50 years, especially in the menopausal and postmenopausal period of women, changes in skin indices begin to be expressed, a deformational type of involutional changes is formed, ptosis, the oval of the face is deformed. Kimball and colleagues demonstrates a wide range of molecular processes in skin affected by aging, providing relevant targets for improving the condition of aging skin at different life stages and defining a molecular pattern of epidermal gene expression in women who appear younger than their chronologic age (18). On the other hand, men are a growing patient population in aesthetic medicine and are increasingly seeking minimally invasive cosmetic procedures. Findings from study Rossi and coauthors conducted in a globally diverse sample may guide clinical discussions with men about the prevention and treatment of signs of facial aging, to help men of all races/ethnicities achieve their desired aesthetic outcomes (19).

Therefore, in order to preserve aesthetic youth, patients of older age groups should be under the constant supervision of specialists, including a plastic surgeon. Newton and coauthors described that cutaneous aging manifests as an observable change in the external appearance of the skin, the major accelerator of the aging process being our interactions with our environment, such as chronic exposure to solar irradiation (UV, IR or visible wavelengths of light) (15). Longo and coworkers suggested that a precise and noninvasive quantification of aging is of outmost importance for *in vivo* assessment of the skin aging "stage", and thus acts to minimize it. Several bioengineering methods have been proposed to objectively, precisely, and non-invasively measure skin aging, and to detect early skin damage, that is sub-clinically observable (16). Other authors, but suggested different methods for diagnosis skin aging (17). The Skin Ageing Index was defined as the normalized projection of the clinical grading values on the first Principal Component Analysis (PCA) axis. Several Skin Indexes were built by grouping specific parameters related to a skin condition such as overall ageing, wrinkles and sagging. All indexes were highly correlated with the real and the perceived age ($0.57 \leq$ Pearson $R \leq 0.92$, P-value ≤ 0.05) (17).

First Consensus on Primary Prevention and Early Intervention in Aesthetic Medicine published guidelines regarding to facial aging as an complex interplay of extrinsic and intrinsic factors leading to progressive changes in the skin, subcutaneous tissue, and bone. Clinical experience suggests that early aesthetic intervention may slow the signs of aging, but treatment in the absence of symptoms or with minimal signs of aging has not yet been properly addressed (20, 21). Preventive measures and early therapeutic interventions that may alter the course of facial aging were defined. Further studies are needed to support these recommendations with the best possible evidence.

CONCLUSION

The work carried out by us demonstrates the possibilities of non-invasive instrumental diagnostic methods used in modern aesthetic cosmetology and plastic surgery. These methods should be used not only at the stages of ascertaining the condition of the skin system, but also for choosing a method of treatment and monitoring the effectiveness of conservative and surgical methods of correcting age-related changes.

It is important to note that even with the best modern plastic methods of treatment, the blood supply function is paramount, ensuring an adequate recovery of the dermal cell pool and eliminating the mechanisms of apoptosis. In this connection, physiotherapeutic and pharmacological methods, which contribute to the expansion of the microcirculatory channel on the face, can be useful. Such complex therapy will help to achieve the best result of treatment of involutional skin changes.

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TGF-B AS A MARKER OF ULCERATIVE COLITIS AND DISEASE SEVERITY

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TGF-B KAO MARKER ULCEROZNOG KOLITISA I TEŽINE BOLESTI

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ABSTRACT

Ulcerative colitis (UC) represents chronic inflammation of the large intestine. Immune response plays an important role in disease genesis and progression. Activated leukocytes secrete several cytokines that actively regulate the inflammatory response in UC. The aim of this study was to determine levels of cytokines IL-17, IL-27, IFN-γ and TGF-β in patients with UC and to test them as biomarkers for disease.

The blood samples of 24 patients with ulcerative colitis without previous treatment and 37 healthy individuals were analyzed. Serum levels of IL-17, IL-27, IFN-γ and TGF-β were measured using sensitive enzyme-linked immunosorbent assay (ELISA) kits.

Serum levels of IL-17, IL-27, IFN-γ and TGF-β were increased in patients with UC, compared to healthy controls (p=0.022; p=0.001; p=0.001; and p=0.002; respectively). Ratios of cytokines IL-27/IL-17, IFN-γ/TGF-β and IL-17/TGF-β were significantly higher in group of patients with UC (p=0.002; p=0.002; p=0.003; respectively). Serum value of TGF-β higher than 20 pg/ml presents a highly sensitive and specific marker for UC.

We believe that increased production and predominance of immunosupressive TGF-β may represent compensatory mechanism for ongoing pro-inflammatory processes in UC.

Keywords: *Ulcerative colitis, IL-17, IL-27, IFN-γ and TGF-β*

SAŽETAK

Ulcerozni kolitis je hronično inflamacijsko oboljenje debelog creva. Imunski odgovor igra važnu ulogu u nastanku i progresiji bolesti. Aktivirani leukociti sekretuju brojne citokine koji modulišu inflamacijski odgovor u ulceroznom kolitisu. Cilj ove studije je određivanje nivoa citokina IL-17, IL-27, IFN-γ i TGF-β i njihove specifičnosti i senzitivnosti kao biomarkera bolesti kod pacijenata sa ulceroznim kolitisom.

Analizirani su uzorci krvi 24 pacijenata obolelih od ulceroznog kolitisa i 37 zdravih kontrola. Serumski nivoi IL-17, IL-27, IFN-γ i TGF-β su mereni ELISA testom.

Serumski nivoi IL-17, IL-27, IFN-γ i TGF-β su povišeni kod pacijenata obolelih od ulceroznog kolitisa u poređenju sa kontrolnom grupom (p=0.022; p=0.001; p=0.001; p=0.002). Odnos IL-27/IL-17, IFN-γ/TGF-β i IL-17/TGF-β je značajno veći u grupi pacijenata sa ulceroznim kolitisom (p=0.002; p=0.002; p=0.003). Serumska vrednost TGF-β veća od 20 pg/ml predstavlja visoko senzitivan i specifičan marker za potvrdu ulceroznog kolitisa.

Smatramo da povećana produkcija imunosupresivnog TGF-β može predstavljati mehanizam kompenzovanja proinflamacijskog procesa u ulceroznom kolitisu.

Ključne reči: *Ulcerozni kolitis, IL-17, IL-27, IFN-γ i TGF-β*

INTRODUCTION

Inflammatory bowel disease (IBD) represents a group of chronic disorders. It includes Crohn's disease (CD) and ulcerative colitis (UC). Main characteristics of disease are chronic inflammation of the gastrointestinal tract and relapsing and remitting clinical course (1). Ulcerative colitis (UC) represents chronic inflammation of the large intestine still with unknown etiology. The disease genesis involves the breakdown of intestinal mucosal homeostasis and subsequent pathological communication between commensal microflora and local immune system which is followed by inadequate immune response (2). Immune response plays a critical role during disease progression. Activated leukocytes secrete several cytokines that actively regulate the inflammatory response in UC (1).

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Cytokines represent a group of various small proteins, secreted by cells and playing crucial role in intercellular signaling and communication. They are involved in various biological process such as embryonic development, disease pathogenesis, non-specific response to infection, specific response to antigen, changes in cognitive functions and progression of the degenerative processes of aging, stem cell differentiation, vaccine efficacy and allograft rejection (3, 4).

IFN-γ is an immunoregulatory cytokine involved in the regulation of many phases of the immune and inflammatory responses, including the activation and differentiation of various leukocytes (5). Cellular effects of IFN-γ include up-regulation of pathogen recognition, antigen processing and presentation, the antiviral state, inhibition of cellular proliferation and effects on apoptosis, activation of microbicidal effector functions, immunomodulation and leukocyte trafficking. $CD8^+$ T cells, $CD4^+$ Th cells and NK cells represent main sources of IFN-γ (6). IFN-γ secretion is a hallmark of Th1 lymphocytes and facilitates type 1 immune response, critical for defense against certain pathogen, such as intracellular bacteria, viruses and fungi (5, 6).

The interleukin-17 (IL-17) family consists of a subset of cytokines. The most widely investigated member of this family is IL-17A. Beside Th17 cells, a major source of this cytokine, innate immune cell also produce IL-17A, in response to pathogens or tissue injury (7-9). The interleukin-17 family participates in both acute and chronic inflammatory responses (10-19). IL-17 elicits protection against extracellular bacterial and fungal infections and plays important roles in biology of various inflammatory conditions such as autoimmune diseases, metabolic disorders, and cancer (10-19).

IL-27 is a member of a family of cytokines, which also includes IL-12, IL-23, and IL-35 (20, 21). IL-27 is mainly produced by cells of myeloid origin such as monocytes, macrophages, dendritic cells, and microglial cells, in response to stimuli acting through Toll-like receptors or TNFR-family members (22, 23). It is produced during the innate phase of the immune response and regulates the quality and size of the adaptive immune response (24, 25).

Transforming Growth Factor β (TGF-β) is a powerful pleiotropic cytokine, with predominantly immune-suppressing and anti-inflammatory properties. Under physiological conditions, TGF-β has a role in embryogenesis, cell proliferation, differentiation, apoptosis, adhesion, and invasion (26, 27).

The aim of present study was to assess the serum level of cytokines IL-17, IL-27, IFN-γ and TGF-β in patients with UC and to test them as biomarkers for disease. The results showed increased systemic values of cytokines IL-17, IL-27, IFN-γ and TGF-β and revealed TGF-β highly sensitive and specific marker for UC.

MATERIALS AND METHODS

Ethical approvals

The study was conducted at the Center for Gastroenterology, University Medical Center Kragujevac, Serbia and the Center for Molecular Medicine and Stem Cell Research, Faculty of Medical Sciences, University of Kragujevac, Serbia. Ethical approvals were obtained from relevant Ethics Committees of Faculty of Medical Sciences, University of Kragujevac, Serbia and University Medical Center, Kragujevac, Serbia. All research procedures were made to the Principle of Good Clinical Practice and the Declaration of Helsinki at all times.

Patients

The study included total of 24 patients with ulcerative colitis (13 males and 11 females; mean age: 45.74 ± 19.00 years). Ulcerative colitis was diagnosed on the basis of endoscopic and histopathological criteria. The study did not involve patients with ulcerative colitis who were previously treated with antibiotics, aminosalicylates, corticosteroids, immunosuppressive agents, and biological therapy. All subjects had a complete medical history, including physical examination, routine laboratory tests and diagnostic imaging (chest X-ray, abdominal ultrasound, abdominal computed tomography scan and endoscopy). Control subjects were selected from volunteer blood donors at the University Medical Center Kragujevac. A control group of 37 healthy individuals was matched with the experimental group based on gender.

Measurement of cytokines in the serum

The blood samples of all patients were collected before any application of therapy. Blood samples were collected from each studied subject. Blood clot was cut and centrifuged for serum separation. All serum samples were stored at -20°C until the time of testing. Repeated freeze–thaw cycles were avoided to prevent loss of bioactive substances. Serum levels of cytokines were measured as described before (28), using sensitive enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems Minneapolis, MN, USA for IL-17, IL-27, IFN-γ and TGF-β), specific for human cytokines, according to the manufacturer's instructions. The optical density of each well at 450 nm was detected by an ELISA microplate reader (Zenyth, 3100).

Statistical analysis

The statistical analyses were performed using SPSS 20.0 software. Data are grouped and shown in graphs. The normality of distribution was tested by Kolmogorov-Smirnov test. Statistical differences between the means for the different groups were evaluated using either the two-tailed Student's t test or nonparametric Mann-Whitney U test where appropriate. ROC curve was used for the review of sensitivity and specificity. All reported P values were 2-sided and the results were considered significantly different when $p < 0.05$ and highly significantly different when $p < 0.01$.

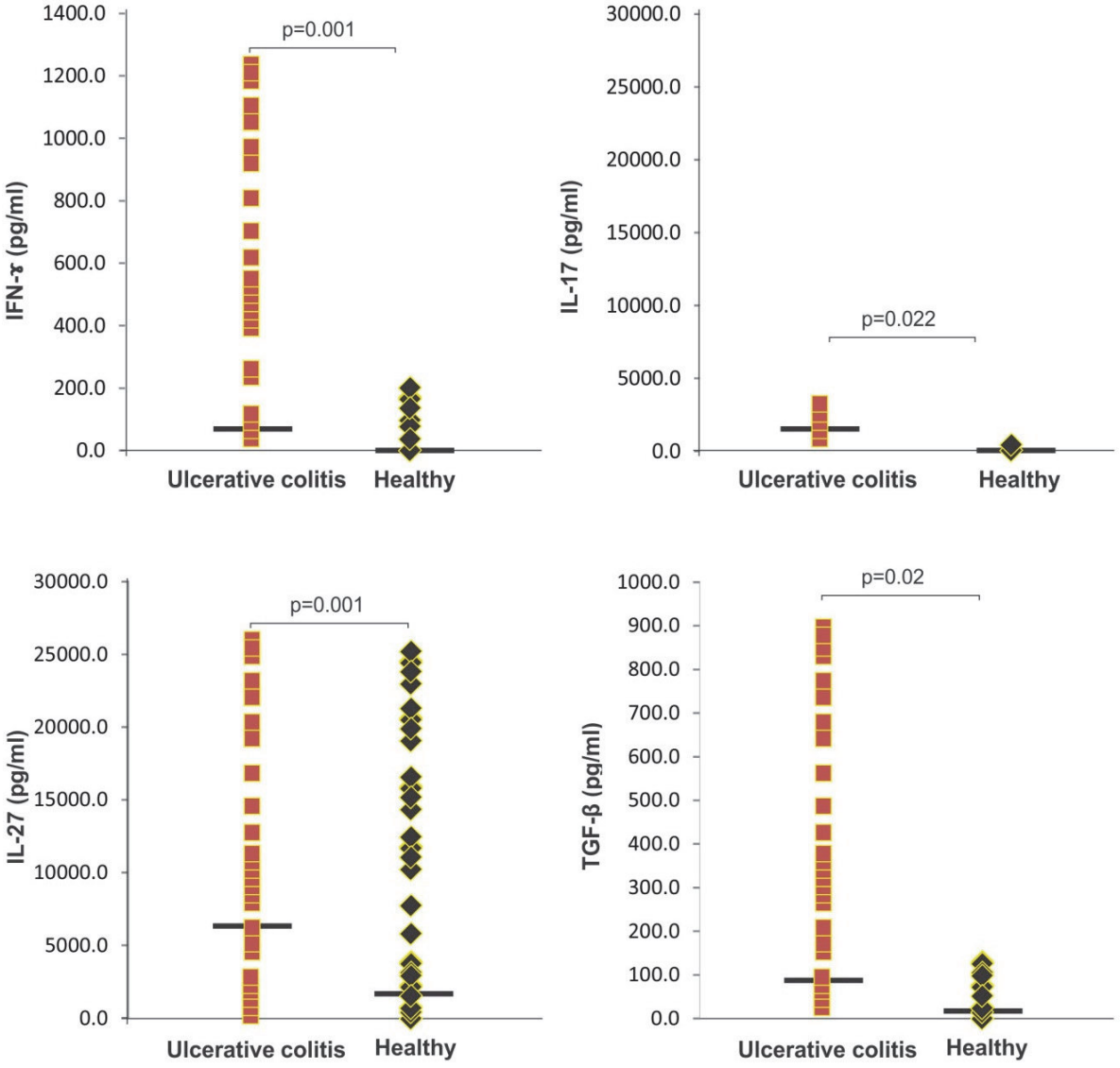

Figure 1. Serum values of IL-17, IL-27, IFN-γ and TGF-β in patients with UC. Increased concentration of IL-17, IL-27, IFN-γ and TGF-β in patients with UC in comparison to healthy control. Serum levels of all mentioned cytokines were determined by ELISA. Statistical significance was tested by

RESULTS

Sera of patients with UC contain higher levels of IL-17, IL-27, IFN-γ and TGF-β

We have compared serum concentrations of cytokines in groups of patients with UC and healthy controls. Serum levels of IL-17, IL-27, IFN-γ and TGF-β were significantly increased in patients with UC in comparison to healthy controls (IL-17: 32,20 (0,00-4021,30) vs. 0,00 (0,00-316,50) pg/ml, p=0.022; IL-27: 6332,60 (914,00-71038,50) vs. 1832,68 (0,00-26094,90) pg/ml, p=0.001; IFN-γ: 71,70 (7,30-1274,90) vs. 0,00 (0,00-219,60) pg/ml, p=0.001; TGF-β: 92,20 (4,93-933,80) vs. 17,70 (0,00-110,60) pg/ml, p=0.002; Figure 1).

IL-27/IL-17, IFN-γ/TGF-β and IL-17/TGF-β ratios were altered in patients with UC

It has been suggested that ratio of counterregulatory cytokines is a relevant marker of the disease process. Therefore, we considered ratios of pro- and anti-inflammatory cytokines studied. The significant difference was found for IL-27/IL-17, IFN-γ/TGF-β and IL-17/TGF-β ratio. IL-27/IL-17, IFN-γ/TGF-β and IL-17/TGF-β ratio was increased in patients with UC, comparing to healthy volunteers (IL-27/IL-17: 116,67 (0,00-679,69) vs. 0,00 (0,00-5895,44), p=0.002; IFN-γ/TGF-β: 0,82 (0,03-16,77) vs. 0,00 (0,00-3,76), p=0.002; IL-17/TGF-β: 0,47 (0,00-34,89) vs. 0,00 (0,00-21.65), p=0.003; Figure 2).

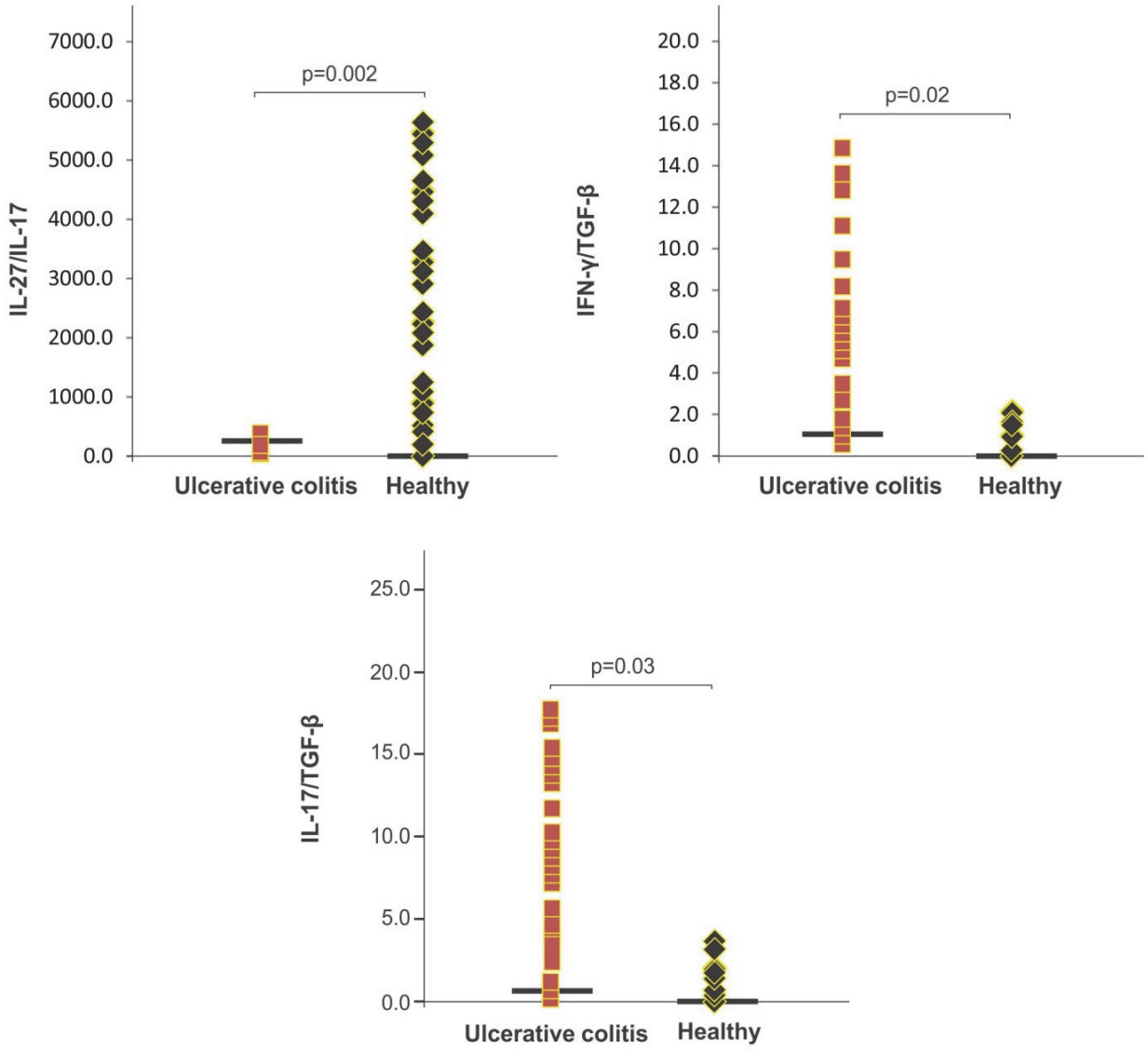

Figure 2. IL-27/IL-17, IFN-γ/TGF-β and IL-17/TGF-β ratios in patients with UC. Increased IL-27/IL-17, IFN-γ/TGF-β and IL-17/TGF-β ratios in patients with UC in comparison to healthy control. Serum levels of all mentioned cytokines were determined by ELISA. Statistical significance was tested by Mann–Whitney Rank Sum test or independent samples t-test, where appropriate.

Logistic regression analyses of IL-17, IL-27, IFN-γ and TGF-β serum level in patients with UC and healthy controls

Binary logistic regression showed that higher serum levels of IL-17, IL-27, IFN-γ and TGF-β strongly correlated with presence of UC (IL-17: sensitivity 85,2%, specificity 71,4%, cut-off=20 pg/ml; p=0.032; IL-27: sensitivity 74,1%, specificity 80,0%, cut-off=1000 pg/ml; p=0.006; IFN-γ: sensitivity 100,0%, specificity 68,6%, cut-off=53 pg/ml; p=0.002; TGF-β: sensitivity 85,2%, specificity 88,6%, cut-off=20 pg/ml; p=0.001; Figure 3). TGF-β can be a valuable marker for distinguishing patients with UC from healthy control. The optimal cutoff value estimated for TGF-β that allows the discrimination was 20 pg/ml.

Serum concentration of TGF-β was altered in UC patients with different disease severity

Patients with UC were categorized in two groups based on presence of extraintestinal manifestations (dermatologic manifestations- pyoderma gangrenosum or erythema nodosum) (29). We analyzed serum level of TGF-β in defined groups. Serum concentration of TGF-β was higher in group of patients with notable extraintestinal manifestations, although this difference did not reach statistical significance (TGF-β: 113,66 (4,39-157,81) vs. 78,08 (11,62-323,41) pg/ml; Figure 4). Further, we divided patients in another two categories: patients with detectable and patients without detectable local complications (stricture and pseudopolyposis) (30, 31) and analyzed them for systemic value of TGF-β. We found increment of TGF-β in pres-

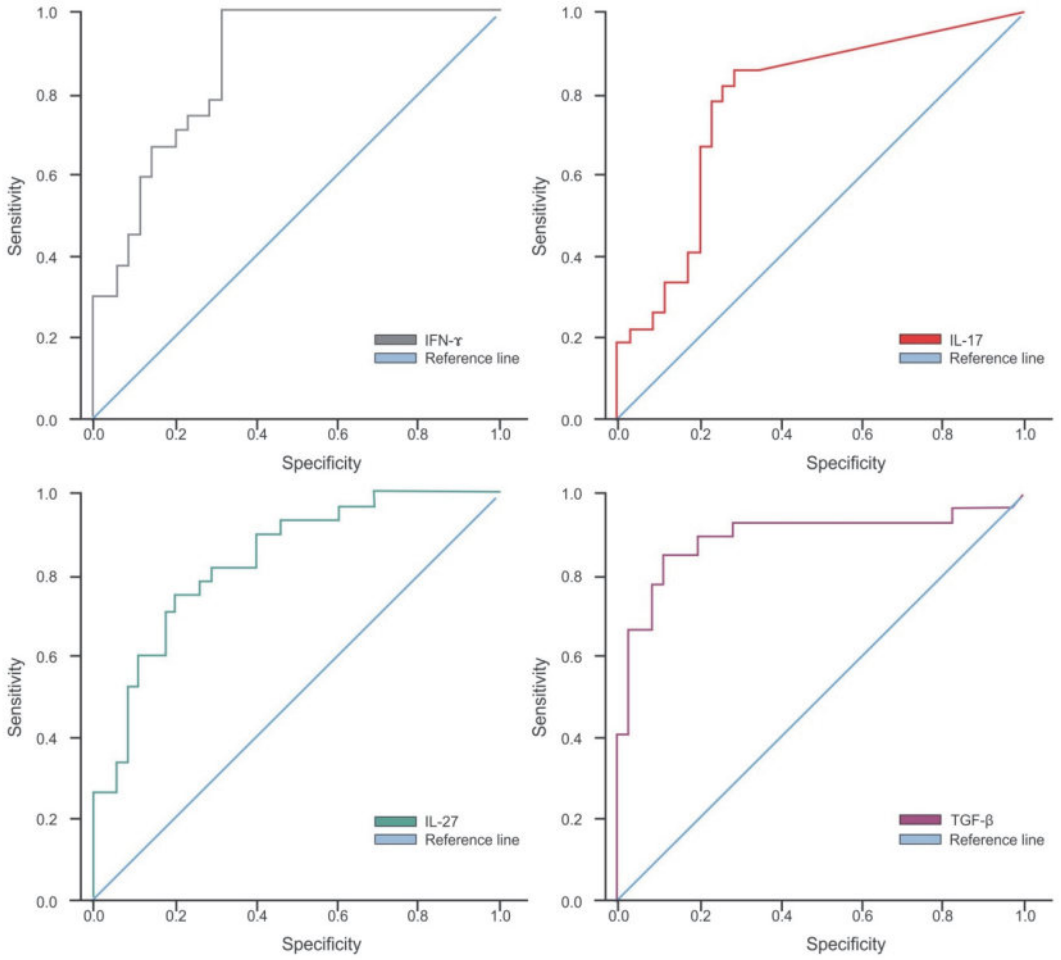

Figure 3. State markers for UC. ROC curves illustrate the specificity and sensitivity of IL-17, IL-27, IFN-γ and TGF-β serum levels in attempt to differentiate control subject from patients with UC.

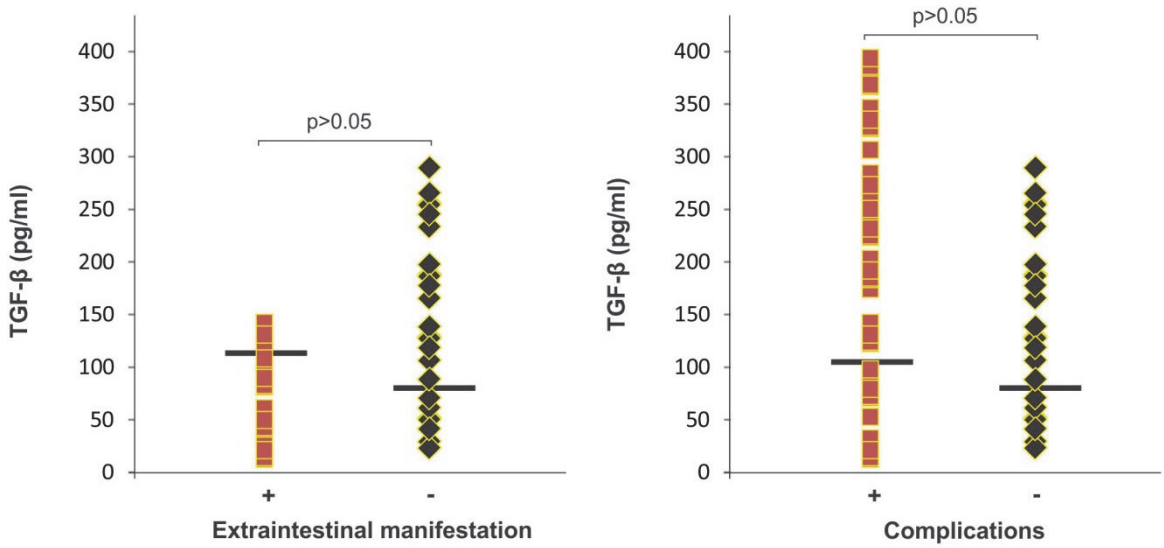

Figure 4. Serum value of TGF-β in patients with UC, based on disease severity.
Left panel. Patients with UC were divided in two groups, based on presence of extraintestinal manifestations. Increased concentration of TGF-β in UC patients with extraintestinal manifestations. **Right panel.** Patients with UC were selected in two groups, based on local complications. Increased concentration of TGF-β in sera of patients with UC with presence of local complications. Serum levels of cytokine were determined by ELISA. Statistical significance was tested by Mann–Whitney Rank Sum test or independent samples t-test, where appropriate.

ence of local complications (TGF-β: 103,89 (4,39-406,66) vs. 78,08 (11,62-323,41) pg/ml; did not reveal statistically significant difference; Figure 4).

DISCUSSION

In the present study we showed increased systemic values of cytokines IL-17, IL-27, IFN-γ and TGF-β in patients with UC in comparison to healthy controls (Figure 1). Patients with UC had significantly higher IL-27/IL-17, IFN-γ/TGF-β and IL-17/TGF-β ratios compared to healthy volunteers (Figure 2). Furthermore, the serum values of TGF-β higher than 20 pg/ml present a highly sensitive and specific marker for UC (Figure 3).

We found significant elevation in levels of IL-17, IL-27, IFN-γ and TGF-β in patients with UC in comparison to healthy volunteers, indicating on robust immune response (Figure 1). Previous studies revealed strong positive correlation between increased synthesis of IFN-γ and disease severity, in UC patients (32). The number of lamina propria IFN-γ positive cells was increased in UC patients compared with controls, but not as remarkably as in CD (33). Increment of IL-17 leads to the induction of many pro-inflammatory mediators, such as TNF-α, IL-6, IL-1β and IFN-γ, indicating on important role of IL-17 in facilitating inflammation (34-36). Although several studies point on protective role of IL-17, by inhibiting Th1 polarization and subsequent IFN-γ dependent inflammation, majority lines of evidence suggest that IL-17 contribute to intestinal inflammation (34-37). IL-27 mediated Th1 polarization and IFN-γ production in naive $CD4^+$ T cells, acting in cooperation with other pro-inflammatory cytokines (38, 39). The fact that both cytokines share STAT1 signaling pathway explains functional overlaps between IL-27 and IFN-γ (38, 39). TGF-β, as an inhibitory cytokine, represents a key regulator of immunological homeostasis and inflammation in UC (40-42). While reduced TGF-β activity is considered to be responsible for the development of autoimmune disorders including UC, increased TGF-β is considered as compensatory mechanism by promoting potent immunosuppressive effect (40-42). Increment of systemic IL-17, IL-27 and IFN-γ induce differentiation and stabilization of polarized Th1 immune response, which is hallmark of Crohn`s disease (1). In majority of studies, in UC, the local immune response is less Th1 polarized. Consistent with these data, the cytokines driving UC were identified with Th2-like characteristics (1). More recently, several studies have shown that levels of pro-inflammatory cytokines including IL-17 were increased in UC (1, 43). Still, this increment was found to be far less than in CD (1, 43).

In line with previous finding, we revealed higher IL-27/IL-17, IFN-γ/TGF-β and IL-17/TGF-β ratios in patients with UC (Figure 2). These finding proves that disease progression correlates with pro-inflammatory immune response, developing toward Th1/Th17 direction. In addition, IFN-γ/TGF-β and IL-17/TGF-β ratios are less than 1 (0,82 and 0,47; respectively; Figure 2), indicating on predominance of immunosuppressive TGF-β above pro-inflammatory IFN-γ and IL-17 in patients with UC. Our results implicate robust immunosupressive response through TGF-β production, in patients with UC. TGF-β concentration in serum of UC patients was elevated, comparing to healthy controls. In line with our results, it was previously noticed that TGF-β serum level was significantly increased in patients with UC, compared with control subjects (40-42). The main sources of TGF-β are mieloid derived supressor cells (MDSCs) and regulatory T cells (Tregs), and it has been noticed that patients with UC display higher number of MDSCs and Tregs in local tissue (44, 45). They are largely responsible for inhibiting host T-cell activity, however, the roles of MDSCs and Tregs in UC are not yet well understood, and there are controversies regarding their immunosuppressive functions (44, 45).

We also envisage the possible role of IL-17, IL-27, IFN-γ and TGF-β as biomarkers of UC. According to our results, TGF-β could be a valuable marker for distinguishing patients with UC from healthy controls, since the level of TGF-β enhanced the risk for UC (Figure 3). We found that optimal cutoff value estimated for TGF-β that allows discrimination form a healthy patients was 20 pg/ml, with 85,2% sensitivity and 88,6% specificity.

The role of TGF-β in UC varies by cell type but also by stage of disease. We analyzed serum level of TGF-β in groups of patients with UC categorized on disease severity: extraintestinal manifestations and local complications (29-31). Serum concentration of TGF-β was increased in patients with severe UC, although this difference did not reach statistical significance (Figure 4). Our previous findings suggest that in UC patients, enhanced systemic pro-inflammatory immune response correlates with disease severity (46). Taken together, during disease progression, robust tissue destruction is associated with strong pro-inflammatory immune response, and increased TGF-β production could be compensatory feedback mechanism.

In conclusion, our data revealed increased systemic values of cytokines IL-17, IL-27, IFN-γ and TGF-β as well as higher IL-27/IL-17, IFN-γ/TGF-β and IL-17/TGF-β ratios in patients with UC in comparison to healthy controls. We believe that enhanced production of pro-inflammatory cytokines IL-17, IL-27, IFN-γ could further facilitate disease progression. In addition, IFN-γ/TGF-β and IL-17/TGF-β ratios less than 1 indicate on predominance of immunosuppressive TGF-β in patients with UC. This may be way to counteract or limit ongoing pro-inflammatory processes and prevent further tissue destruction. Furthermore, the serum values of TGF-β can be used as a valuable marker for UC.

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IS THERE A RELATIONSHIP BETWEEN AUDIOGRAM SHAPE AND THE INTENSITY AND DURATION OF TINNITUS?

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DA LI POSTOJI VEZA IZMEĐU IZGLEDA AUDIOGRAMA I JAČINE I DUŽINE TINITUSA

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ABSTRACT

Chronic tinnitus is often associated with hearing impairment, but it cannot be asserted that only hearing loss causes tinnitus. Audiograms of patients with tinnitus show that hearing loss occurred more often at high frequencies than at low frequencies.

The aim of this study was to analyse the audiogram shapes of patients with chronic tinnitus and to identify the relationship between the shape of the audiogram and intensity and duration of tinnitus.

This investigation was a cross case series study conducted at a general hospital in Kraljevo on patients with chronic subjective tinnitus. The study included 43 patients of both genders and of different ages. We used audiometry (measuring the threshold of hearing for frequencies from 250, 500, 1000, 2000, 4000 and 8000 Hz) and tympanometry. Each patient reported the intensity of tinnitus in each ear on a visual analogue scale (VAS[1]) and stated the duration of tinnitus for each ear.

Our research showed that patients with chronic tinnitus had a characteristic audiogram with progressive hearing loss to high frequencies. This difference was significantly increased starting from lower to higher frequencies, and the most hearing-decreased range ("edge") was between 2000 and 4000 Hz. We did not find a strong link between the tinnitus intensity measured by the visual analogue scale and tinnitus duration on one side and hearing loss in the studied patients and audiogram shape on the other side. The duration of tinnitus was most associated with hearing loss at 2000 Hertz, but even that was not significant.

Keywords: *tinnitus, audiogram, visual analogue scale*

SAŽETAK

Hronični tinitus je često udružen sa oštećenjem sluha ali se se ne može tvrditi da samo oštećenje sluha uzrokuje zujanje u ušima. Na osnovu audiograma pacijenata sa tinitusom, češće se uočava oštećenje sluha za visoke nego za niske frekvencije.

Cilj ove studije je bio analiziranje izgleda audiograma pacijenata sa hroničnim tinitusom i ispitivanje povezanosti između izgleda audiograma i intenziteta i trajanja tinitusa.

Studija praćenja, rađena u Opštoj bolnici u Kraljevu kod pacijenata sa hroničnim subjektivnim tinitusom. U istraživanje je uključeno 43 pacijenta oba pola različite životne dobi (meren je prag sluha na oba uva na frekfencijama 250, 500, 1000, 2000, 4000 i 8000 Hz). Koristili smo audiomerijsko testiranje i timpanometriju. Pacijenti su se izjašnjavali o jačini tinitusa za svako uvo na vizuelno analognoj skali, kao i o dužini tinitusa za svako uvo.

Rezultati našeg istraživanja su pokazali da kod pacijenata sa hroničnim tinitusom postoji karaketrističan audiogram sa progresivnim oštećenjem sluha idući ka visokim frekfencijama sa najvećim padom u opsegu između 2000 i 4000 Hz. Ta razlika se statistički povećava idući od nižih ka višim frekfencijama. Nismo našli jaku vezu između jačine tinitusa merenog vizuelno analognom skalom i dužine tinitusa sa jedne strane i ukupnog gubitka sluha kod ispitivanih pacijenata i izgleda audiograma sa druge strane. Dužina tinitusa najviše je povezana sa gubitkom sluha na 2000 herca ali ni ona nije značajna

Ključne reči: *tinitus, audiogram, vizuelno analogna skala*

[1] VAS/ visual analogue scale

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INTRODUCTION

Tinnitus is defined as a phantom sound perception, i.e., the perception of sound without appropriate acoustic or mechanical correlations in the cochlea (1). The American Tinnitus Association estimates that approximately 50 million Americans suffer from tinnitus and that 12 million people have sought medical help because of tinnitus (2). Approximately 15% of people experience tinnitus, and in persons over 65 years of age, the incidence of tinnitus reaches 30% (3). Within approximately 6 to 25% of affected persons, tinnitus causes a considerable amount of stress (4-6), which leads to a seriously impaired quality of life in approximately 2-4% of the population (7). Tinnitus can interfere with sleep, concentration, social contact and work (8). Persons with tinnitus have an increased prevalence rate of anxiety and depression (9-10). Many experts differ regarding subjective and objective tinnitus (11). Objective tinnitus is created in the body and can be spread to the ear. It is mostly generated in the vascular system and can be heard by another person. An individual with subjective tinnitus hears the sound although an external source of sound is absent. Subjective tinnitus is much more common and reflects some insufficiently known disorders of the auditory pathway. It can be of a different intensity and tonality, permanent or intermittent. The aetiology and pathogenesis are unclear. Tinnitus is accompanied by hearing loss, but not always. Hearing loss in aging is sometimes accompanied by tinnitus. Tinnitus is the first symptom of an acoustic neurinoma and persists after removal of the tumour. Numerous pharmacological agents, such as salicylates, diuretics, quinine, indomethacin, antidepressants, antihistamines, beta-adrenergic blocking agents, local anaesthetics, and corticosteroids, can cause tinnitus. There is often a vitamin B12 deficiency in patients with tinnitus (13). The mechanism of how these agents cause tinnitus is not well understood. In most patients, tinnitus is accompanied by hearing loss. However, contrary to expectations, tinnitus is not associated with total hearing loss but with the appearance of the audiogram (14,15). Recent studies indicate that hearing loss at high frequencies and a steeply sloping audiogram with reduced plasticity of the brain cause a phantom sound. A similar mechanism causes phantom pain. In the second part, we wanted to determine whether the intensity and duration of tinnitus correlated with the appearance of the audiogram. There are additional scales, mainly in English, that classify the intensity and severity of tinnitus, but there are no validated scales in the Serbian language that estimate the severity of tinnitus; therefore, we used a visual analogue scale and the tinnitus duration for each ear individually. We assumed that there is a link between these factors and the appearance of the audiogram. We have not found similar research in the literature. The existence of these links would be useful for further research on the deficiencies of domestic scales. Therefore, the aim of this study was to determine whether there is a specific audiogram for persons with chronic subjective tinnitus, and if so, whether there is a relationship to the tinnitus duration and intensity measured on the visual analogue scale.

MATERIALS AND METHODS

This case series study, which was conducted on patients who appeared to have tinnitus in the ENT department of the general hospital in Kraljevo. The study was approved by the ethics committee of the general hospital (20-2/5-a, 28.9.2015.), and informed consent was obtained from all subjects. The study included only patients with subjective tinnitus and patients with tinnitus longer than six months, bilateral or unilateral. The study excluded patients with acoustic neurinomas and Meniere's disease as well as patients with objective or pulsatile tinnitus. The patients underwent audiometry (Amplaid A321 Twin channel) and certain thresholds of hearing for frequencies from 250 to 8000 Hertz, and the level of hearing loss in each ear and the total hearing loss were determined using Fowler-Sabin (FS) tables and tympanometry (Amplaid Tympanometer A756 Screening). All of the patients used a visual analogue scale (VAS) from 1 to 10 to report the severity of tinnitus for each ear; the visual analogue scale correlates with the tinnitus handicap index THI scores (16). We measured the tinnitus duration in each ear; every patient stated the continuance of tinnitus for each ear.

STATISTICS

The study used the following statistical methods: the independent samples test and Mann-Whitney test, the correlation method and the regression model. We compared whether there was a statistically significant difference in hearing loss between adjacent frequencies in each ear (250 Hz and 500 Hz, 500 Hz and 1000 Hz, 1000 Hz and 2000 Hz, 2000 Hz and 4000 Hz, 4000 Hz and 8000 Hz). The second part showed that there was a link between the intensity of subjective tinnitus measured by the visual analogue scale (VAS) and the audiogram shape and hearing loss in the same ear (FS[2] table) as well as whether there was a link between the tinnitus duration and audiogram shape and hearing loss in this ear (FS table). We used the method of correlation analysis.

RESULTS

Forty-three patients participated in the study. There were 23 male patients and 20 female patients. The average age (mean) of the patients was 63 years; the standard deviation (SD) was 11,163. The tympanogram was normal in all the patients (Type A). The audiogram indicated sensorineural hearing loss (41 patients) or was normal hearing (2 patients). The values of the hearing thresholds at different

[2] Fowler Sabine

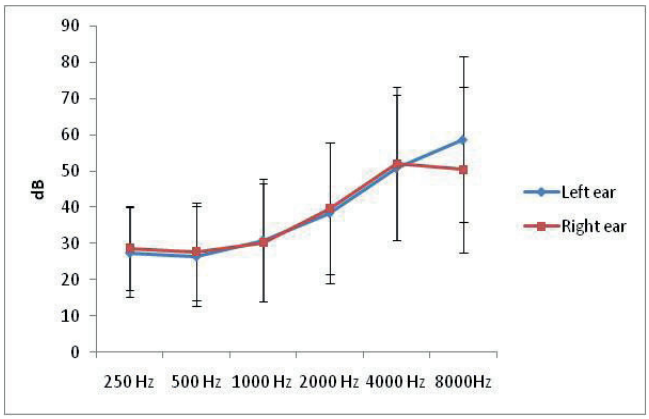


Figure 1. Values of hearing loss at different frequencies in the left and right ear. Each value is presented as the mean±SD. (* p<0,05)

frequencies for the left and right ear are shown in Figure 1. Each value is presented as the mean±SD.

The average hearing loss[3] in both ears of a given patient (43) was 20.68%. The average hearing loss in the right ear was 22.68% and in the left ear was 25.88%. The composed audiogram of hearing loss in the left and right ear is shown in Picture 1.

We compared the hearing threshold between the two neighbouring frequencies groups (43 thresholds per group) on the audiogram for each ear separately using the independent samples test and the Mann-Whitney test. The results of the statistical study are shown in Table 1).

The average values of the tinnitus intensity (VAS) and the tinnitus duration (years) for each ear are listed in Table 2).

The coefficient of correlation linking the hearing loss and its corresponding frequency for the left ear was 0,963 and for the right ear was 0,958. The regression curve for the left ear was y = 27,587 + 0,04x and for the right ear was y = 28,289 + 0,004x.

[3] Calculated by Fowler Sabine tab.

Table 1. Statistical values for comparing the hearing threshold between the two neighbouring frequencies for the left and right ear, separately. p<0.005, probability of the null hypothesis for the difference between frequency groups, MW - Mann-Whitney test

Independent samples test and Mann-Whitney test					
frequency (Hz)	250-500	500-1000	1000-2000	2000-4000	4000-8000
p[§] for the left ear	0,817	0,23	0,087	0,03	0,119[2MW]
p for the right ear	0,89	0,57	0,007	0,006[MW]	0,068[MW]

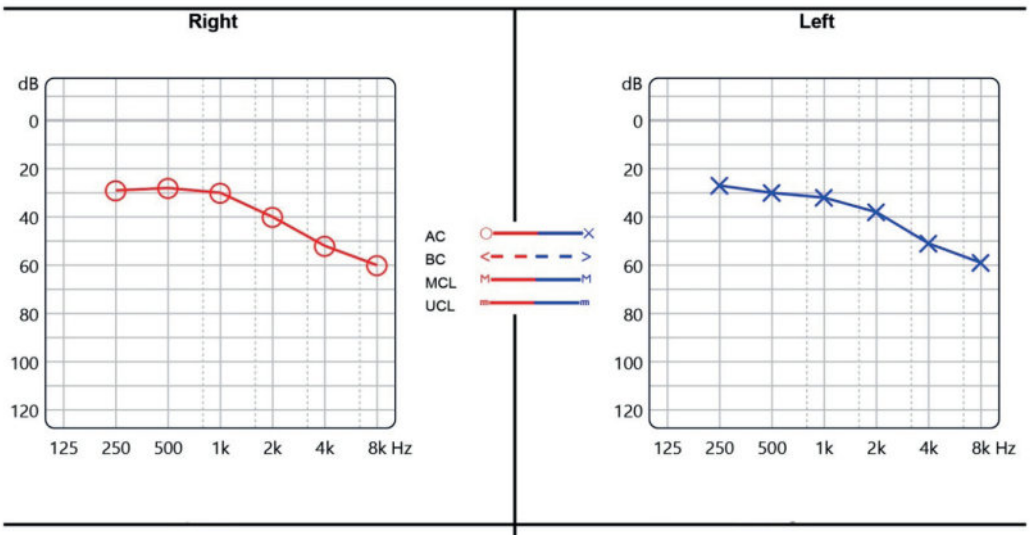
[§]p<0.005, probability of the null hypothesis for the difference between frequency groups
[MW]Mann-Whitney test

Table 2. Mean and median of the tinnitus intensity and duration. VAS, visual analogue scale;SD, standard deviation

		VAS[**] Left	VAS Right	Duration Left (years)	Duration Right (years)
N	Valid	41	41	43	43
	Missing	2	2	0	0
Mean (r)		5,24	4,83	4,298	3,942
Median		5,00	5,00	2,000	2,000
SD[4]		1,969	2,024	5,0710	4,7273[5]

[**] SD, standard deviation

We compared the total hearing loss and hearing threshold for each frequency on one hand and the intensity of tinnitus (measured by the VAS) on the other side of each ear. We compared the average hearing loss and hearing threshold for each frequency on the one hand and the duration of tinnitus (expressed in years and months) on the other side of each ear. We used the method of correlation analysis. The research results are presented in Tables 3 and 4. Picture 2 shows a graphical schedule, the correlation



Picture 1. Composed audiogram of hearing loss in the left ear.

Table 3. Correlation analysis between the hearing threshold group and the tinnitus intensity and duration in the right ear. S - Spearman's correlation coefficient

Pearson and Spearman's correlation coefficients for the right ear							
Hearing threshold	**250 Hz**	**500 Hz**	**1000 Hz**	**2000 Hz**	**4000 Hz**	**8000 Hz**	**average**
Tinnitus intensity	0,287^{S}	0,288^{S}	0,280^{S}	0,237^{S}	0,214^{S*}	0,169^{S}	0,342^{S}
Hearing threshold	**250 Hz**	**500 Hz**	**1000 Hz**	**2000 Hz**	**4000 Hz**	**8000 Hz**	**average**
Tinnitus duration	0,110	0,062	0,043	0,239	0,235^{S}	0,175^{S}	0,263

* Spearman's coefficient

Table 4. Correlation analysis between the hearing threshold group and the tinnitus intensity and duration in the left ear. S - Spearman's correlation coefficient

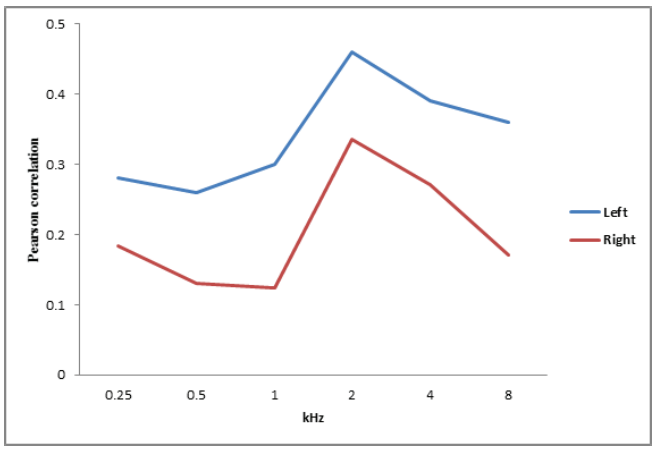
Pearson and Spearman's correlation coefficient for the left ear							
Hearing threshold	**250 Hz**	**500 Hz**	**1000 Hz**	**2000 Hz**	**4000 Hz**	**8000 Hz**	**Average***
Tinnitus intensity	0,225	0,262	0,289	0,356	0,245^{S}	0,257^{S}	0,312
Hearing threshold	**250 Hz**	**500 Hz**	**1000 Hz**	**2000 Hz**	**4000 Hz**	**8000 Hz**	**Average**
Tinnitus duration	0,207	0,241	0,3	0,388	0,347	0,415^{S}	0,294

* average hearing threshold calculated by the FS table

coefficient obtained by comparing the duration of tinnitus and hearing threshold at the measured frequencies.

DISCUSSION

Tinnitus is a symptom with various aetiologies. Most likely, all levels of the nervous system in various degrees are involved in the tinnitus event (17, 18). The opinion has been posed that tinnitus is a result of an increased spontaneous discharge rate of individual auditory nerve fibres. This view has been encouraged by the fact that electrical stimulation of the cochlea mitigates tinnitus. Some diseases such as Meniere's disease accompanied by tinnitus are localized to the ear. However, hearing loss, which is often associated with tinnitus, is accompanied by hypoactivity of nerve fibres. Tinnitus is thought to be caused by hyperactivity of nerve fibres. Currently, cochlear damage is usually considered to be affected by the outer hair cells,



Picture 2. Graphical schedule. The correlation coefficient was obtained by comparing the tinnitus duration and hearing threshold at the measured frequencies.

whose activity can be measured by otoacoustic emissions, as detected by Kemp (19). These emissions are rarely observed in patients with hearing impairments and much cannot be expected from them in the diagnosis of tinnitus. Patients with unbearable tinnitus have only discrete abnormalities on the BERA test. This is contrary to the assumption that tinnitus causes an increase in spontaneous activity of the auditory nerve fibres. Today, there is growing evidence that decreased activity of auditory nerve fibres leads to hyper-reactivity in the central auditory pathways. In animal experiments, ear injuries and removal of the cochlea cause increased activity of the lower colliculi and auditory nuclei (20), in contribution to the central mechanisms of the occurrence of tinnitus, which persists even after surgical resection of the auditory nerve in most experiments (21). Using NMR and SPECT in patients with tinnitus increases the activity of the associative auditory area for more than just the primary area (22). The limbic and autonomic nervous system exclusively determines each tinnitus event. There is much evidence about the role of stress in the pathogenesis of tinnitus. Stress in patients with tinnitus causes a sympathetic reaction (23).There is an increasing integration of the frontal cortex, hippocampus and other structures. The problem of tinnitus is much more complex than previously thought. In this paper, we aimed to study the audiological profile of people with chronic tinnitus and its relationship to the intensity and duration of tinnitus. More authors believe that the audiogram shape holds some answers to the aetiology of tinnitus. Most publications favour a steep audiogram in patients with tinnitus (14, 15). Our results are consistent with the assertions of Moller, Demeeseter and Konig (24, 14, 15). The difference in the hearing threshold, measured between the adjacent frequencies progressively increases with a higher frequency. The results are statistically significant for the left ear between 2000 and 4000 Hz ($p = 0.03$) and for the right ear between 1000 and 2000 Hz (p

= 0.007). Between 2000 and 4000 Hz (p=0,006), the analysed population in our study had a steeply sloping audiogram. Our audiogram had an audiometric edge between 1000 and 4000 Hz. The analysis included patients with tinnitus who had normal hearing. We did not separately analyse the patients who worked in noisy environments. This steeply sloping audiogram associated with reduced brain plasticity may be important for the aetiology of tinnitus (tonotopic reorganization theory) (25).

In the second part of our research, we did not find a strong link between the loudness of the tinnitus measured by VAS on one side and the average hearing threshold for each ear individually and the hearing threshold for each frequency measured on the other side. We started from the assumption that measured subjective tinnitus is associated with hearing loss or hearing loss at certain frequencies. This connection is weak (correlation coefficients less than 0.3) or medium (correlation coefficients less than 0.5 and greater than 0.3.). VAS is a subjective assessment volume and cannot fully indicate the severity of tinnitus but shows results for monitoring the therapeutic effect of a drug or therapeutic method. Currently, there are more scales that assess the intensity and severity of tinnitus, none of which have been validated in the Serbian language, for example: The Tinnitus Severity Scale (TSS) (26), the Tinnitus Handicap Questionnaire (THR) (27), the Tinnitus Handicap Inventory (THI) and the Tinnitus Questionnaire (TQ) (28). The work of certain authors shows a connection between the VAS and the THI (29).

We did not find a strong link in our research between the duration of tinnitus on one side and the average hearing threshold in each ear and the hearing threshold at each of the measured frequencies in the other ear (correlation coefficient of less than 0.5). The relationship between tinnitus duration and hearing threshold at each frequency was weak (correlation coefficient less than 0.3) or medium strong (correlation coefficients between 0.3 and 0.5). This relationship exhibited a certain regularity and was strongest at 2000 Hz and gradually decreased going towards both ends of the audiogram (Table 3, Table 4 and Picture 2). Notably, the greatest hearing decrease occurred at this frequency range (between 2000 and 4000 Hz), which had an audiometric edge. This is a very important frequency range in the pathogenesis of tinnitus. One zone in the auditory cortex is significantly less active than others. It creates a compensatory sound in the brain (phantom sound) with a frequency range between 2000 and 4000 Hz. The limitation of this research is that we did not separately assess different categories of chronic subjective tinnitus.

CONCLUSION

Tinnitus and hearing loss are not related measures in persons with chronic subjective tinnitus, but tinnitus is associated with the audiogram shape. Hearing in people with tinnitus rapidly decreases with higher frequencies. The most hearing-decreased range is between 2000 and 4000 Hz. The severity of tinnitus experienced by the patient is not related to the degree of hearing loss and is not associated with hearing loss at some frequencies (audiometric shape). The relationship between tinnitus duration and hearing loss and audiometric shape is not strong. It is the strongest at 2000 Hz and decreases towards each end of the audiogram.

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TEAR FILM STABILITY IN PATIENTS WITH PSEUDEXFOLIATION

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STABILNOST SUZNOG FILMA KOD PACIJENATA SA PSEUDOEKFOLIACIJAMA

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ABSTRACT

Pseudoexfoliation syndrome is an age related disorder, characterized by abnormal fibrous fiber production and accumulation in different visceral organs as well as in the eye and periocular tissues. Hystological examination recorded the presence of the pseudoexfoliation in the conjunctiva, and they can disturb the accessory lacrimal gland and goblet cell function. This can explain tear film instability in patients with pseudoexfoliations. In our study, we examined the tear film stability in patients with and without pseudoexfoliation, using Schirmer test and tear break up time test. Our results indicated that patients with pseudoexfoliation had lower values of Schirmer and tear break up time tests than patients without it. Pseudoexfoliation is the main reason for the instability of the tear film, because of its negative impact on the conjucntival goblet cells. In conclusion, ophthalmologists must have these data on their mind in the process of the pseudoexfoliation glaucoma treatment and controlling.

Keywords: *pseudoexfoliation, tear film, antigluacomatous drug*

SAŽETAK

Pseudoeksfolijativni sindrom je poremećaj vezan za starost bolesnika, koji se karakteriše abnormalnom proizvodnjom fibroznih vlakana i njihovom akumulacijom u različitim visceralnim organima, kao i u oku i periokularnim tkivima. Histološkim pregledom utvrđeno je prisustvo pseudoeksfolijacija u vežnjači, kao i da mogu dovesti i do poremećaja funkcije pomoćne suzne žlezde i peharastih ćelija. Ovim se može objasniti nestabinost suznog filma kod pacijenata sa pseudoeksfolijacijama. U našoj studiji, ispitivali smo stabilnost suznog filma kod pacijenata sa i bez pseudoekfolijacija, koristeći Schirmer test i test pucanja suznog filma. Naši rezultati su pokazali da su pacijenti sa pseudoeksfolijacijama imali niže vrednosti Schirmer testa i testa pucanja suznog filma, od pacijenata bez pseudoeksfolijacija. Pseudoeksfolijacije su glavni razlog za nestabilnosti suznog filma zbog svog negativnog uticaja na peharaste ćelije vežnjače. U zaključku, oftalmolozi moraju imati na umu ove podatke u procesu lečenja i kontrole pseudoekfolijativnog glaukoma.

Ključne reči: *pseudoeksfolijacije, suzni film, antiglaukomatozni lek*

ABBREVIATIONS

IOP - intraocular pressure
PACG - primary angle closure glaucoma
PEX - Pseudoexfoliation
TBUT test - tear break up time test
XFG - pseudoexfoliative glaucoma
XFS - pseudoexfoliative syndrome

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INTRODUCTION

Pseudoexfoliation syndrome (PEX) is an age-related generalized disorder of the extracellular matrix with increased production and accumaltion of abnormal extracellular material in different tissues of the body (skin, connective tissue portions of visceral organs, eye) (1). Biomicroscop eye examination indicated that pseudoexfoliation material can be found on corneal endothelial, pupilar margin, iridocorneal angle, lens anterior capsule, iris surface and on ciliary body (2). Also, data indicated for PEX material presentation in periorbital tissues as well as on conjunctiva (3). Pathohystological examination of the conjunctiva suggested that pseudoexfoliation can be found on the conjunctiva surfaces with affection on the accessory lacrimal gland and goblet cells functions (4). These findings can indicate on the possibilities of tear film instability. Pseudoexfoliation syndrome is one of the most common cause of elevated intraocular pressure and developing of pseudoexfoliative glaucoma, which is the final step in the process of production and accumulation of PEX material (5).

MATERIAL AND METHODS

The study was performed in Clinic of Ophthalmology, Clinical Centre Kragujevac, from 1st January 2015 until 1st January 2016. The patients included in our study underwent routine ophthalmological examination in the Outpatient Department of the Clinic. All patients (n=150) were divided into three groups according to PEX presentations: PEX syndrome, PEX glaucoma, and age and sex matched control group (without PEX). Detailed slit lamp examination in mydriasis was performed for every patient, as well as Schirmer and TBUT tests. The patients with previous history of intraocular surgery, laser treatment or intraocular inflammation, PACG, contact lens wearer, ocular surface diseases, with lid abnormality or pterygium were excluded from the study. Also, excluding criteria was previous using of artificial tear eyedrops. Glaucoma diagnosis was determined on the earlier clinical examination (elevated IOP, optic head structural changes, visual field defect), without previous antiglucomatous drugs using. The measurement of intraocular pressure was performed by applanation tonometry. Tear break up time, indicator of the lipid lay of the tear film, was determined using fluorescein strips before the other planned intervention (measuring of the IOP, Schirmer test) and using some ophthalmic drug. Under the cobalt blue light, we noticed time until the appearance of the dry spot on the corneal surface. Local anesthetic (generic tetracain 0.5%) was applied before the test. Tear secretion test was done using Schirmer paper, applied in the lateral 1/3 of inferior fornix. Wet part of the paper was recorded for every eye.

Statistical analysis was performed with SPSS 19.0 software (SPSS Inc., Chicago, IL). The distribution of the data was determined by Shapiro–Wilks or Kolmogorov–Smirnov test. The continuous variables were expressed as mean ± standard deviation and the categorical variables as frequency and percent. Pearson chi-square test was used to determine the difference among three groups; and, differences among the groups were analyzed by Kruskal–Wallis test. The dual comparisons among groups with significant values were evaluated with Bonferroni adjusted Mann–Whitney U-test. p value of less than 0.05 was considered statistically significant for all tests.

RESULTS

Our study covered 269 eyes in 150 patients, divided in three groups (n=50). PEX syndrome group included 13 patients with PEX presentation only in one eye, and 37 patients with PEX presented in both eyes (totally 87 eyes). We also examined 82 eyes in XFG group (18 patients with affected only one eye, 18 patients with affected both eyes, and 14 patients with mixed PEX presentation-XFS on eye, and XFG on the other eye).

Patients' characteristics

The mean age of the participants among the group did not show the statistically significant differences, because of its matching. In the XFS group mean age was 69.2±4.3, in XFG group 69.8±3.9, control group 70.0±2.1. Our results indicated that was no statistically significant differences in gender distribution (male:female- XFS-26:24, XFG-24:26; control group-25:25), Table1.

Schirmer tests results

Groups with PEX (XFS and XFG) measurement indicated that patients with XFS and XFG had statistically significant lower values than control group ($p<0.05$), but without statistically significant differences among PEX groups ($p>0.05$). Our results indicated that control group had mean value of Schirmer test 13.5±4.2mm; XFS group had mean value 9.75±2.7mm (range: 5-18mm) and XFG had mean value 8.6±2.5mm. XFS group showed some differences between the participants: participants with one PEX presented eye had lower value of Schirmer test in affected eye (8.4±2.3mm) than in the other eye without PEX (9.2±2.6mm) but without statistically significant differences ($p>0.05$). In XFG group also, we noticed differ-

Table1. Patients` charcteristics

	XFS	XFG	control
ages	69.2±4.3	69.8±3.9	70.0±2.1
female/male	26/24	24/26	25/25

ences between the participants: participants with PEX material in the eye recorded lower Schirmer tests than other without the PEX without statistically significant differences ($p>0.05$), but also it was statistically significant lower than in the patients form control group ($p<0.05$).

TBUT tests results

TBUT tests results showed that PEX groups had statistically significant lower values than the group without PEX ($p<0.001$), with statistically significant differences between the two PEX groups ($p<0.05$). Control group patients had TBUT- 15±2 sec (range 9-15 sec); XFS group showed mean TBUT result10±2 (range 6-15 sec), and XFG- 8±1 (range 5-12 sec). In XFS group, participants with one eye affected with PEX had lower values of TBUT 9±2 sec compare with TBUT results on the other eye (10±2). Participants from the XFG group also had different values of TBUT according to the PEX presentation: eyes without PEX presentation recorded TBUT value- 10±1, with XFS on one eye-10±2 sec, and with both XFG- 8±2 sec.

DISCUSSION

Pseudoexfoliation syndrome is generalized fibrillopathy, characterized by abnormal production and accumulation of the pseudoexfoliative material in the whole body (6). Also, it is well known that PEX material can be detected by histological examination in the conjunctival tissue (3). Presentation of the PEX in conjunctival tissue can have the influence on the tear film stability (4). Tear film stability is predictive factor for the health of the ocular surface (7). If one of the components of the tear film is disturbed, ocular surface disturbances can be observed (8). Kozobolis et al. proved that PEX material in conjunctival tissues provoke the changes of the basic features of the morphology of the goblet cell (4). Though, it was established that density of the goblet cells in the patients with or without the PEX are similar, the alterations in their morphology can cause the changes in the tear film quality, decreasing the basic part of the tear secretion. Hystological examination of the conjunctival tissue discovered the presentation of the PEX (9). Our results indicated that basic tear secretion was decreased in patients with PEX presented in the eye, nevertheless to the stage of its presentation (XFS/XFG) compared with PEX negative patients. The stage of the PEX presentation may be determined by the Schirmer test values: lower values indicated for the advanced stage.

Oncel et al. established that tear film osmolarity was higher in patients with PEX, which can be explained by the dysfunction of the goblet conjunctival cells (10). TBUT test results from our study recorded lower in patients with PEX (XFS/XFG) compared with control group patients with statistically significant difference. XFG and XFS had different values of the TBUT without statistically significant difference. This fact can be useful in clinical practice to determine the stage of the PEX production and accumulation. So, patients with PEX own the dry eye symptoms more often than patients without PEX, as well as inflammation and tissue damage of the ocular surface.

According to the definition of the dry eye The Jones et al., indicated that tear secretion is divided into basic (fundamental tree layers tear film) and reflex (additional secretion peripheral sensory, retinal or psychogenic stimulated) (8). Anesthesia applied before the Schirmer test remains mainly basic secretion, which is the indicator for the tear secretion and goblet cells function (11).

These facts must be accepted and considered in the treatment of the XFG. Earlier studies indicated that XFG is one the most difficult form of glaucoma for controlling, because of its unknown nature (12). So, many antiglaucomatous drugs must be prescribed for its control. Ophthalmologists must have all of these informations when they prescribe the drugs. Particularly, if the two or more antiglaucomatous drugs are needed for the treatment, ophthalmologist must choose the preservative free drugs as well as the artificial tears for protecting the ocular surfaces (13). Patients discomfort when using antiglauocmatous drugs can be the reason for bed compliance in the glaucoma treatment (14). So, the results of the treatment can be also bed and the disease can be in the progression.

The very important thing for caring out the study is that group must be matched. Age matching is important because tear film is more disturbed in older patients (15). So, gender is very significant for the quality of the tear film, because females have lower tear secretion after the age of forty, caused by hormonal disturbances (16).

In conclusion, we can advise the ophthalmologist that they must consider the antigluaocmatous drugs for the treatment of the glaucoma, because of its negative effect on the damaged ocular surface caused by the disturbed tear film. PEX is the main reason for the instability of the tear film, because of its negative impact on the conjucntival goblet cells. Some future studies must establish correlation between the PEX conjunctival deposition and the mechanism of the PEX production. The results of this analysis can be the key for the successful treatment of the XFG.

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ANALYSIS OF GLYCODELIN LEVELS BEFORE AND AFTER HYSTEROSCOPIC POLYPECTOMY IN INFERTILE PATIENTS

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ANALIZA NIVOA GLIKODELINA PRE I POSLE HISTEROSKOPSKE POLIPEKTOMIJE KOD INFERTILNIH PACIJENTKINJA

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ABSTRACT

Glycodelin (or placental protein 14) is a glycoprotein located in the glandular and thin epithelium of the endometrium. It is considered an important factor in the implantation process, and its traces can be found in elevated concentrations in the uterine flushing obtained at the time of implantation, while in the proliferative phase of the cycle, its levels are low. A certain concentration has been found to inhibit the binding of spermatozoids to the zona pellucida of the oocites therefore, it effects conception. It has a role in angiogenesis and is in high concentrations in the tissues of both benign and malignant gynaecological tumours.

The aim of this study is to analyse and display the glycodelin level changes before and after hysteroscopic polypectomy in infertile patients in the uterine flushing fluid and serum. This survey covers 80 infertile patients, who were divided into two groups. The first group, the experimental group, consisted of 50 infertile patients with endometrial polyps, and a control group of 30 infertile patients without endometrial polyps was also included.

The results primarily indicate the existence of changes in glycodelin levels preoperatively in the flushing and venous blood in infertile patients with endometrial polyps compared with the levels after surgery. In the control group of patients, no significant change in the glycodelin levels was detected in the flushing and venous blood. When comparing these two groups, statistically significant differences in the glycodelin levels in the flushing and venous blood were noted. We conclude that the presence of endometrial polyps in the cavum uteri affects the increase in the glycodelin concentration in the flushing fluid and in the plasma. Increased glycodelin concentrations complicate fertilization and implantation.

Keywords: *cytokine, glycodelin, endometrial polyp, flushing and serum.*

SAŽETAK

Glikodelin (ili placentni protein 14) je glikoprotein koji se nalazi u glandularnom i površinskom epitelu endometrijuma. Smatra se da je jedan od bitnih faktora u procesu implantacije i nalazi se u povišenim koncentracijama u ispirku uterusa dobijenom u vreme implantacije, dok su u proliferativnoj fazi ciklusa njegove vrednosti niske. U određenoj koncentraciji nađeno je da inhibira vezivanje spermatozoida za zonu pelucidu ovuuma, tako da utiče i na koncepciju. Ima ulogu u angiogenezi, te se nalazi u povišenim koncentracijama u tkivu i benignih i malignih ginekoloških tumora.

Cilj ovog rada je analiza i prikaz promena nivoa glikodelina, pre i posle histeroskopske polipektomije kod infertilnih pacijentkinja, u ispirku uterusa i serumu. Istraživanje obuhvata 80 infertilnih pacijentkinja, koje su podeljene u dve grupe. Prvu grupu, eksperimentalnu, čini 50 infertilnih pacijentkinja sa polipom endometrijuma, a kontrolnu grupu 30 infertilnih pacijentkinja bez polipa endometrijuma.

Dobijeni rezultati prvenstveno ukazuju na postojanje promena nivoa glikodelina preoperativno, i u ispirku i u venskoj krvi, kod infertilnih pacijentkinja sa endometrijalnim polipom u odnosu na nivoe nakon operativnog zahvata. U kontrolnoj grupi pacijentkinja nema značajnih promena nivoa glikodelina u ispirku i venskoj krvi. Poređenjem ove dve grupe prisutne su statistički značajne razlike u nivoima glikodelina i u ispirku i u venskoj krvi. Možemo zaključiti da prisustvo endometrijalnog polipa u kavumu uterusa utiče na porast koncentracije glikodelina, i u ispirku i u plazmi. Povećana koncentracija glikodelina otežava i oplodnju i implantaciju.

Ključne reči: *citokin, glikodelin, endometrijalni polip, ispirak i serum.*

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INTRODUCTION

Infertility is a problem that affects approximately 80 million couples worldwide. It is believed that approximately 15% of the general population is affected by this problem. Endometrial polyps occur when the endometrium is hypertrophic, due to oestrogen stimulation (1). A diagnosis is based on sonographic, hysteroscopic or hysterosonographic findings, when a polyp is spotted in the cavum uteri (2).

Cytokines are a large family of protein molecules that function as a mediator and regulator of cellular communications, in both physiological and pathological conditions (3). Different cells produce a variety of cytokines, which act on the chemotaxis, activation, proliferation and differentiation of other cells. Cells that secrete the highest amounts of cytokines are leukocytes. Glycodelin (or placental protein 14) is a cytokine and is a 28kDa glycoprotein that contains 180 amino acids (4). Glycodelin is encoded by a single gene that is located in the chromosomal region 9q34; it is derived from the endometrium. It consists of a single (GaINAcβ1-4GlcNAc) oligosaccharide series and is located in the glandular and surface epithelium of the endometrium. Glycodelin is the primary product during the secretion phase of endometrial cells. Maximum levels are produced in the secretory endometrium and early pregnancy decidual. It is produced in the lumen of the endometrial glands and can be detected in circulation. The highest concentration of glycodelin is produced during the secretory phase of the endometrial cycle, while at the early proliferative phase, its concentration is low. There are strong indicators that, in conjunction with other cytokines, glycodelin represents an important factor that facilitates implantation (5). It is found in elevated concentrations in the uterine flushing obtained at the time of implantation. During conception, glycodelin levels remain elevated. Glycodelin has an immunosuppressive effect and probably has a protective role in the preservation of embryos from natural killer (NK) cell destruction. Additionally, it has been found that in certain concentrations, glycodelin inhibits the binding of sperm to the zona pellucida of the oocites therefore, it effects conception. Recently, it has been shown that it plays a role in angiogenesis and is elevated in the tissues of both benign and malignant gynaecological tumours (6).

MATERIAL AND METHODS

The survey was conducted as a study of 80 infertile patients. The two groups of female patients were compared. The first group of patients, the experimental group, consisted of 50 infertile patients diagnosed with endometrial polyps. The second group was a control group of 30 infertile patients without endometrial polyps. The research was carried out at the Gynaecology and Obstetrics Clinic "National Front" in Belgrade, from May 2012 to November 2013, and in the Center for Molecular Medicine and Testing of Stem Cells, Faculty of Medical Sciences, in Kragujevac.

This study was conducted on patients with childbearing potential (aged 20-43 years) who were diagnosed with endometrial polyps as a cause of infertility. The polyp diagnoses were determined by transvaginal ultrasound examinations during the first phase of the cycle, by hysterosonographic examinations, or during an actual hysteroscopy in patients with suspected endometrial polyps, based on anamnesis. In addition to the group who participated in the study, there was a control group of patients of the same age who were treated for infertility did not have endometrial polyps. The patients were evaluated for one month after the intervention in terms of peripheral venous blood sampling, cytokine level determinations, and control ultrasound examinations.

Diagnostic and operative hysteroscopies were performed under general anaesthesia in the operating room with appropriate equipment and instruments. The preoperative preparation included a complete diagnosis and the necessary routine analyses including cervical and vaginal smears, chlamydia, mycoplasma and ureaplasma evaluations, Pap test, ultrasound, blood typing, blood count and biochemical analysis with an anaesthetic examination and surgical treatment approval. Surgical procedures were performed in infertile patients to mid-proliferative phase immediately after menstrual bleeding.

Hysteroscopic examination and intervention-polypectomy are done during the first phase of the menstrual cycle (9). During hysteroscopy, the uterine cavity is viewed at a 30-degree angle, which allows for visualization of both mouths of the fallopian tube, fundus, anterior and posterior wall of the uterus as well as the lateral sides of the uterine cavity (10). At the height of the internal uterine mouth with the hysteroscope, a panoramic image of the uterine cavity is displayed, and the presence of any pathological findings, such as endometrial polyps (which are often observed), submucosal fibroids, septa, and adhesions, can be easily visualized. Polyps are removed with hysteroscopic graspers and hysteroscopic scissors followed by electrocoagulation of their base (11).

To determine the glycodelin (PP14) concentration in the uterus flushing during hysteroscopic polypectomy, the operation sequence was as follows: 10 ml injection of saline into the cavum uteri and immediate aspiration without contamination. Then, the flushing was centrifuged at 2500 rpm for 10 minutes. The supernatant was collected and stored at -20° C. The glycodelin concentration was determined with the ELISA method at the Center for Molecular Medicine and Testing of Stem Cells, at the Faculty of Medical Sciences in Kragujevac (15).

To determine the glycodelin (PP14) serum concentration, approximately 5 ml of peripheral venous blood was taken from patients in both groups, before hysteroscopic surgery and one month after surgery. The sample processing methodology is as follows: the blood was collected in a vacutainer with heparin and centrifuged for

10 minutes at 2500 rpm. The supernatant was collected and stored at -20 ° C. Detection and determination of the glycodelin concentration was then determined with the ELISA method (16).

The study inclusion criteria were women with endometrial polyps who were verified as 20-43 years of age and treated for primary or secondary infertility as well as infertile women without endometrial polyps of the same age (16).

Criteria for exclusion from the study for both groups of respondents were the existence of submucosal fibroids, endometriosis, endometrial cancer, uterine anomalies, and patients who had surgery on their uterus and tubes, as well as patients with a previous failed ovulation following stimulation.

Statistical data analysis was based on use of the t-test. As the sample distribution in this study (with a minimum group of 30 patients) was mildly leptokurtic and skewed, for this study, t-tests were successfully implemented, and even for similar mean values of the sample, the t-test provided more accurate results than the Wilconxon Mann-Whitney test (17).

RESULTS

We analysed the glycodelin levels in the uterus flushing and venous blood from both test and control group patients (8). The levels of this cytokine were examined before and one month after hysteroscopic polypectomy.

Statistical analysis of the patient groups was based on testing the null hypothesis with t-tests of group differences according to the mean value and variance (7). The groups were significantly different if $p < 0.05$.

Figure 1 shows the glycodelin values in the uterine flushing from patients with endometrial polyps compared with the control group during surgery. It can easily be seen that the glycodelin levels in the uterine flushing were significantly decreased in the control group subjects compared with the experimental group. Using the t-test and analysis of the obtained results, it can be concluded that there was a statistically significant difference ($t=1.992$; $p=0.0144$).

Analysis of the venous blood glycodelin levels in the endometrial polyp patients is shown in Figure 2. Notably, the venous blood glycodelin levels that were collected a month after hysteroscopic polypectomy were significantly lower. By applying the t-test, it can be concluded that the groups differ statistically ($t=2.01$; $p=0.00017$).

The venous blood glycodelin values in the endometrial polyp and control group patients before hysteroscopic polypectomy are shown in Figure 3. Applying a t-test leads to the conclusion that the groups differ statistically and that the glycodelin levels were significantly higher in the endometrial polyp patients ($t=1.996$; $p=0.0036$).

Figure 4 provides the venous blood glycodelin values in the control group before and one month after hysteroscopy. By applying the t-test, it can be concluded that the groups did not differ statistically, i.e., the glycodelin levels did not significantly differ in the control group before and after hysteroscopy ($t=2.048$; $p=0.851$).

The venous blood glycodelin values in patients one month after hysteroscopic polypectomy and in the control group one month after hysteroscopy are shown in Figure 5. These levels were found to not be significantly different using the t-test ($t=2.004$; $p=0.838$).

DISCUSSION

The glycodelin concentrations in the flushing and serum were significantly higher in patients with endometrial polyps in comparison with the control group. A month af-

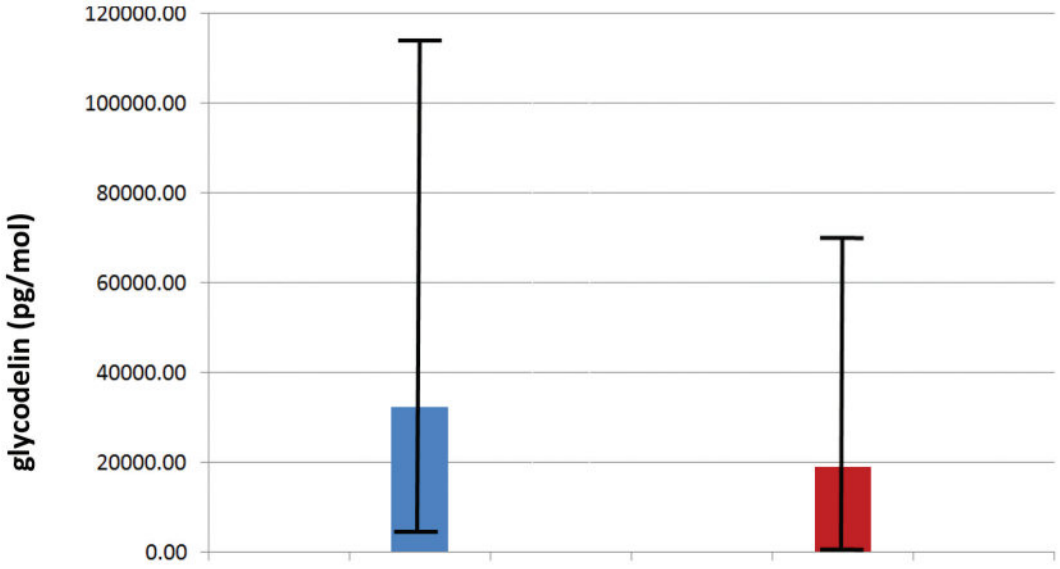

Figure 1. Glycodelin average values in the uterine flushing from patients with endometrial polyps compared with control group patients during hysteroscopy. These are the t-test parameters, the critical average values for accepting the null hypothesis.

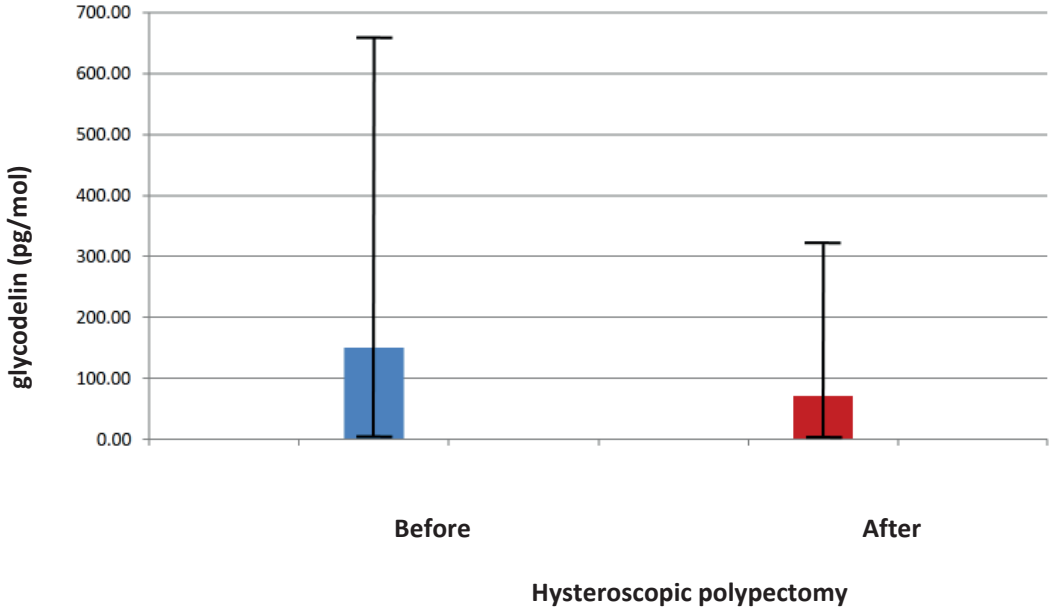

Figure 2. Venous blood glycodelin average values in patients with endometrial polyps before hysteroscopic polypectomy and one month after surgery.

ter hysteroscopic polypectomy, the serum glycodelin levels were reduced in the experimental group and reached values very similar to the those of the control group. These results were confirmed in most studies that examined glycodelin levels in the uterine flushing and plasma of infertile patients.

Endometrial polyps are common in infertile patients who are preparing for in vitro fertilization, and their presence does not affect pregnancy rates, but there is a higher risk of pregnancy loss in these patients (18, 19). Polyps are endometrial tissue growths covered with epithelium. They have glands, stroma and blood vessels that contribute to increased glycodelin secretion in the cavum uteri (12, 13). The common assumption is that glycodelin may "leak" from blood vessels near polyps and dissolve in saline, which is used during hysteroscopy uterine flushing (14, 20). Glycodelin levels in uterus flushing fluid are then determined using ELISA measurements, and in most surveys, it was confirmed that the values were significantly higher in uterine flushing with endometrial polyp or submucosal fibroids compared with the control group (21).

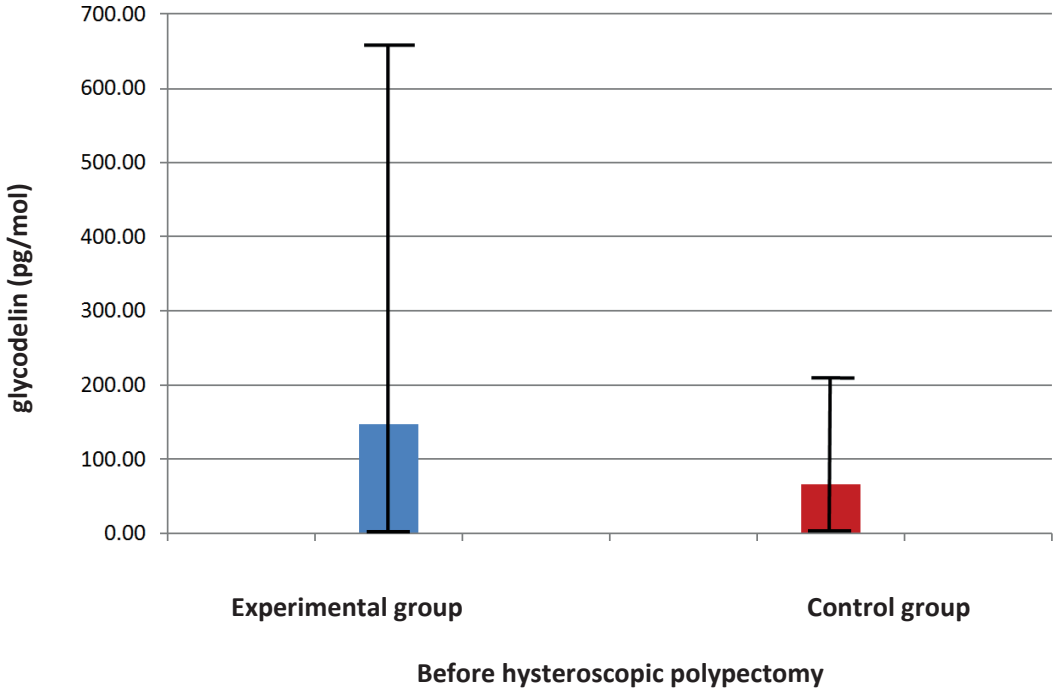

Figure 3. Venous blood glycodelin average values in the endometrial polyp and control group patients before hysteroscopic polypectomy.

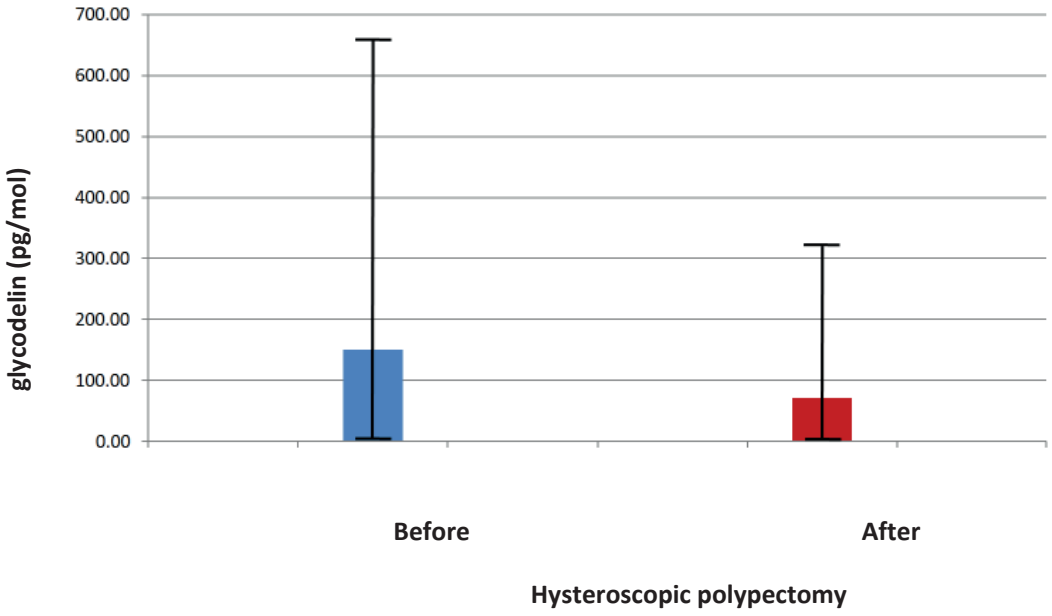

Figure 4. The venous blood glycodelin average values in the control group patients before hysteroscopy and one month after surgery.

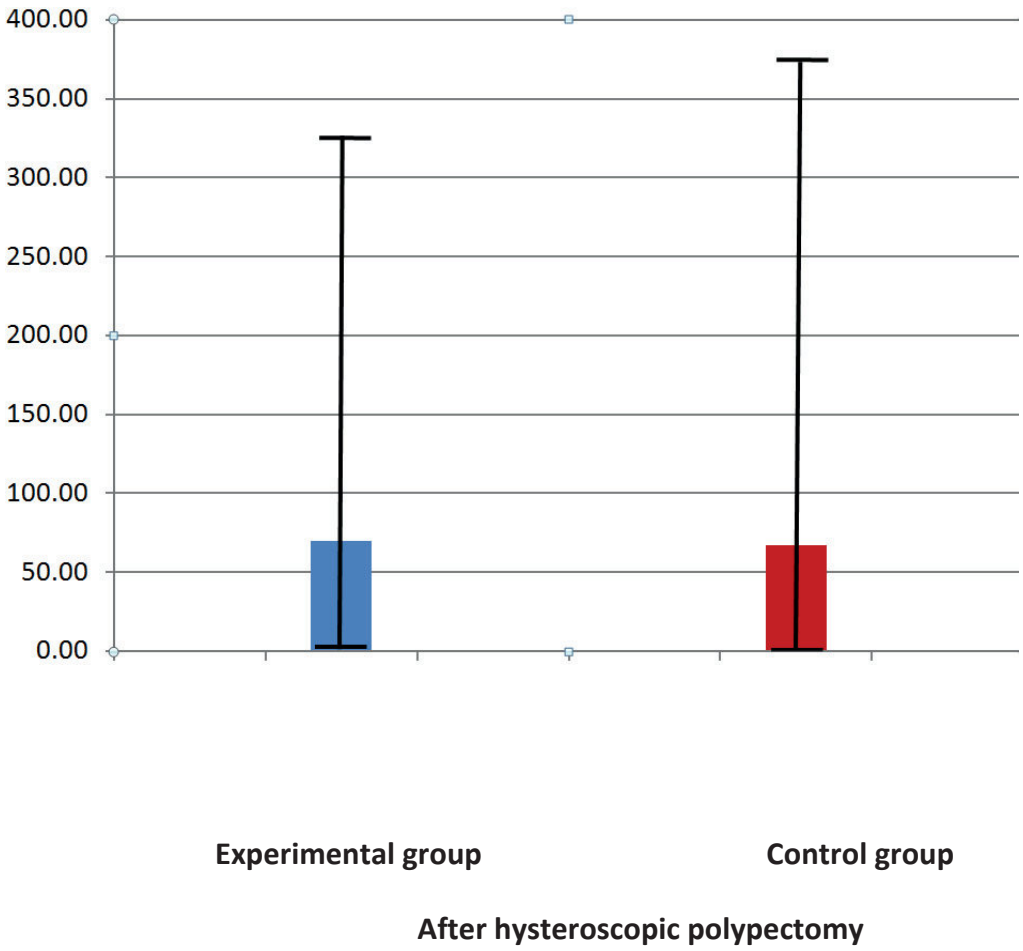

Figure 5. Venous blood glycodelin average values in the endometrial polyp and control group patients one month after surgery.

Glycodelin contributes to polyp growth by encouraging neovascularization and thus allowing a better flow of the nutrients necessary for polyp growth. It can be detected in the circulation; therefore, in various studies, its concentration in plasma was often determined. In most of these studies, elevated glycodelin levels in the plasma of patients with endometrial polyps was observed compared with control group patients (22).

It has been proven that glycodelin inhibits sperm and oocyte binding as well as NK cell activity (23). Glycodelin values are very low in the period 6 days before and 5 days after ovulation (periovulatory period). Thus, low glycodelin levels allow for fertilization. Then, 6 days after ovulation, glycodelin secretion increases significantly and becomes a key to the formation of a receptive endometrium and suppresses NK cell activity. The results of this study indicate that the glycodelin levels measured during the middle and late proliferative phase of the menstrual cycle were elevated when they should have been at their lowest values, or even absent; however, they significantly affected fertilization and altered endometrial receptivity in patients with endometrial polyps (20).

Changes in glycodelin concentrations are registered in the endometrium, or uterine flushing and are correlated with the values obtained in the serum of the experimental group patients. We conclude that changes in the glycodelin values can be tracked by just specifying the venous blood in patients in whom a hysteroscopic polypectomy was performed and can thus monitor the success of the operational procedures and limit possible polyp recurrences by avoiding administering complicated uterus flushings, which require preoperative preparation and general anaesthesia during a hysteroscopy.

CONCLUSION

Two groups of infertile patients were analysed, one with endometrial polyps and a second control group that did not have polyps. The glycodelin values in the uterus flushing fluid and in the venous blood before surgery were significantly higher in the patients with endometrial polyps. The uterus flushing fluid and venous blood glycodelin values in the polyp group were significantly decreased one month after surgery. The glycodelin levels in the serum of the control group patients who were detected preoperatively remained similar, even one month after surgery. Additionally, there was no significant difference in the glycodelin levels one month after surgery in a group of patients where the polyps were removed compared with the control group patients.

The results of this study clearly indicate that the glycodelin levels in infertile patients with endometrial polyps were elevated; therefore, we can safely conclude that glycodelin is a cytokine that has a crucial impact on endometrial miles and can significantly disturb homeostasis at the molecular level, thus affecting fertility.

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RISK FACTORS FOR CARBAPENEM-RESISTANT *KLEBSIELLA PNEUMONIAE* HOSPITAL INFECTION IN THE INTENSIVE CARE UNIT

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FAKTORI RIZIKA ZA NASTANAK BOLNIČKIH INFEKCIJA UZROKOVANIH *KLEBSIELLA PNEUMONIAE* REZISTENTNOM NA KARBAPENEME U JEDINICAMA INTENZIVNE NEGE

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ABSTRACT

Carbapenem-resistant Klebsiella pneumoniae (CR-Kp) has become a major threat to patients in hospitals, increasing mortality, length of stay and costs.

The aim of this study was to discover risk factors for the development of hospital infections (HIs) caused by CR-Kp.

A prospective cohort study was conducted in the Medical-Surgical Intensive Care Unit of the Clinical Centre in Kragujevac, Kragujevac, Serbia, from January 1, 2011, to December 31, 2015. The "cases" were patients with HIs caused by CR-Kp, while the "controls" were patients infected with carbapenem-sensitive Klebsiella pneumoniae (CS-Kp). The significance of multiple putative risk factors for HIs caused by CR-Kp was tested using multivariate logistic regression.

Although univariate analyses pointed to many risk factors, with a significant influence on the occurrence of hospital CR-Kp infections, the multivariate logistic regression identified five independent risk factors: use of mechanical ventilation (OR=6.090; 95% CI=1.030-36.020; p=0.046); length of antibiotic therapy before HIs (days) (OR=1.080; 95% CI=1.003-1.387; p=0.045); previous use of carbapenems (OR=7.005; 95% CI=1.054-46.572; p=0.044); previous use of ciprofloxacin (OR=20.628; 95% CI=2.292-185.687; p=0.007) and previous use of metronidazole (OR=40.320; 95% CI=2.347-692.795; p=0.011)

HIs caused by CR-Kp are strongly associated with the use of mechanical ventilation and the duration of the previous use of certain antibiotics (carbapenems, ciprofloxacin and metronidazole).

Key Words: *Klebsiella pneumoniae; Antimicrobial drug resistance; Hospital infections; Risk factors*

SAŽETAK

Klebsiella pneumoniae rezistentna na karbapeneme (CR-Kp) postala je velika opasnost za hospitalizovane pacijente, obzirom da povećava smrtnost, produžava boravak u bolnici i dovodi do većih troškova lečenja.

Utvrditi potencijalne faktore rizika za razvoj bolničkih infekcija uzrokovanih sa CR-Kp.

Sprovedena je prospektivna kohortna studija u Intenzivnoj nezi Kliničkog centra Kragujevac (Kragujevac, Republika Srbija) u periodu od 1. januara 2011. godine do 31. decembra 2015. godine. "Slučajevi" su bili pacijenti sa bolničkim infekcijama uzrokovanim CR-Kp, dok su "kontrole" činili pacijenti inficirani Klebsiella pneumoniae (CS-Kp) patogenom osetljivim na karbapeneme. Značaj potencijalnih faktora rizika za razvoj bolničke infekcije uzrokovane sa CR-Kp ispitan je logističkom regresijom.

Mada je univarijantna analiza ukazala na značajan uticaj brojnih faktora rizika na razvoj intrahospitalnih infekcija uzrokvanih sa CR-Kp, multivarijantnom analizom je, pak, identifikovano pet nezavisnih faktora rizika za razvoj infekcije sa CR-Kp: upotreba mehaničke ventilacije (OR=6.090; 95% CI=1.030-36.020; p=0.046), dužina primene antibiotske terapije pre nastanka bolničke infekcije (OR=1.080; 95% CI=1.003-1.387; p=0.045), prethodna upotreba karbapenema (OR=7.005; 95% CI=1.054-46.572; p=0.044), prethodna upotreba ciprofloksacina (OR=20.628; 95% CI=2.292-185.687; p=0.007) i prethodna upotreba metronidazola (OR=40.320; 95% CI=2.347-692.795; p=0.011)

Bolničke infekcije izazvane sa CR-Kp udružene su sa primenom mehaničke ventilacije kao i dužinom prethodne primene određenih antibiotika (karbapenemi, ciprofloksacin i metronidazol).

Ključne reči: *Klebsiella pneumoniae; rezistencija na antibiotike, bolničke infekcije; faktori rizika*

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INTRODUCTION

In recent years, due to uncritical use of carbapenems in the Intensive Care Unit (ICU), there are numerous reports of a progressive increase in the incidence of severe and life-threatening hospital infections (HIs) by gram-negative bacteria that are resistant to these antibiotics. An important example of such a microorganism is the carbapenem-resistant *Klebsiella pneumoniae* (CR-Kp), which has become a major threat to hospitals, increasing mortality, length of stay, and cost (1-4). An additional difficulty is the multi-resistant nature of such isolates, which further complicates the treatment of HIs due to limited choices for effective antibiotics.

Recent studies shed light on some risk factors for CR-Kp acquisition (5-8). However, the importance of the prior use of antibiotics has been little investigated. Modern medicine requires additional knowledge for prevention of these infections and for the optimal choice of empirical antibiotic therapy.

The aim of our study was to discover the risk factors for the development of HIs caused by CR-Kp. These may be crucial for implementing efficient infection control measures to decrease the spread of these pathogens. In addition, this study analysed the sensitivity of isolates to the most frequently used antibiotics.

MATERIALS AND METHODS

Hospital settings and the study population

This study was conducted in the 18-bed Medical-Surgical ICU of the Clinical Centre Kragujevac, Kragujevac, Serbia, during the 5-year period from January 1, 2011, to December 31, 2015. The inclusion criteria for the study participants were (a) a stay in the ICU >48 hours, (b) development of HIs caused by *Klebsiella pneumoniae* with any localization and (c) age >18 years. The exclusion criteria were (a) isolation of pathogens within 48 hours of admission and (b) repeated sampling from the same location of infection.

Study design and definition of the study groups

A prospective cohort study design was used that involved construction of two study groups. The "cases" were patients with HIs caused by CR-Kp, while the "controls" were patients infected with carbapenem-sensitive *Klebsiella pneumoniae* (CS-Kp).

Microbiology and susceptibility testing

The isolates were identified to the species level using conventional biochemical methods in the Microbiology Laboratory of Clinical Centre Kragujevac (9). Antimicrobial susceptibilities were determined by the disk diffusion method or with a Vitek-2 automated system (BioMerieux, France) for the following antibiotics: amoxicillin+clavulanic acid (30 μg/mL), piperacillin- tazobactam (110 μg/mL), cefotaxime (30 μg/mL), ceftriaxone (30 μg/mL), ceftazidime (30 μg/mL), cefepime (30 μg/mL), imipenem (10 μg/mL), meropenem (10 μg/mL), gentamicin (10 μg/mL), amikacin (30 μg/mL), ciprofloxacin (5 μg/mL), trimethoprim-sulfamethoxazole (2.5 μg/mL) and tigecycline (15 μg/mL). All isolates with intermediate sensitivity or resistance to the tested antibiotic were considered resistant. The results were interpreted according to the guidelines issued by The Clinical and Laboratory Standards Institute (CLSI) (10).

CR-Kp was determined if *Klebsiella pneumoniae* isolates were resistant to both tested carbapenems (imipenem and meropenem). In cases of discrepancy, imipenem resistance served as the reference. According to the latest recommendations, multi-resistance was defined as acquired non-susceptibility to at least one agent from three or more different antibiotic groups, and pan-resistance as non-susceptibility to all antimicrobial categories (11).

Definition HIs and data collection

Diagnosis and anatomical localization of HIs caused by *Klebsiella pneumoniae* were determined by the Centers for Disease Control (CDC) criteria (12). Surveillance of HIs included daily clinical examination of patients and daily review of the patients' medical records. To exclude the patients colonized with *Klebsiella pneumoniae,* each of the cases was analysed by an independent expert group composed of an infectologist, epidemiologist, specialist of intensive medicine and clinical pharmacologist. The patients were followed to the final outcome, either cure and discharge from the hospital or death.

Patient data and potential risk factors were extracted from the medical records and the recording order on the standardized questionnaire for each patient, which had two sets of data:

a. related to patient characteristics: demographics (age and gender) and co-morbid conditions (hypertension, diabetes mellitus, cancer of different localization, injury), and
b. related to health care: urgent admission, dates of admission and discharge (to the hospital and to the ICU), diagnostic and therapeutic procedures performed (venous catheters, mechanical ventilation, surgery within a month), date of the first isolation of positive *Klebsiella pneumoniae* culture, and data about administered antibiotics. Prior exposure to antibiotics was defined as administration of a systemic antimicrobial agent for at least 24 hours during the 14-day period before isolation of *Klebsiella pneumoniae*.

The Ethics Committee of the health institution approved the study.

Statistical analysis

The data obtained were processed using descriptive statistics with measures of central tendency (mean), variability (standard deviation from the mean) and relative numbers. The significance of differences in the values of the continuous variables between the study groups was

Table 1. Hospital infection sites caused by carbapenem-resistant *Klebsiella pneumoniae* (CR-Kp) and carbapenem-sensitive *Klebsiella pneumoniae* (CS-Kp) in Intensive Care Unit

Anatomical site	CR-Kp n (%)	CS-Kp n (%)	Total n (%)	p value
Surgical site infection	7 (10.3)	3 (8.1)	10 (9.6)	0.715
Nosocomial pneumonia	53 (77.9)	31 (83.8)	84 (80.0)	0.475
Blood stream infection	8 (11.8)	3 (8.1)	11 (10.4)	0.559
Total	68 (100.0)	37 (100.0)	105 (100.0)	

tested by Student's T-test for independent samples. The chi-square test or Fisher's test (for values in cells of contingency tables lower than 5 or zero) were used for comparison of categorical variables between the groups. Independent variables detected as significant predictors in univariate analysis were later entered into the multivariate logistic regression model. The hypotheses were tested at the 0.05 level of statistical significance. All calculations were performed using SPSS (Statistical Package for Social Science for Windows) software, version 18.

RESULTS

During the study period in the ICU, there were 105 patients who developed HIs caused by *Klebsiella pneumoniae*, according to the pre-defined criteria. Three-quarters were male (n=78; 74.3%) and the average age of the patients was 59.37±15.37 years (range 20-85 years). In 68 patients (64.8%), the HIs were caused by CR-Kp, while 37 (35.2%) of the HIs were caused by CR-Kp. The predominant anatomical location of infection in both groups was

Table 2. Risk factors for hospital infections caused by carbapenem-resistant *Klebsiella pneumoniae* (CR-Kp) (univariate analysis)

Variable	CR-Kp n=68 (%)	CS-Kp n=37 (%)	p value
Age	61.01±14.68	56.35±16.54	0.140
Male gender	50 (73.5)	28 (75.7)	0.810
Comorbidities			
Hypertension	13 (19.1)	4 (10.8)	0.270
Diabetes mellitus	6 (8.8)	1 (2.7)	0.230
Cancer	18 (26.5)	3 (8.1)	0.025*
Injury	21 (30.9)	16 (43.2)	0.205
Concomitant HIs	33 (48.5)	9 (24.3)	0.016*
Invasive procedures			
Central venous catheter	57 (83.8)	18 (48.6)	<0.001*
Periferal venous catheter	63 (92.6)	35 (94.6)	0.702
Mechanical ventilation	58 (85.3)	24 (64.9)	0.016*
Surgical intervention	57 (83.8)	24 (64.9)	0.027*
Hospitalization			
Emergency admission	62 (91.2)	34 (91.9)	0.900
Hospital (days)	37.68±19.84	24.95±11.56	0.001*
Hospital > 1 month	43 (63.2)	11 (29.7)	0.001*
ICU (days)	27.91±12.94	15.78±8.54	<0.001*
ICU > 1 month	32 (47.1)	3 (8.1)	<0.001*
Lenght before HIs (days)	18.21±12.54	10.83±5.86	0.001*
Previous antibiotics use			
Days of antibiotic therapy before HIs	14.15±7.17	7.86±14.91	<0.001*
Piperacillin-tazobactam	4 (5.9)	0 (0)	0.133
Carbapenems	28 (41.2)	3 (8.1)	<0.001*
2nd gen. cephalosporin	18 (26.5)	10 (27.0)	0.951
3rd gen. cephalosporin	22 (32.4)	12 (32.4)	0.993
Aminoglycosides	30 (44.1)	8 (21.6)	0.022*
Ciprofloxacin	18 (26.5)	2 (5.4)	0.009*
Vancomycin	18 (26.5)	1 (2.7)	0.003*
Metronidazol	20 (29.4)	1 (2.7)	0.001*

Results are presented as $\overline{X}$±SD, or n (%);
* significant difference
CR-Kp - carbapenem-resistant *Klebsiella pneumoniae*
CS-Kp - carbapenem- sensitive *Klebsiella pneumoniae*
ICU - Intensive Care Unit

Table 3. Multivariate analysis (logistic regression) of risk factors for hospital infection caused by carbapenem-resistant *Klebsiella pneumoniae*

Risk factors	B	p value	OR	95% CI
Mechanical ventilation	1.807	0.046	6.090	1.030-36.020
Days of antibiotic therapy before HIs	0.165	0.045	1.080	1.003-1.387
Previous Carbapenems use	1.947	0.044	7.005	1.054-46.572
Previous Ciprofloxacin use	1.121	0.007	20.628	2.292-185.687
Previous Metronidazol use	3.697	0.011	40.320	2.347-692.795

Only significant factors are presented for the sake of clarity.
B - coefficient of logistic regression analysis;
OR- Odds Ratio
CI - Confidence Interval

pneumonia (80.0%), followed by bloodstream infections and surgical-site infections (10.4% vs. 9.6%, respectively). The difference between study groups relative to the site of HIs was not statistically significant (p>0.05) (Table 1).

Table 2 shows the results of the univariate analysis of potential risk factors for HIs caused by CR-Kp relative to clinical characteristics, invasive procedures and prior therapy. According to this analysis, risk factors significantly associated with the development of these infections were co-morbidities such as cancer or concomitant HIs, the use of invasive medical procedures such as placement of central venous catheter or mechanical ventilation or surgical intervention, a prolonged stay in hospital/ICU, the use of antibiotics before emergence of infection, and previous administration of carbapenems, aminoglycosides, ciprofloxacin, vancomycin or metronidazole (p<0.05).

Multivariate logistic regression identified five independent risk factors for CR-Kp infections: the use of mechanical ventilation (OR=6.090; 95% CI=1.030-36.020; p=0.046), length of antibiotic therapy before HIs (days) (OR=1.080; 95% CI=1.003-1.387; p=0.045), and previous use of carbapenems (OR=7.005; 95% CI=1.054-46.572; p=0.044), ciprofloxacin (OR=20.628; 95% CI=2.292-185.687; p=0.007) and metronidazole (OR=40.320; 95% CI=2.347-692.795; p=0.011) (Table 3). The Hosmer-Lemeshow Goodness-of-Fit Test for this logistic regression model was χ^2=5.223; p=0.734.

Resistance rates (%) of the tested isolates of *Klebsiella pneumoniae* to other antibiotics are shown in Table 4. For the majority of antibiotics, it was over 85%, except for piperacillin-tazobactam (59.8%) and tigecycline (14.0%). However, isolates of CR-Kp had a significantly higher incidence of resistance to the following antibiotics than the CS-Kp isolates: piperacillin-tazobactam (80.4% vs. 25.8% respectively; p<0.001), amikacin (93.7% vs. 71.4% respectively; p=0.003), ciprofloxacin (98.4% vs. 80.0% respectively; p=0.001) and trimethoprim-sulfamethoxazole (95.0% vs. 76.2% respectively; p=0.029). Nearly two-thirds of the isolates were multi-resistant (n=67; 63.8%), while 7 (6.7%) were pan-resistant.

Lethal outcomes occurred in the 36 (52.9%) patients with CR-Kp and 14 (37.8%) with CS-Kp infections, but the difference was not statistically significant (p=0.139).

In this period, the outbreaks of HIs caused by *Klebsiella pneumoniae* were not recorded.

Table 4. Comparison of antimicrobial resistance of *Klebsiella pneumoniae* to selected antibiotics

Antimicrobial agent	CR-Kp n/N (%)	CS-Kp n/N (%)	p value
Amoxicillin+clavulanic acid	43/44 (97.7)	31/32 (96.9)	0.819
Piperacillin-tazobactam	41/51 (80.4)	8/31 (25.8)	<0.001*
Cefotaxime	44/44 (100.0)	27/28 (96.4)	0.207
Ceftriaxone	52/52 (100.0)	34/34 (100.0)	-
Ceftazidime	61/61 (100.0)	33/33 (100.0)	-
Cefepime	57/64 (89.1)	28/34 (82.4)	0.351
Gentamicin	35/35 (100.0)	23/23 (100.0)	-
Amikacin	59/63 (93.7)	25/35 (71.4)	0.003*
Ciprofloxacin	62/63 (98.4)	28/35 (80.0)	0.001*
Trimethoprim-sulfamethoxazole	38/40 (95.0)	16/21 (76.2)	0.029*
Tigecyclin	6/42 (14.3)	2/15 (13.3)	0.927

n=number of resistant isolates and N=number of isolates with available results
* significant difference
CR-Kp - carbapenem-resistant *Klebsiella pneumoniae*
CS-Kp - carbapenem- sensitive *Klebsiella pneumoniae*

DISCUSSION

Klebsiella pneumoniae has become an important challenge in health-care settings due to its frequency in HIs and the incidence of resistance to the entire spectrum of antibiotics (13). We still do not know what predisposes patients to acquire infections with resistant strains of *Klebsiella pneumoniae*. Therefore, we conducted a prospective cohort study to analyse the association between numerous potential risk factors and HIs caused by CR-Kp among patients in the ICU. Most previous studies were retrospective, which imposed a bias in the interpretation of the results (differentiation between infection and colonization), and data derived from mixed medical and critical care populations are biased because the prevalence of patients was small. Our study showed that the CR-Kp is essentially a "healthcare-associated infection". Multivariate analysis pointed to five significant risk factors, none of which were patient-related.

When overviewing the role of invasive medical procedures in the development HIs caused by CR-Kp, only mechanical ventilation emerged as an independent risk factor, which was expected because previous studies also pointed to this factor (14). According to our results, mechanical ventilation increased the risk of these infections 6.1-fold (95% CI=1.030-36.020; p=0.046). Patients in the ICU often require mechanical ventilation due to respiratory failure, poor gas exchange, and lung and neurologic injury, but this procedure also opens the pathway for the entrance of infectious agents. Microorganisms from the environment adhere to respiratory support equipment and form a biofilm, which is out of reach of antibiotics and neutrophils. Patients with unrecognized CR-Kp colonization are especially important since they are reservoirs for the further transmission of these bacteria (15,16). To reduce these infections, it is prudent to use non-invasive ventilation instead, such as nasal continuous positive pressure ventilation or nasal synchronized intermittent mandatory ventilation.

Previous exposure to antibiotics is often associated with the occurrence of HIs caused by resistant pathogens. For this research, it was defined as at least 24 hours of therapy with antibiotics within 14 days before the isolation of *Klebsiella pneumoniae*. Multivariate analysis showed that the duration of the previous use of antibiotics was associated with a risk of CR-Kp infection. Longer exposure to antimicrobial therapy can be considered an additional factor in the destruction of protective barriers because it promotes colonization and infection by drug-resistant pathogens. The selection of these strains contributes to the broad-spectrum antibiotics widely used in many ICUs. In patients with prolonged antimicrobial therapy, normal microbiological flora are replaced with endemic bacterial strains from the hospital environment, which are often multi-resistant.

In our study, previous exposure to carbapenems predisposed patients for HIs caused by CR-Kp by 7.0-fold (95% CI=1.054-46.572; p=0.044) consistent with the results of previous studies (17,18). According to the case-control study by Wu and associates (14), an independent risk factor for contracting CP-Kp infection is the previous use of carbapenems (OR=12.69, 95% CI=2.09–77.10; p=0.006). These beta-lactam antibiotics with broad-spectrum activity destroy the susceptible bacterial strains that colonized patients and facilitate the selection of multi-resistant pathogens.

Introduction of a new generation of fluoroquinolones with improved activity against respiratory pathogens and improved pharmacokinetics led to the wide use of these antibiotics for empirical therapy in critically ill patients. However, we must better understand their role in the development of bacterial resistance. Our study showed that the risk of HIs caused by CR-Kp after the use of ciprofloxacin was 20.6-fold higher (95% CI=2.292-185.687; p=0.007). Schwaber and associates (19) found that the prior use of fluoroquinolones was related to CR-Kp isolation, and only the strength of association was low (OR=7.2, 95% CI=1.1–49.4; p=0.04). This should be considered when evaluating the potential benefits and harms of administering ciprofloxacin to patients in the ICU.

The previous use of metronidazole is also associated with HIs caused by CR-Kp, which is of broad relevance since it is frequently used in patients residing in the ICU. Metronidazole decreases the burden of anaerobic bacteria and creates room for invasion by multi-resistant hospital flora, including *Klebsiella pneumoniae*.

Broad-spectrum antibiotics select multi-resistant bacterial strains because they eradicate concurrent, yet sensitive, microorganisms. However, in our study, previous use of piperacillin-tazobactam, second- and third-generation cephalosporins, aminoglycosides or vancomycin were not associated with HIs caused by CR-Kp, after adjustment for other variables (aminoglycosides and vancomycin showed significant influences when taken separately, at p=0.022 and p=0.003, respectively). The finding is unexpected, considering the generally accepted link between antimicrobial use and resistant bacteria. This should probably be attributed to the recent administration of a variety of agents to the majority of patients included in the study. Some recent studies have presented results similar to ours (5). All of this suggests that the correlation between antimicrobial use and resistance is a much more complex phenomenon.

Klebsiella pneumoniae became resistant to almost all available antimicrobial agents (20). This is alarming because a high resistance rate for most tested antibiotics was recorded (over 85%), while only tigecycline sensitivity was still preserved (rate of resistance is 14.0%). In addition, two-thirds of isolates were multi-resistant (63.8%). In a recent study similar to ours (21), the rates of antibiotic resistance were lower, as were the percentages of multi- and pan-resistant isolates (47.1% and 1.2%). Treatment of patients with these infections is prolonged, expensive and bears increased mortality (22).

An especially worrying result of this study is the high rate of carbapenem-resistant isolates (n=78; 74.3%). These antibiotics are often used as a drug of last resort in combating gram-negative infections that are resistant to other antibiotics. The most important mechanism of resistance of carbapenems is the production of a carbapenemase enzyme, blaKPC. The gene for this enzyme is carried on a mobile piece of genetic material, which increases the risk for dissemination, even to other *Enterobacteriaceae* (23,24). The emergence and spread of CR-Kp underlines the need for immediate aggressive detection and control strategies to preserve their efficacy in the future (25).

In conclusion, HIs caused by CR-Kp are strongly associated with the use of mechanical ventilation and the duration of the previous use of certain antibiotics (carbapenems, ciprofloxacin and metronidazole). These results should help doctors to identify at-risk patients to implement measures to prevent the onset of CR-Kp infections. The results can also contribute to crafting appropriate institutional policy for antibiotics utilization and the development of effective strategies for the prevention of these infections.

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EFFECT OF VARIOUS AGENTS ON THE DIRECTION OF THP-1 CELL DIFFERENTIATION

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UTICAJ RAZLIČITIH AGENASA NA SMER THP-1 ĆELIJSKE DIFERENCIJACIJE

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ABSTRACT

The ability of physiological (1α,25-dihydroxyvitamin D3, retinoic acid) and non-physiological (various LPS) agents and their combinations to influence the direction of pro-monocytic THP-1 cell differentiation was studied.

The differentiating activity of the agents was evaluated by the expression and the ratio of surface receptors (TLR4, CD11b, and CD14) as well as by the change in THP-1 cell phagocytic activity of different degree of differentiation by Flow cytometry.

The THP-1 cell differentiation by VD3 was shown to lead probably to the formation of classical monocytes.

Summarizing we can conclude that VD3 induces the THP-1 cells differentiation with the formation of classical monocytes and the sequence of 1α, 25-dihydroxyvitamin D3 and non-toxic LPS R. capsulatus PG causes the THP-1 cells differentiation with the formation of inflammatory or intermediate monocytes.

Keywords: *THP-1 cells, vitamin D3, retinoic acid, lipopolysaccharides, receptor expression, phagocytosis*

SAŽETAK

Ispitivama je sposobnost fiziološkog (1alfa, 25-dihidroksivitamina D3, retinoinska kiselina) i ne-fizioloških (različitih LPS) agenasa i njihove kombinacije i uticaj na smer promonocita i diferencijaciju ćelija THP-1.

Diferencijalna aktivnost agenasa je prikazana odnosom površinskih receptora (TLR4,CD11b i CD14), kao i promenom THP-1 ćelije i fagocitne aktivnosti pri cemu je razlicit stepen diferencijacije odredjen protocnom citometrijom.

Rezultati su pokazali da je doslo do diferencijacije ćelija THP-1 pomoću VD3 i verovatno do formiranja klasičnih monocita.

Sumirajući možemo zaključiti da VD3 indukuje diferencijaciju ćelija THP-1 sa formiranjem klasičnih monocita i sekvence 1a, 25-dihidroksivitamina D3 kao i da netoksični LPS R. capsulatus PG uzrokuje diferencijaciju THP-1 ćelije sa formiranjem inflatornog ili međuproizvoda -monocita.

Ključne reči: *TNR-1 ćelije, vitamin D3, retinoinska kiselina, lipopolisaharidi, ekspresija receptora, fagocitoza*

ABBREVIATIONS

CD — cluster of differentiation;
CR3 — complement receptor;
MHC II — major histocompatibility complex,
MD-2 — myeloid differentiation protein 2;
MyD88 — myeloid differentiation protein;
PMA — phorbol myristate acetate;
TLR4 — toll-like receptor 4;
TRIF — adaptor protein;
VDR — vitamin D receptor.

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INTRODUCTION

THP-1 is a line of acute monocytic leukemia cells and good model system for studying differentiation mechanisms and TLR4-mediated cellular response to bacterial lipopolysaccharides (LPS, endotoxins) (1). THP-1 cells express some typical monocyte surface receptors such as TLR4, CD11b, CD14, receptors to Fc-fragment of immunoglobulins and C3b component of complement (2-4).

To obtain a convenient model system of the innate immune cell response to various infectious agents THP-1 cells are differentiated into monocytes or macrophages. During differentiation both physiological agents are used, as vitamin D3 (VD3) and retinoic acid (RA), and nonphysiological agents, as LPS and PMA (5, 6).

LPS as non-physiological agents can induce differentiation of leukemia cells by themselves and also reinforce the differentiating activity of some physiological agents (7, 8). The toxic LPS *E. coli* are shown to reinforce the CD14 and CD11b receptors expression on THP-1 cells differentiated by vitamin D3 (9).

The differentiation is characterized by a change in various cell parameters such as cell morphology, TLR4, CD11b, and CD14 receptors surface expression (4, 10), as well change in the functional activity under various stimuli including LPS (11) compared to the undifferentiated phenotype.

The CD14 was described initially as a marker of monocytes differentiation. CD14 is expressed on 10-15% of the THP-1 cells with activation of expression during the differentiation (12). Thus the increase of CD14 expression on a cell surface is a good marker for cell maturation (13).

CR3 (β_2-integrin, CD11b) is another classical marker of monocytic differentiation. The CD11b/CD18 is expressed in mature myeloid cells and is widely used as an early marker of monocyte differentiation (14). This complex is expressed predominantly on macrophages as a marker of differentiation and provides adhesion (15-17).

Macrophages can be distinguished from monocytes directly in culture by the following strict criteria: change of the cell shape, increase of the cytoplasm volume, and adhesion cell capacity (18).

The populations of macrophages and monocytes are heterogeneous. Based on the primary monocyte markers (CD14 and CD16 receptors) expression the monocytes are divided into classical, inflammatory, and non-classical subpopulations. The classical monocytes with $CD14^{+}CD16^{-}$ phenotype form about 90% of all blood monocytes and are phagocytes (19, 20). Their phagocytic activity is higher than that of macrophages (18). Inflammatory monocytes ($CD14^{++}CD16^{+}$) have a low phagocytic activity but are highly active concerning IL-1β and TNF-α induction in response to LPS (21). Non-classical monocytes ($CD14^{+}CD16^{++}$) synthesize IL-1β and TNF-α in response to DNA and RNA and participate in the development of autoimmune diseases such as rheumatoid arthritis (21). These monocytes express a large number of MHC II and CD32 molecules and are closest to mature tissue macrophages (19, 22). In blood it is difficult to distinguish these monocyte subpopulations, because the development of inflammation is accompanied by a consistent differentiation of classical monocytes to the non-classical subpopulation (23). During the differentiation of monocytes to macrophages the expression of the primary monocyte CD14 marker is decreased. Thus the phagocytic activity seems to lower during the differentiation of monocytes from classical to the non-classical subpopulation.

The discovery of the heterogeneity of the monocyte and macrophage populations caused new studies of the structure and functions of these cells (20, 24-27). However, the literature search showed no data on the subtypes of monocytes obtained from the D3-differentiated THP-1 cells.

An essential feature of the cell differentiation stage is the CD11b/CD14 receptors ratio. The dominance of one of these receptors can indicate the direction of THP-1 cell differentiation. One of the criteria of phagocyte physiological activity is their ability for phagocytosis. The preliminary results of the work are published in the Proceedings of the Conference (28) and patent application (29).

This was aimed to evaluate the ability of physiological (VD3 and RA) and non-physiological (various LPS) agents and their combination to influence the direction of THP-1 cell differentiation. The differentiation stage was evaluated by the expression and the ratio of surface receptors (TLR4, CD11b, and CD14) as well as by the cell phagocytic activity under various differentiating agents.

MATERIALS AND METHODS

Materials

Retinoic acid, 1α,25-dihydroxyvitamin D3, PBS, endotoxin-free cell culture medium RPMI-1640, supplemented with 25 mM HEPES, $NaHCO_3$, L-glutamin-penicillin-streptomycin solution was obtained from Sigma (USA). FBS Standard was obtained from Hyclone (USA). Cell Staining Buffer, Human TruStain FcX™, RBS Lysis buffer was obtained from Biolegend (USA). BODIPY FL conjugate *E. coli* (K-12 strain) BioParticles, *E. coli* BioParticles opsonizing reagent, Trypan blue in citrate-balanced salt solution, pH=4.4 was obtained from Invitrogen (USA).

Toxic LPS *Escherichia coli* 055:B5, *Salmonella enterica* serotype Typhimurium was obtained from Sigma (USA). The non-toxic LPS was isolated from the phototrophic bacterium of the strain *Rhodobacter capsulatus* PG deposited in VKM IBPM RAS № B-2381D (RF) by phenolic extraction as described previously (30).

Alexa Fluor 488-labeled anti-human CD284 (TLR4) Clone HTA125 (eBioscience, USA), anti-human CD14 Clone HCD14, Anti-Human CD11b Clone ICRF44 (BioLegend, USA) monoclonal antibodies (mAb) and isotype matched mouse IgG2a k isotype Crtl (FC) Clone MOPC-173, IgG1 k isotype Crtl (FC) Clone MOPC-21 (BioLegend, USA) controls were used.

THP-1 cell cultivation and differentiation

THP-1 cell line from the ATCC®TIB™202 collection (USA) were cultivated in RPMI 1640 media, containing 2 mmol/l of L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% inactivated FBS in CO_2-incubator (Jouan, France) at 37°C and in humidified air containing 5% (v/v) CO_2. The cells viability determined by the trypan blue staining averaged 94%.

The THP-1 cells (10^6 cells/ml) in RPMI 1640 media containing antibiotics and 10% serum were differentiated by either 10^{-7} M VD3, 10^{-7} M RA, or combination of these two differentiation-stimulating agents of the same concentrations; 500 ng/ml LPS *E. coli*, *S. enterica*, or *R. capsulatus* PG for 72 h at 37°C and in 5% (v/v) CO_2. To study the combined VD3 and LPS action on cell differentiation the THP-1 cells pre-differentiated by VD3 for 72 h were differentiated additionally for 24 h by LPS *S. enterica* or *R. capsulatus* PG. The cells viability averaged 92%.

Expression of TLR4, CD11b, and CD14 receptor on the THP-1 cells and whole blood monocyte

After incubation with the corresponding differentiating agents THP-1 cells were separated from the cultivation media by centrifugation and resuspension in Cell Staining Buffer (10^6 cells/100 μl). To eliminate the non-specific linking of antibodies to the studied receptors Human TruStain FcX buffer was added to each sample and incubated for 10 min at room temperature and then the corresponding mAb (5 μl) was added and incubated in the dark for 30 min at 4°C. The cells were washed twice with Cell Staining Buffer and resuspended in 400 μl of the same buffer.

The blood from healthy volunteers (mean age 25±2 years) of both sexes was collected into tubes (BD, UK) with heparin sodium (17 U/ml) in clinical conditions. Written informed consent was provided by each volunteer. Blood samples (100 μl) were incubated with 5 μl of the corresponding mAb in the dark for 30 min at 4°C. Then erythrocytes were lysed by RBS Lysis buffer and remaining leucocytes were washed twice with Cell Staining Buffer and resuspended in 400 μl of the same buffer.

The receptor expression of the THP-1 cells and monocytes was measured as mean fluorescence intensity (MnI) cells were gated on the basis of FS-SS using EPICS XL-MCL Flow cytometer (Beckman Coulter, United States). At least 6000 events were collected for each experimental variant.

Phagocytic activity of the THP-1 cells

BODIPY FL conjugate *E. coli* (K-12 strain) BioParticles (6×10^9 cells/ml PBS) are used to determine the phagocytic activity of THP-1 cells of various differentiation degrees. The bacteria were opsonized for 1 h at 37°C by opsonizing reagent and added to THP-1 cells of different differentiation degree with the cell:bacteria ratio equal to 1:6. The samples were incubated for 30 min at 37°C. The external fluorescence was extinguished by cold trypan blue solution washed twice by cold Cell Staining Buffer and resuspended in 400 μl of the same buffer.

The phagocytic activity of the cells was measured as mean fluorescence intensity (MnI) of THP-1 cells were gated on the basis of FS-SS using EPICS XL-MCL Flow cytometer (Beckman Coulter, United States). At least 6000 events were collected for each experimental variant.

Statistical analysis

The results are presented as averages with standard errors. The significance of the differences between the sample mean values was assessed by paired Student t-test. The Flowing Software, Microsoft office Excel 2010 (Attestat plug-in), and OriginPro 7.5 were used for statistical analysis and graphical presentation of the data.

RESULTS

Expression of TLR4, CD11b, and CD14 receptors on THP-1 cells.

Results of the influence of physiological (VD3 and RA) and non-physiological (various LPS) agents on TLR4, CD11b, and CD14 expression on THP-1 cells are presented as relative units compared to receptor fluorescence (MnI) of the control undifferentiated THP-1 cells taken as 1 and in Figures 1 and 2.

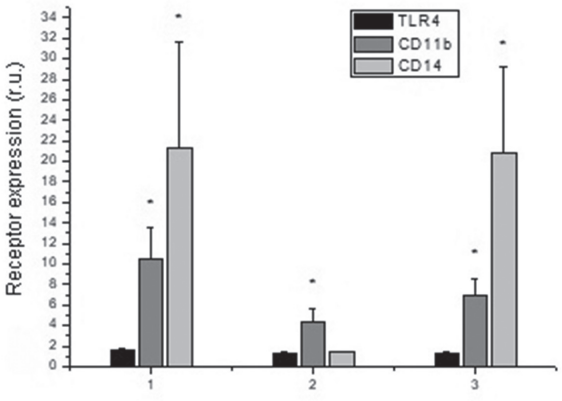

Figure 1. Change in the TLR4, CD11b, and CD14 receptors expression on the surface of THP-1 cells differentiated by 1. VD3, 2. RA, 3. VD3 and RA (n=6). *P<0.05 vs undifferentiated cells.

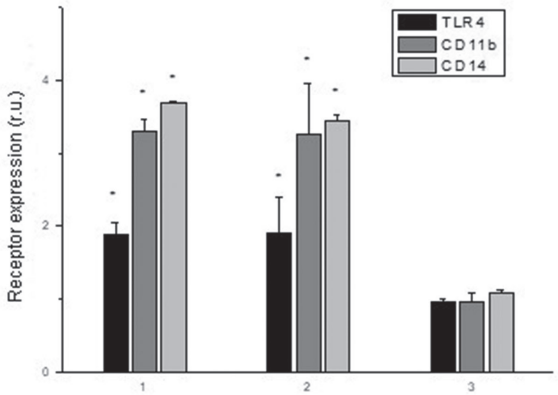

Figure 2. Change in the TLR4, CD11b, and CD14 receptors expression on the surface of THP-1 cells differentiated by 1. LPS *E. coli*, 2. LPS *S. enterica*, 3. LPS *R. capsulatus* PG (n=6). *P<0.05 vs undifferentiated cells.

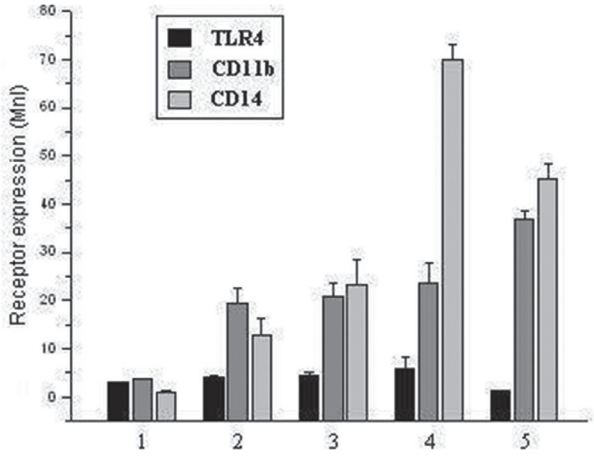

Figure 3. Figure 3. TLR4, CD11b, and CD14 receptor expression on THP-1 cells 1. undifferentiated, 2. VD3-differentiated, 3. VD3- and *R. capsulatus* LPS-differentiated, 4. VD3- and *S. enterica* LPS-differentiated, 5. human blood monocytes (n=6).

We have founded that VD3 and RA had virtually no influence on the TLR4 expression on the THP-1 cells but significantly activated the CD11b expression (in 10 and 4 times, respectively) (Fig. 1).

The combined use of VD3 and RA caused a decrease in CD11b expression compared to the VD3. A notable change in the CD14 expression was observed under the VD3 action. A weaker influence of RA compared to VD3 on the studied receptor expression can be explained by the retinoids action on the myeloid cell lines with the induction of mostly granulocyte formation. The results show that VD3 is the best of the THP-1-differentiating physiological agents since it caused the highest expression of receptors marking the differentiation into monocytes and macrophages.

Furthermore, we studied the influence of toxic LPS *E. coli* and *S. enterica* and of non-toxic LPS *R. capsulatus* PG on TLR4, CD11b, and CD14 receptor expression (Fig. 2). The figure shows that *E. coli* and *S. enterica* endotoxins induced the similar changes in the expression of the studied receptors on the cell surface: the TLR4 amount increased approximately twice and the CD11b and CD14 amount is increased 3-4 times as compared to that in undifferentiated THP-1 cells. Non-toxic LPS *R. capsulatus* PG did not show a significant influence on the expression of the receptors.

In this study we examined the influence of endotoxin *S. enterica* and the non-toxic LPS *R. capsulatus* PG on chang-

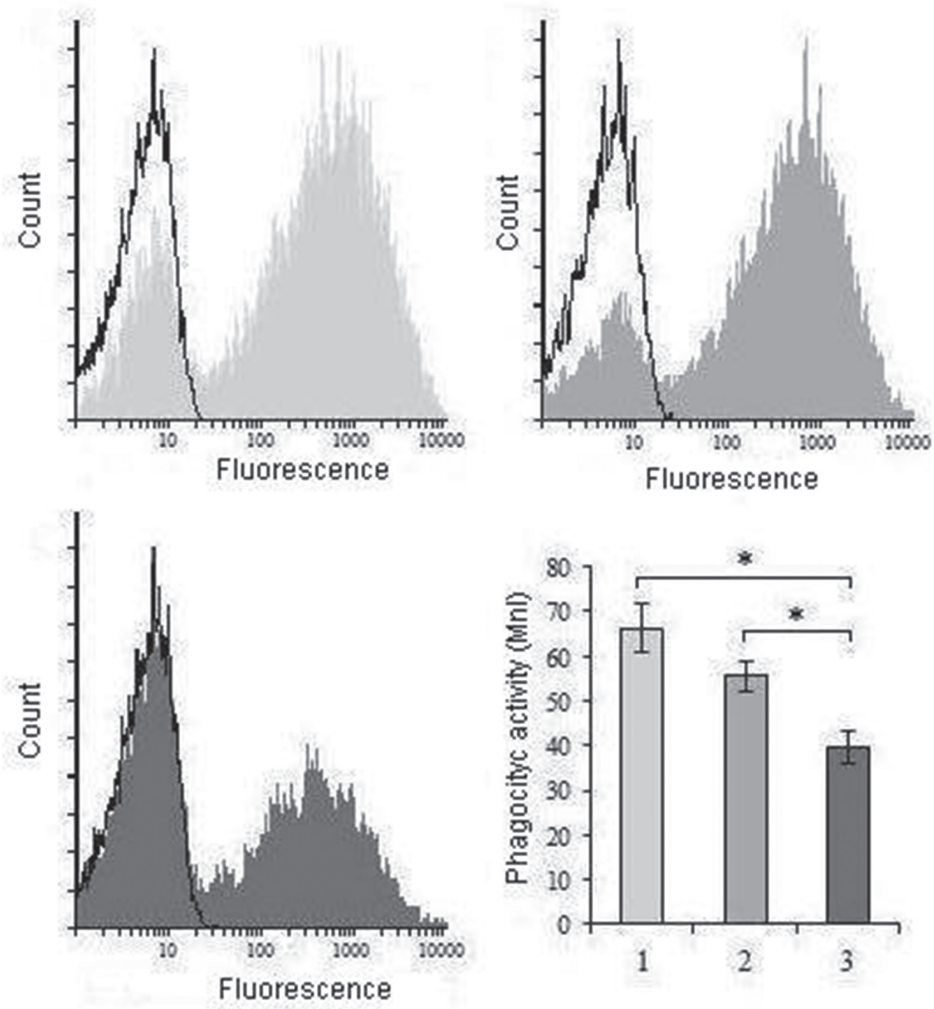

Figure 4. Phagocytic activity of THP-1 cells *a)* undifferentiated, *b)* VD3-differentiated, *c)* VD3- and *R. capsulatus* LPS-differentiated, *d)* average values of the phagocytic activity (n=6). 1. undifferentiated, 2. VD3-differentiated, 3. VD3- and *R. capsulatus* LPS-differentiated. Unshaded area – isotypic control; shaded area – fluorescence of phagocytic cells. *$P<0.05$ between experimental variants.

ing of surface receptor level of the pre-differentiated THP-1 cells by VD3 (Fig. 3). The results show that the THP-1 cells are differentiated by VD3 with the predominance of CD11b receptors expression. Against the backdrop of VD3 both the toxic LPS *S. enterica* and the non-toxic LPS *R. capsulatus* PG changed the ratio of receptor expression to increasing the CD14 level. Concerning the number and ratio of the receptors none of the differentiation agent versions used was sufficient to cause a complete differentiation of THP-1 cells in normal monocytes.

To assess the direction of THP-1 cell differentiation by various agents their CD11b/CD14 receptor ratio was compared with that in the human whole blood monocytes. The decrease in the CD11b/CD14 receptor ratio indicates the direction of THP-1 cells differentiation into monocytes. Table 1 shows that the receptors ratio closest to that on the human whole blood monocytes (0.81 ± 0.05) was observed under the combined action of VD3 and LPS *R. capsulatus* PG on THP-1 cells (0.89 ± 0.33).

Phagocytic activity of THP-1 cells

Phagocytic activity *of THP-1 cells* was determined by absorption of BODIPY FL conjugate *E. coli.* THP-1 cells differentiated by VD3 and additionally differentiated by LPS *R. capsulatus* PG were compared to control undifferentiated cells using the Flow cytometry. The results of cell phagocytic activity measured by the average intensity of fluorescence (MnI) of the cells are presented in Fig. 4 and the data show that LPS *R. capsulatus* PG reduce the phagocytic activity of THP-1 cells previously differentiated by VD3.

DISCUSSION

The promonocyte cells of the THP-1 line are widely used as a model system for the study of monocytic line cells (monocytes and macrophages) responses to various stimuli. To obtain the cells closest to the native ones THP-1 cells are differentiated using various agents and investigated by Flow cytometry using the surface labeling (18, 27). The most commonly used agents are $1,25(OH)_2D3$ and PMA. The study of THP-1 cell differentiation based on morphology, adhesion and phagocytosis ability, loss of proliferation, and expression of CD11b and CD14 showed that PMA stimulates THP-1 differentiation into macrophage-like cells and VD3 causes differentiation into monocytic cell type (5, 13, 18). However the question remains whether the differentiated THP-1 cells are close to the native monocytes and macrophages.

The receptors we investigated play the principal role in the cellular immune responses on endotoxins and are the primary markers of differentiation. TLR4 is identified as a signal molecule essential to the LPS recognition (32). CD14 is considered as receptor transporting LPS to the TLR4/MD-2 complex (33, 34). The TLR-stimulated active CD11b integrin is shown to be involved in MyD88 and TRIF signaling pathways with subsequent inhibition of TLR-signaling in the innate immune responses (35). LPS activate a signal transduction from the TLR4-MD2/CD14/CD11b receptor complex through some kinases to the nuclear factor NF-κB (36). NF-kB-dependent transcription response of differentiated THP-1 cells to LPS is similar to that of the human mononuclear cells (37). The $1,25(OH)_2D3$ is known to modulate the responses of the human monocyte line cells through the NF-kB-dependent activation of the anti-inflammatory target genes (9).

As promonocytes THP-1 cells can differentiate to monocytes (with increased CD14 expression) and macrophages (with increased CD11b expression). Each of the differentiation stages: promonocyte → monocyte → macrophage is characterized by a change in the TLR4, CD14, and CD11b receptor ratios. To determine the THP-1 cells maturity and differentiation degree we assessed the CD11b/CD14 receptor ratio on the cell surface.

The results show that under the VD3 action both the non-toxic LPS *R. capsulatus* PG and the toxic LPS *S. enterica* changed the CD11b/CD14 receptor ratio to increase the CD14 level (Fig. 3, Table 1) which confirms the literature data (9). The non-toxic LPS *R. capsulatus* PG has a weak differentiating capacity but increases the pro-differentiating activity of VD3 in monocyte-like cells. Neither endotoxins nor VD3 nor a combination of the agents cause a complete differentiation of THP-1 cells in native monocytes (Fig. 3). The receptor ratio closest to those in monocytes is observed after the combined action of VD3 and LPS *R. capsulatus* PG (Table 1).

Expression of CD16 is absent on the undifferentiated THP-1 cells (38). The change in the CD11b and CD14 receptor expression on VD3-differentiated THP-1 cells as well as the phagocytic activity allowed us to discover a significant increase in the CD14 level (Fig. 1) and a slight decrease in phagocytic activity (Fig. 4) possibly indicating the formation of the classical monocytes of $CD14^{+}CD16^{-}$ phenotype.

The THP-1 cells differentiation by combination of VD3 and non-toxic LPS *R. capsulatus* PG caused a significant decrease in phagocytic activity and a further increase of CD14 level on the THP-1 cells. Activation of CD14 expression simultaneously with reduction the phagocytic activity of the THP-1 cells differentiated by the combination of VD3 and LPS *R. capsulatus* PG suggests the formation of a phenotype of intermediate or inflammatory $CD14^{++}CD16^{+}$ monocytes involved in response to bacteria and viruses.

It is known that neither CD11b nor CD14 are NF-κB target genes (39). However our findings show that the VD3 and LPS increase the expression of these receptors on the surface of THP-1 cells. This conclusion is also confirmed by the literature data on the mRNA pool increase correlating quantitatively with the CD14 and CD11b protein expression in response to these agents (9, 40). It is possible that the expression of these receptors is regulated through other nuclear factors and alternative receptors to LPS (5). Studies of recent years have shown that LPS in addition to the NF-kB activation also

Table 1. The CD11b/CD14 receptors ratio on the THP-1 cells and human whole blood monocytes (n=6)

Options	CD11b/CD14 ratio
Undifferentiated THP-1 cells	3.42±0.56
THP-1+LPS *R. capsulatus*	4.03±0.38
THP-1+VD3	1.51±0.55
THP-1+VD3+LPS *R. capsulatus*	0.89±0.33
Human monocytes	0.81±0.05

induce activation of PU.1 (41), which is considered to be the central regulator of TLR4 expression (42, 43). PU.1 is also an essential modulator of signals transduction from VDR in the monocytic line cells (44). VD3 induces the expression of the CD11b receptors through the activation of the PU.1/C-Jun transcription factors and the CD14 receptors expression through CEBP/Sp-1 (45). An additional differentiating action of LPS *R. capsulatus* PG on the VD3 background can be due to an increased synthesis of PU.1 which is the principal transcription factor of monopoiesis.

Conclusion

Summarizing we can conclude that VD3 induces the THP-1 cells differentiation with the formation of classical monocytes and the sequence of 1α, 25-dihydroxyvitamin D3 and non-toxic LPS *R. capsulatus* PG causes the THP-1 cells differentiation with the formation of inflammatory or intermediate monocytes.

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PRECLINICAL AND CLINICAL EVIDENCE OF SAFETY OF ANTIVIRAL DRUG WITH IMMUNOMODULATORY ACTIVITY

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PRETKLINIČKI I KLINIČKI DOKAZI O BEZBEDNOSTI ANTIVIRUSNOG LEKA SA IMUNOMODULATORNOM AKTIVNOŠĆU

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ABSTRACT

Antiviral drug Kagocel is widely used in Russia for prevention and treatment of acute respiratory infection, influenza, and herpes. The drug belongs to the group of interferon inductors. The article contains the review and analytical evaluation of safety of antiviral drug Kagocel. Kagocel is registered in the Russian Federation and some CIS countries and refers to the group of interferon inducers. This is a chemical compound of carboxymethyl cellulose and low-molecular natural polyphenol gossypol common in cotton-plant (Gossypium spp.) which protects the plant from depredators and diseases. Authors pay a special attention to the analysis and generalization of data from preclinical and clinical studies including the control of related substances. Absence of free gossypol impurities guaranteed by highly sensitive and specific quality control methods. Preclinical studies data was analyzed and the results were presented with focus on reproductive safety of Kagocel® in immature and mature animals.. No negative effect on animals' reproductive function was revealed including spermatogenesis and generative function. No long-term product effect on reproductive system or next generations of animals was recorded both at therapeutic doses and at doses 10 times their exceeding. The safety of the drug demonstrated on data obtained from numerous clinical trials, including those involving children aged 2 years and older. This confirms the safety of antiviral drug Kagocel usage in clinical practice, including pediatrics.

Keywords: *antiviral drug, interferon inducer, Kagocel, reproductive safety, pediatrics, preclinical and clinical studies*

SAŽETAK

Antivirusni lek Kagocel ima široku primenu u Rusiji za prevenciju i lečenje akutne respiratorne infekcije, gripa i herpesne infekcije. Ovaj pregledni članak sadrži pregled i analitičku procenu bezbednosti antivirusnog leka Kagocel-a. Kagocel je registrovan u Ruskoj Federaciji i nekim zemljama ZND. Lek pripada grupi induktora interferona i predstavlja hemijsko jedinjenje karboksimetil-celuloze i niskomolekularnog prirodnog polifenola gosipola, čestog u biljci pamuk (Gossipium spp.), koji štiti biljku od predatora i bolesti.

Autori posvećuju posebnu pažnju analizi i generalizaciji podataka iz pretkliničkih i kliničkih ispitivanja uključujući kontrolu srodnih supstanci. Odsustvo slobodnih gošipolnih nečistoća garantovano visoko osetljivim i specifičnim metodama kontrole kvaliteta. Podaci o pretkliničkim studijama su analizirani i rezultati su predstavljeni sa fokusom na reproduktivnu bezbednost Kagocela® kod mladih i adultnih životinja. Nije otkriven negativan uticaj na reproduktivnu funkciju životinja, uključujući spermatogenezu i generativnu funkciju. Pored toga, kod primene u terapijskim dozama i u dozama deset puta većim od terapijske nije zabelezen dugoročan efekat Kagocel®-a na reproduktivni sistem ili naredne generacije životinja Bezbednost leka zasnovana je i na podacima dobijenim iz brojnih kliničkih ispitivanja, uključujući i one koji su uključivali decu uzrasta od 2 godine i više. Ovo potvrđuje sigurnost upotrebe Kagocela protiv virusa u kliničkoj praksi, uključujući i pedijatriju.

Ključne reči: *antivirusni lek, induktor interferona, Kagocel, reproduktivna bezbednost, pedijatrija, pretkliničke i kliničke studije*

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INTRODUCTION

Prevention and therapy of influenza and other acute respiratory viral infections (ARVIs) is a critical current issue. Thus, the data obtained from October 2015 to March 2016 for 10 Russian cities evidence that the most affected were children aged 3-6 years old, while hospitalization rate reached maximum in 15-65-year-old age group (65 %). Moderate and severe forms with high hospitalization rate prevailed, viral pneumonia including bilateral ones were found in 10 % hospitalized children and 30 % hospitalized adults (1, 2). World Health Organization currently recommends vaccination to fight influenza, however its efficacy may vary due to extreme influenza virus variability (3). For example, it has been shown that repeated annual vaccinations with the same vaccine strain of the H1N1 virus improve cellular and humoral immunity (4). Therefore, development of an effective antiviral product is of vital importance, and its safety based on comparative analysis of its efficacy and health risk is relevant as well. Safety assessment should be carried out throughout the whole life cycle of the product from development until recall from market (5). Meanwhile, while at preauthorization stage special attention is paid to pharmaceutical safety aspects (quality and stability of the substance and formulation), investigation of toxicity on laboratory animals, efficacy and tolerability in clinical studies, safety evaluation after the product implementation into clinical practice is made within post-marketing clinical studies and pharmacovigilance system. Therefore, safety assessment shall be performed both at preclinical and clinical stages and during clinical use of the product.

HISTORY

Starting from the second half of the last century, interferon inducers were the most demanded medicinal products meeting criteria of safety, multifunctional nature and lack of viral resistance with minimum adverse effects. Interferon (IFN) system forms part of immune system responsible for antiviral defense (6). Medicinal products belonging to interferon inducers possess wide range of antiviral effect (etiotropic effect) and expressed immunomodulating activity. It should be stressed that synthesis of endogenous interferons is balanced and regulated by the body itself providing lack of adverse effects typical for their exogenous administration. Most viruses are known to have no resistance to endogenous interferons (7).

Long-term targeted screening by national researching virologists managed to form a group of original interferon inducers with high therapeutic index suitable for both prevention and therapy of a number of viral infections. Russian drug Kagocel belongs to this class of the products (8). Its single use was associated with longer (120 h) interferon blood circulation as compared to other similar products (9). Oral administration provided maximum production of interferons (alpha/beta) as soon as 4 hours post dosing, and their blood circulation was observed for 4-5 days. The founder of school for development of national interferon inducers, academician of RF RAMS F.I. Ershov reports that Kagocel is one of the most well-studied antiviral agents (10). It causes production of "late" interferons representing a mixture of alpha- and beta-interferons and stimulates production of physiological amounts of gamma-interferons. A certain advantage of Kagocel is its verified efficacy against various viral pathogens. Kagocel was found to be effective against A(H1N1)v, H1N1, H5N1, H3N2 viruses, herpes type 1 and 2 virus (9-11).

Most scientific data on Kagocel are well-known in medical practice. These researches predominantly cover the studies of clinical efficacy of the product. It should be noted that specific weight of publications on safety is limited since the available works are frequently printed in extremely specialized experimental medicine editions. The purpose of the review was to provide data on Kagocel safety to a broad population of practitioners.

Antiviral drug Kagocel is widely used in Russia for prevention and treatment of acute respiratory infection, influenza, and herpes. The drug belongs to the group of interferon inductors.The active ingredient of drug Kagocel is a highly molecular compound synthesized using carboxymethyl cellulose sodium salt and low molecular natural polyphenol, i.e. gossypol (12-15). Carboxymethyl cellulose is a polymeric carrier, a macromolecule conventionally used in alimentary and medical industries. Gossypol is contained in cotton plant, predominantly in free form, and defends the plant from pests and diseases. Numerous multinational studies demonstrated that gossypol produces antitumour, antioxidant and immunomodulating effect. (8-11). However, it should be noted that gossypol use as a medicinal product is limited by low therapeutic breadth of the doses applied, especially systemic ones. Gossypol is known to exert negative effect on erythro- and myelopoiesis, have hepatotoxicity, suppress spermatogenesis, therefore being limited in medicine for long time (16-18). It caused interest due to identification of new properties of its derivatives. A number of scientific studies demonstrated that, by molecular cross-links with polymeric carriers, gossypol loses its toxic properties while retaining antiviral and immunomodulating effects (19-22). It is this approach upon which Kagocel development is based. Kagocel shows expressed antiviral and immunomodulating effects being absolutely safe. Kagocel manufacturers pay special attention to quality control, especially control of impurities of natural polyphenol. So, throughout the life cycle of the product, investigation of its safety at both chemical and biological levels are ongoing.

MANUFACTURING QUALITY CONTROL

Highly sensitive spectrophotometric assay has been generally used to ensure reliable control of impurities of residual gossypol in the substance at safe level. It is noteworthy that the current RF State Pharmacopoeia based on global prac-

tice recommends integrating more specific methods into control and quality systems including high-performance liquid chromatography (HPLC). To implement the method in the manufacturing of pharmaceutical substance of Kagocel, the current HPLC method with spectrophotometric detection was developed and validated to ensure accurate and selective determination of gossypol in Kagocel substance. This method ensures reliable detection of free gossypol impurity with high accuracy and precision at minimum amounts starting from 1.56•10-5 mg/mL. The specified method sensitivity is in line with the international publications on HPLC assay of gossypol (11, 23). It should be mentioned that the method validation demonstrated that whole gossypol amount added to the substance from outside is reliably and accurately identified in this complex evidencing lack of nonspecific sorption of its free molecules on polymeric matrix. HPLC with UV detection revealed that the contents of residual impurities of free gossypol in the product, both after manufacturing and after storage during the established shelf life, is at 0.0002-0.0030 % of the substance weight being 20-100 times lower vs. minimum thresholds for trace constituents in drug substances defined by international pharmacopoeias including the RF State Pharmacopoeia XIII. Such a low level of gossypol impurity guarantees lack of any untoward effect of Kagocel on humans. It should be noted that unfavorable physiological effects of free gossypol are observed when it is administered per os by humans in free form at doses > 0.12 mg/kg (16). Currently, HPLC method is used for Kagocel manufacturing ensuring effective complete elimination of unbound residues of free gossypol. Each batch of the substance is subject to monitoring.

Research unit of the company jointly with researchers from Federal Research Center of Biotechnology of RAS considered potential dissociation of molecules of bound gossypol due to long-term exposure by the components model medium simulating gastrointestinal media. These simulating systems may modify dissolution parameters or cause changes in the proper pharmacological active ingredient. The study results demonstrated that long-term incubation of Kagocel (24-hour) in the model media specified above as well as in a special medium containing microbial cellulase capable of destroying cellulose and its derivatives do not increase levels of free gossypol (19). Therefore, the current methods of purification of the substance from gossypol impurities, hydrolytic stability of its molecule secondary to gastric and intestinal juice exposure and in the medium containing microbial cellulose suggest no toxicity of Kagocel typical of natural polyphenol. However, the information ensuring Kagocel safety may only be obtained in experimental *in vivo* and *in vitro* studies as per current RF MoH regulations.

PRECLINICAL STUDIES

Toxicological characteristics of Kagocel are well-studied, both preclinically and clinically. Pre-authorisation preclinical studies included experiments investigating acute, subchronic and chronic toxicity and specific types of toxicities in the leading research centers of Russia and CIS countries. The experiments involved mice, rats, rabbits and dogs. Acute toxicity study allows to determine tolerable, toxic and lethal doses of test product administered inragastrically, establish the causes of animal mortality during 14-day follow-up period, investigate its effect on general condition and a number of functional and morphological parameters. It should be noted that intoxication signs in acute toxicity study of free gossypol has been investigated thoroughly including respiratory distress, body weight changes, anorexia, weakness, apathy, signs of cardiac failure and death several days later (11). At that, the data from acute toxicity study of Kagocel revealed no animal mortality and no signs of acute intoxication were detected either. In subchronic and chronic toxicity studies, all the study parameters were not different from the ones in control group of animals. The results of studies of specific toxicities revealed that the product has no immunotoxic, genotoxic, allergenic, mutagenic or carcinogenic potential. The experiments showed that Kagocel does not produce negative effect on any aspects of reproductive system of animals and does not affect generative function of males or females, does not cause teratogenic or embryotoxic effect, does not have fetotoxic potential in postnatal or antenatal periods. Therefore, preclinical experiments demonstrated that Kagocel is absolutely safe. Meanwhile, free gossypol in toxicological experiments proved to be a highly toxic compound.

Preclinical studies investigated reproductive toxicity (at the stage of germ cell development) of Kagocel on mature animals (19). Such experiments are mandatory stage of preclinical studies. The results demonstrated that daily 70-day Kagocel administration at therapeutic dose and at the dose exceeding therapeutic one 25-fold does not deteriorate reproductive function of male rats. Embryonic loss parameters in intact female rats coupled to them did not exceed the control values. The litter of male rats receiving Kagocel did not show abnormal changes or retarded physical development; at that, the litter had high survival rate. Investigation of morphological and functional spermatogenesis parameters revealed that weight factor of testicles and tail region of epididymis as well as average sperm count, relative count of its immobile forms, maximum mobility time and number of its abnormal forms were similar to control values. Morphological examination of testicles of rats receiving Kagocel did not detect reduced spermatogenesis index. No suppression of proliferative activity was observed in testicular tissue. The number of sources of proliferative pool of spermatogenesis (normal spermatogonia) was in line with the values in control group. Therefore, Kagocel did not exert negative effect on rat spermatogenesis, while free gossypol at high doses inhibits maturation of male germ cells (24). The data obtained evidence that Kagocel does not exert toxic effect on reproductive system of mature male rats. Given that Kagocel is indicated for prevention and therapy of pediatric viral infections, evalu-

ation of its effect on immature gonads sensitive to various toxic effects is certainly important (25). Thus, reproductive system laboratory under RI of Pharmacology and Regenerative Medicine named after E.D. Goldberg, National Research Medical Center, carried out a number of experiments investigating long-term reproductive safety of Kagocel on immature sex glands (24). These studies were fully in line with the requirements of FSBI "Scientific Center for Evaluation of Medical Products". The experiments included 3 series of studies. The first series investigated potential long-term toxic effect of Kagocel on reproductive system of infantile rats (males, females; aged 10 days). The product was administered for 12 days. The second series investigated reproductive system of rats after administration (48-hour) in pubertal period (aged 52-54 days). The third series investigated potential toxicity of the product after its triple course administration (for 4 days) throughout the whole process of maturation of sex glands (infantile, prepubertal, pubertal periods). In all series of the experiments Kagocel was administered at therapeutic dose and at the dose exceeding therapeutic one 10-fold. Evaluation of reproductive safety was made when the animals reached reproductive age, i.e. long after the product exposure. Kagocel did not reduce fertility of male or female rats. Kagocel does not induce cytogenetic changes in germ cells leading to embryonic loss based on embryonic loss rates. Furthermore, the product does not increase rate of DNA breaks in germ cells in DNA comet assay. Administration of the study product did not exert toxic effect on the litter of animals receiving the product. Body weight of fetuses and infant rats, survival rate, condition of visceral organs and ossification process were similar to control values. Morphometric analysis of testicles did not reveal atrophy of convoluted seminiferous tubules. Seminiferous epithelium of male rats in the study and control groups was represented by spermatogonia, spermatocytes, spermatides, spermatozoa. Thinning of spermatogenous tissue was not observed. Tubular lumens were free, and no reinforced desquamation of dead cells was reported. Spermatocytes and spermatogonia showed active processes of cell division. The number of sources of proliferative pool of spermatogenesis was similar to control values. The latter suggests that spermatogenesis suppression is unlikely within 3 months after the experiment. Lumens between spermatogonia in study and control groups contained Sertoli cells. Their cell membranes did not look damaged suggesting integrity of blood-testis barrier. Testosterone-synthesizing cells (Leydig cells) were found between convoluted seminiferous tubules of all experimental animals. Most of them had specific granularity which is known to be typical of functionally active cells. As reported previously, gossypol administration exerts toxic effect on Leydig's cells and Sertoli's cells causing spermatogenesis suppression at which maturing germ cells stop proliferating. The resulting data evidence that Kagocel does not induce any abnormal changes in testicles of immature animals after reaching maturity. Meanwhile, bibliographic sources suggest that administration of free gossypol to prepubertal and pubertal male rats results in epididymis cysts being potential cause of infertility (26). Therefore, thorough external examination of the tail region of epididymis was performed in experiments investigating potential reproductive toxicity of Kagocel, their weight was determined and weight factor was calculated. The study results demonstrated that male rats receiving Kagocel did not have any cysts. Weight factors of epididymes were in line with the control values (placebo). Three batches of the experiments also investigated morphology of female sex glands of rats receiving Kagocel. It was also similar to that in control animals. Hemodynamic changes were not reported. Glandular tissues contained follicles at various stages of maturity: primordial, with two or more layers of granular cells, Graafian vesicles. In a number of cases, the follicles were atresic. Developing yellow bodies were distinctly visualized. Thecal membranes did not look deorganized. Interstitial cells were intact. Present ovulating follicles in ovaries and the fact that rat fertility did not decrease suggest that Kagocel, unlike gossypol, does not suppress folliculogenesis.

So, administration of Kagocel to immature animals does not produce detrimental effect on their sex glands, reproductive system or litter when reaching reproductive age. The resulting data revealed no long-term sequelae of Kagocel effect on sex glands of immature animals and characterize the product as having high reproductive safety profile. It may be used in pediatrics.

CLINICAL STUDIES

Assessment of safety of a new medicinal product is not limited with experimental trials only. All adverse effects of a new product should be traced at each stage of clinical studies in accordance with the current laws and approved by state regulatory authorities. Phase I clinical studies of Kagocel established its good tolerability at the study doses in healthy volunteers, revealed no allergic reactions or toxic effect on hepatic, renal functions, homeostasis or immunocompetent cells. Further studies of therapeutic and preventive efficacy of Kagocel at specific diseases (ARVI/influenza, herpes) and simultaneously safety of the product were included in phase 2 and 3 clinical trials. Registration randomized, blind, placebo-controlled, multicenter studies of efficacy and safety of Kagocel in adult subjects in the treatment and prevention of influenza and other ARVIs were performed by the leading Russian research institutes: RI of Influenza, RAMS (Saint-Petersburg), D.I. Ivanovskiy RI of Virology, RAMS (Moscow) and S.M. Kirov Military Medical Academy (Saint-Petersburg) in 2000-2002. The study results along with efficacy findings revealed no side events. The subjects administering Kagocel for therapeutic and preventive purposes reported its good tolerability, lack of adverse events of allergic reactions, gastrointestinal complaints or other organ system complaints. According to the results of laboratory tests, Kagocel did not exert nega-

tive effect on hepatic, renal function or hematopoiesis (27). The safety data obtained in clinical studies on adults (lack of adverse effects and good tolerability) as well as lack of adverse reactions in clinical studies according to pharmacovigilance service of the company in addition to preclinical data justified initiation and performance of clinical studies in children. Pediatric studies investigating therapeutic and preventive efficacy of the product in influenza and ARVI and safety were carried out consecutively in 2 steps obtaining data on safety with gradual reduction of children's age: at ≥ 6 years old (2007-2009) and at 2-6 years old (2010-2011). Multicenter, blind, randomized, placebo-controlled clinical studies were performed by test facilities of .I. Ivanovskiy RI of Virology, RAMS, Russian State Medical University, Institute of Immunology, FMBA, Moscow RI of Pediatrics and Pediatric Surgery of Rosmedtechnologies. Adverse events, toxic or allergic reactions to Kagocel in clinical studies were not reported. The children receiving therapy showed good tolerability of Kagocel with no adverse reactions verified by lack of negative changes in peripheral blood, urinalysis and biochemistry over time (27-30). Despite the fact that clinical study in children > 2 years of age was authorized and safety data were obtained for children of this age, patient information leaflet for Kagocel authorizes its use in children > 3 years of age. This is due to the fact that Kagocel is manufactured in tablets which are approved for use in children > 3 years old only. Therefore, starting from 2008, the product is recommended for the treatment of influenza and ARVI in children > 6 years old, from 2011 – in children > 3 years old.

More than 2000 subjects including adults and children > 2 years old were enrolled in blind randomized placebo-controlled clinical studies in 2000-2011. The results of these studies demonstrated high safety of Kagocel along with its high therapeutic and preventive efficacy, both for influenza caused by various types and subtypes of virus (including pandemic one) and ARVI. The data obtained in clinical studies on populations with specific nosologies selected in accordance with strict inclusion/exclusion criteria and performed on a small sample (hundreds, thousands of patients) are critical. However, they do not provide the full pattern of peculiarities of use and tolerability of the product in various populations who may have co-morbidities which are found commonly in actual clinical practice. For this purpose, phase IV studies are carried out which are initiated after the product has obtained state registration for use in broad practice. These post-marketing studies pursue a number of purposes, one of them being to identify previously unknown or potential side effects of the product and risk factors.

In 2016, the results of a large-scale international prospective observational study were published in which therapy of ARVI and influenza with Kagocel was investigated for the first time in outpatient practice involving large number of adult patients (17,266) from 262 medical centers of several countries: Russia, Armenia, Moldova, Georgia. These studies demonstrated good tolerability and efficacy of Kagocel over time, regardless of the time of therapy prescription. The product was found to be combined well with other medicinal products including those for ARVI and influenza therapy, and arising complications were detected (30, 31). The information on adverse effects was obtained in 14 subjects receiving Kagocel. At that, mild to moderate allergic reactions were most common. Based on the composition of excipients of Kagocel tablets, contraindications include individual hypersensitivity to its components, lactase deficiency, lactose intolerance and glucose-galactose malabsorption as well as pregnancy and lactation, pediatric use < 3 years of age (31, 32).

CONCLUSION

Therefore, the published materials suggest that the Russian antiviral medicinal product Kagocel has high safety profile evidenced by experiments on animals and results of clinical studies. Special attention is paid by the manufacturer to quality control of the substance using innovative methods of the substance purification from impurities including gossypol as well as advanced control techniques. The data available suggest that the Russian drug Kagocel is a highly effective and safe antiviral product both for adults and children > 3 years old.

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TANYCYTIC EPENDYMOMA OF THE FILUM TERMINALE REGION; A CASE REPORT

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TANICITIČNI EPENDIMOM FILUM TERMINALE REGIONA; PRIKAZ SLUČAJA

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ABSTRACT

Tanycytic ependymoma is a very rare spindle-cell variant of ependymoma derived from tanycytes, which are part of the primitive nervous system. This paper is presenting 48-year old woman who presented with low back and right-sided leg pain of moderate intensity. MRI showed spinal intradural tumor at the level of the L1 vertebral body. Right-sided L1 hemilaminectomy and en bloc tumor resection were performed. Neuroradiological and intraoperative diagnosis of schwannoma was revised to tanycytic ependymoma after careful immunohistochemical analysis. Six months post-operativly, MRI did not show tumor recurrence. Tanycytic ependymoma at the region of filum terminale is extremely uncommon and only three cases have been described in the literature. The low incidence of this tumor and atypical histological image, which is distinct from the typical features of commonly encountered ependymomas, can present a challenge in terms of making an accurate diagnosis. Awareness of this transitional form of ependymoma among neurosurgeons and pathologists may avoid incorrect surgical approaches and postoperative treatment course.

Key words: *tanycytes, ependymoma, filum terminale*

SAŽETAK

Tanicitični ependimom je vrlo retka forma ependimoma koja vodi poreklo od tanicita, ćelija koje su deo primitivnog nervnog sistema. Ovde je prikazan klinički slučaj pacijentkinje stare 48 godina, kod koje je bolest počela umerenim bolovima u donjem delu leđa i desnoj nozi. MR pregled je ukazao na spinalni intraduralni tumor u nivou L1 prršljenskog tela. Nakon hemilaminektomije na L1 nivou sa desne strane, tumor je uklonjen u celosti. Nakon pažljive analize imunohistohemijskih preparata, neuroradiološka i intropeorativna dijagnoza švanoma je revidirana u tanicitični ependimom. MR pregled nakon 6 meseci od operacije nije ukazao na sigurne znake recidiva tumora. Tanicitični ependimom u filum terminale regionu je izuzetno redak i do sada je u literaturi opisano 3 slučaja. Niska inidenca ovog tumora i atipična histološka slika, koja se razlikuje od drugih čestih ependimoma u ovoj regiji, može biti veliki izazov pri pokušaju donošenja precizne dijagnoze. Podizanjem svesti o postojanju ove forme ependimoma među neurohirurzima i patolozima mogu se izbeći pogrešni hirurški pristupi, kao postoperativna evaluacija i tok lečenja.

Ključne reči: *taniciti, ependimom, terminalni filum*

ABBREVIATIONS

MRI - magnetic resonance imaging
L – lumbar
HE – hematoxylin-eosin

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INTRODUCTION

Ependymomas are tumors of neuroectodermal origin which usually arise from the ependymal cells in the central canal of the spinal cord, the filum terminale region, choroid plexus or white matter adjacent to the ventricular surface of the brain (1). The annual incidence rate of all ependymomas in Europe is around 2 cases per million, occurring more often in men than women (2), and approximately 15% of all patients are children younger than 5 years (3). Spinal cord and filum terminale lesions are typically associated with back pain of long duration, and motor or sensory deficits of lower and upper extremities. Tanycytic ependymoma is an even more rare spindle-cell variant of ependymoma derived from tanycytes, which are part of the primitive nervous system. By reviewing the scientific papers that have been published so far, it is possible to find three similarly described cases of tanycytic ependymoma occurring at the region of filum terminale (4-6).

In the present paper we report a rare case of a tumor of the cauda equina region in a 48-year-old woman in whom the intraoperative diagnosis of schwannoma was revised to tanycytic ependymoma after the application of immunohistochemical stains and careful interpretation. The identification of ependymoma is of a particular significance not only in this case but in the similar cases as well because of the postoperative treatment course of patients and further evaluation.

CASE REPORT

In this paper, we present the case of a 48-year-old female patient, who was admitted to the Centre for Neurosurgery, Clinical Centre "Kragujevac", Kragujevac, because of the spinal intradural tumor at the level of the L1 vertebral body, that is the region of filum terminale, diagnosed by means of a lumbosacral spine MRI. The lesion showed isointensity on the T1-weighted image and slightly higher signal intensity than the spinal cord on the T2-weighted image with minimal enhancement after gadolinium administration.

The patient's discomfort in terms of low back pain of moderate intensity had lasted for a couple of years before she felt the pain in her right leg five months prior to the hospitalization.There was no presence of a neurological deficit in the patient verified on hospital admission and there was no bowel or bladder dysfunction either.

After preoperative preparation the patient underwent surgery on the sixth day of hospitalization. We performed L1 right-sided hemilaminectomy, after which the strictly restricted intradural tumor was removed. The tumor was friable, its colour was gray-white and it was adherent to the filum terminale and spinal nerve. The entire tumor was removed under operative magnification with surgical microscope, the spinal nerves remained undamaged and the resection of the filum terminale was not performed.

The early postoperative course was uneventful. The patient had neither motor nor sensory deficits nor sphincter disturbances. Sutures were removed on the eighth postoperative day, after which liquorrhea occurred in the cranial part of the postoperative wound. The liquorrhea was managed by means of one secondary suture and the restriction of fluid intake.

Pathohistological analysis (HE staining and immunohistochemistry) indicated the presence of the moderate cellular tumor tissue of glial origin and solid and fascicular structure. Spindle-shaped cells formed perivascular rosettes and they were characterized by round to oval, moderately pleomorphic nuclei and grainy chromatin. In the pathohistological sample the cells showed diffuse immunoexpression of glial fibrillary acidic protein (GFAP) and individual expression of S100 protein. The lesion was characterized by the pathologists as a grade II tanycytic ependymoma according to the classification of World Health Organization (WHO) (7).

In the further course of treatment the patient was referred to physical therapy. No adjuvant radiotherapy was offered to the patient. In order to exclude the possibility that the removed ependymoma had occurred due to the liquor dissemination – MRI of the endocranium was performed during a postoperative period, showing no sure signs of expansive lesions.

Three months after the surgery, at the first control examination the patient did not claim to feel any level of pain and discomfort. Six months after the surgery a control MRI of the lumbosacral spine was performed, showing no signs of the recurrence of tumor which had previously been operated on. A control MRI is planned to be performed again in a one-year period.

DISCUSSION

Tanycytes are specialized ependymal cells which line the floor of the third ventricle and provide structural and functional links between cerebrospinal fluid and the perivascular and neural space. They can also be found in the spinal cord and represent the common progenitor cells of both ependymal cells and astrocytes (8).

Tanycytic ependymoma is a form of ependymoma that was initially described by Friede and Pollak in 1978, who represented it as neoplasm of low-to-moderate cellularity characterized by a flow of elongated cells with moderate nuclear pleomorhism and usually absent mitotic figures (9). In these lesions, the classic ependymal rosettes and perivascular pseudorosettes are replaced by more fibrillar cells (10). Neoplastic cells usually do not exhibit anaplastic cytological features, and although it has been assigned for grade II lesions in the current WHO classification (7), it is generally a slow-growing and noninvasive tumor (11).

Just like in the case presented here, the clinical presentation correlates with the anatomic location of the neoplasm. Contrast-enhanced MR imaging remains the

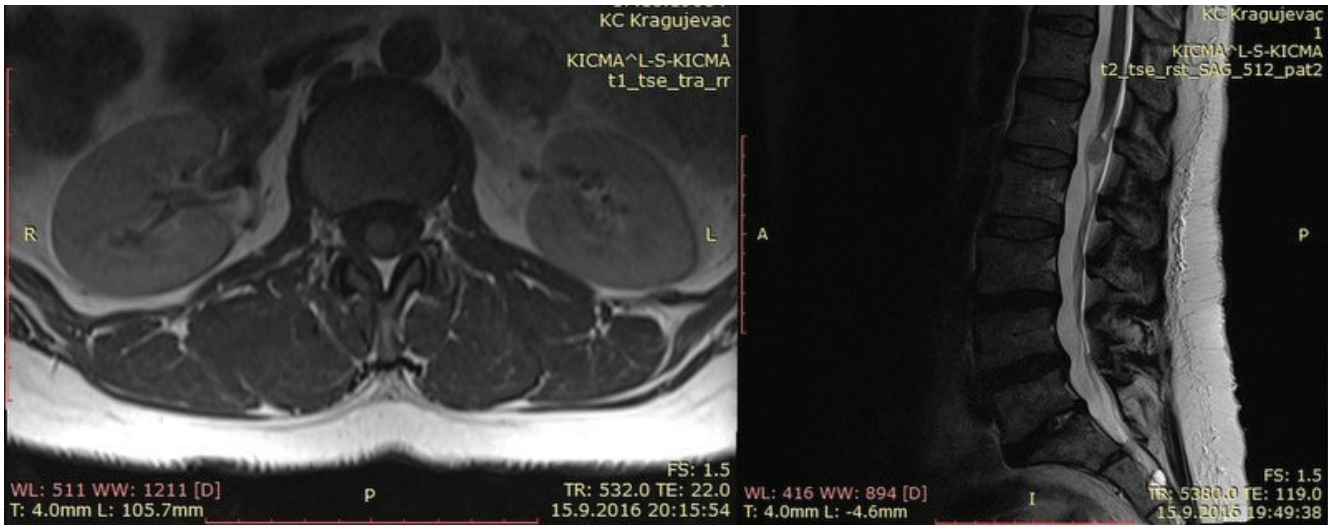

Figure 1. Lesion showed isointensity on the MRI T1-weighted image (1a, transversal plane) and slightly higher signal intensity than the spinal cord on the T2-weighted image (1b, sagittal plane) with minimal enhancement after gadolinium administration.

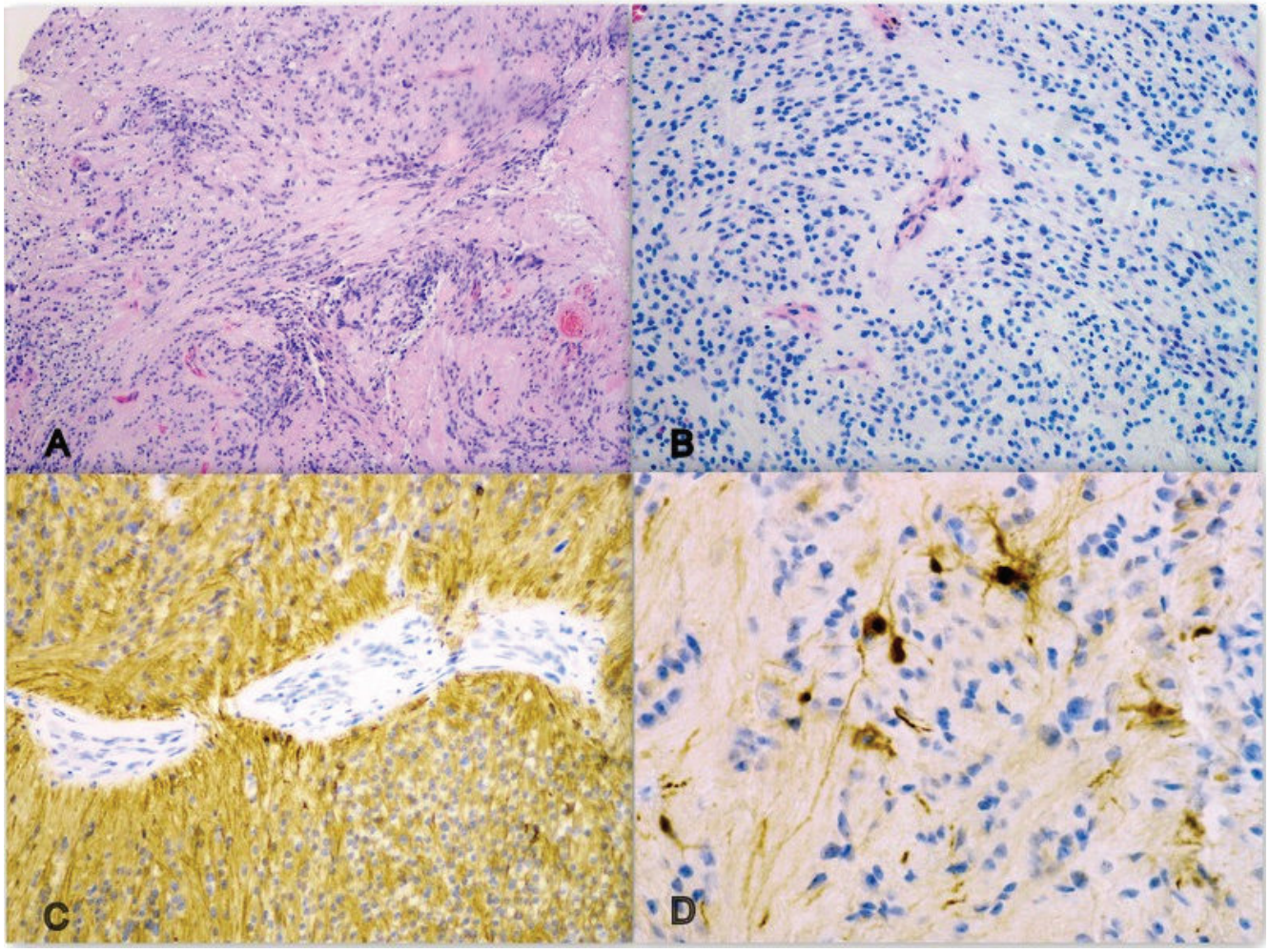

Figure 2.

A. HE x100 1 - The moderate cellular tumor tissue of glial origin and solid and fascicular structure.
B. HE x200 1 - The tumor cells contain round to oval nuclei with the grainy chromatin and form rare structures such as perivascular pseudorosettes.
C. GFAP x200 3 –The tumor cells show diffuse immunoexpression of glial fibrillary acidic protein (GFAP).
D. S100 x400 3 – The tumor cells show individual expression of S100 protein.

radiological investigation of choice. Due to the similar radiological picture as in myxopapillary ependymomas and cystic schwannomas, the final decision in resolving the diagnostic suspense rests with the pathologist.

Intraoperatively, the tumors have a clear cleavage in regard to neural structures but require a microneurosurgical technique for their removal. Tumors usually have minimal vascularity with cystic component, which contain dark-colored fluid. Among cases reported so far (4, 5), no increase in neurological deficits has been noted. Additionally, no tumor recurrence has been detected in the patients, which indicate favorable outcomes, without adjuvant therapy.

The low incidence of these tumors and atypical histological image, which is distinct from the typical features of commonly encountered ependymomas, can present a challenge in terms of making an accurate diagnosis. However, the presence of spindle cells, eosinofil cytoplasm, oval isomorphic nuclei and the absence of Rosenthal fibers indicate the ependymoma rather than other similar tumors (pilocytic astrocytoma, schwannoma, fibroblastic meningioma) (11). Careful immunohistochemical and ultrastructural analyses are necessary to establish the diagnosis of tanycytic ependymoma. Considering the fact that among other ependymomas in the cauda equine region the most frequent type is myxopapillary ependymoma, the diagnosis of the afore mentioned ependymoma can be excluded in this particular case due to the absence of papillary architecture and myxoid degeneration.

CONCLUSION

Tanycytic ependymoma at the region of filum terminale is extremely uncommon and only three cases have been described in the literature. The treatment of tanycytic ependymomas should be conducted in the same way as ordinary ependymomas, since there is no current evidence suggesting that these morphologically distinct tumors differ in terms of biological behavior. A careful histological inspection with utilization of immunohistochemical stains and ultrastructural microscopy may be necessary to distinguish tanycytic ependymoma from other neoplasms such as schwannoma and pilocytic ependymoma. Awareness of this transitional form of ependymoma among neurosurgeons and pathologists may avoid incorrect surgical approaches and postoperative course.

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FOCAL MYOCARDITIS IN PROFESSONAL FEMALE ATHLETE: A CASE REPORT

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FOKALNI MIOKARDITIS KOD PROFESIONALNE ATLETIČARKE: PRIKAZ SLUČAJA

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ABSTRACT

A 35-year-old female athlete appealed to her sports physician on new onset of frequent palpitations, just before an important competition. Initial electrocardiography revealed unifocal premature ventricular complexes in the form of bigeminy. Echocardiography revealed fine-granulated hyperdensic changes in septum. Global strain rate was within a range normal, as well as pulsed tissue Doppler ultrasound. Patient was referred for cardiac MRI, which revealed interventricular septum with rougher compounds, but with preserved continuity, with thickness of 10 mm, which is in the middle of the LV, in length of 5 mm, thinned to a thickness of 4 mm. ELISA laboratory test demonstrated an increased titer of IgM antibodies for adenovirus. Six months later, the patient was referred for control MRI of the heart, which showed pronounced trabeculation of infero-lateral wall of the left ventricle, but without certain criteria for non-compaction cardiomyopathy. There was T1 oedema component in apical septal segment and apical segment of the left ventricle. There was increase of the signal in late gadolinium enhancement in the medial parts of the same segments but also in the segment of the basomedial septum, with previous focal myocarditis. These findings suggest myocardial fibrosis in the segments that were stricken by myocarditis, now without active ongoing myocarditis, but without consequent myocardial fibrosis.

Keywords: *focal myocarditis, sudden cardiac death, athlete, cardiac MRI*

SAŽETAK

Sportistkinja, starosti 35 godina, se javila svom sportskom lekaru zbog osećaja čestih palpitacija, neposredno pre početka važnog takmičenja. Inicijalnom elektrokardiografijom je utvrđeno postojanje unifokalnih preuranjenih ventrikularnih kompleksa po tipu bigeminije. Ehokardiografskim pregledom su uočene finogranulirane, hiperdenzične promene u septumu. Globalni strain rate (naprezanje miokarda u jedinici vremena) je bio u okviru fizioloških vrednosti, kao i vrednosti pulsnog tkivnog Doppler ultrazvuka. Pacijent je podvrgnut MRI ispitivanju srca, čime je otkriveno postojanje grubljeg sadržaja u intervetrikularnom septumu, koji je bio nepromenjenog kontinuiteta i debljine 10 mm, koja se u sredini leve komore u dužini od 5 mm smanjila na 4 mm. ELISA testom je pokazano povećanje titra IgM antitela na adenoviruse. Šest meseci kasnije urađen je kontrolni MRI pregled srca, na kome je uočena izražena trabekulacija infero-lateralnog zida leve komore, ali bez dovoljno kriterijuma za dijagnozu nekompaktne kardiomiopatije. Takođe, postojala je T1 komponeta edema apikalnih delova septuma i leve komore. Postojalo je pojačanje signala uz upotrebu gadolinijuma u medijalnim delovima istih segmenata, ali i u segmentu bazomedialnog septuma, sa prethodnim fokalnim miokarditisom. Ovi nalazi sugerišu fibrozu miokarda u segmentima koji su pogođeni miokarditisom, sada bez aktivnog miokarditisa, ali bez posledične fibroze miokarda.

Ključne reči: *fokalni miokarditis, iznenadna srčana smrt, sportisti, MRI srca*

ABBREVIATIONS

ARVC- Arrhythmogenic right ventricular cardiomyopathy
ECG – Electrocardiogram
LGE - Late gadolinium enhancement
LV – Left ventricle
MRI - Magnetic resonance imaging
MSCT - Multislice computed tomography
nsVT - Non-sustained ventricular tachycardia
PVC - Premature ventricular complexes
RV – Right ventricle
SCD – Sudden cardiac death
SPECT - Single positron emission computed tomography

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INTRODUCTION

Athletes are often perceived as the healthiest portion of the society; however, they are not exempt from sudden cardiac death (SCD). Furthermore, athletes with cardiovascular abnormalities are at greater risk of SCD compared with their non-athletic counterparts (1). Those cardiovascular abnormalities represent a substrate for the development of ventricular arrhythmias that lead to SCD. The most important cause of SCD in athletes older than 35 years coronary artery disease while in the athletes younger than 35 years, the most common cause of the SCD are hypertrophic and dilated cardiomyopathy, arrhythmogenic right ventricular cardiomyopathy (ARVC), electrical cardiac abnormalities (such as Wolf-Parkinson-White syndrome, long QT syndromes and Brugada syndrome) and acquired cardiac abnormalities, such as myocarditis and performance-enhancing drugs (2-3).

Identification of athletes with potentially serious cardiac diseases plays an important role. Pre-participation screening with a 12-lead electrocardiogram (ECG) is effective and may rise suspicion for cardiomyopathies (4). Organizations and sports governing bodies, such as European Society of Cardiology, American Heart Association and International Olympic Committee are positive in the attitude of the obligatory pre-participation cardiovascular screening (5-7). However, not all causes of SCD can be excluded with pre-participation screening, whereas we are still witnessing tragic SCD.

Myocarditis, an inflammation of the myocardium, is one of the major causes of sudden unexpected death in athletes - children and young adults (8). This inflammation may cause acute heart failure and life-threatening arrhythmias (9). Regarding an increased risk of SCD and development of dilated cardiomyopathy (10-11), myocarditis is one of the most challenging diagnoses in cardiology, often due to broad spectrum of its clinical presentation, which overlaps with other cardiac diseases (12). Viral infections of the myocardium are the most common, with up to more than 50 well known cardiotropic viruses, while the most frequent are adenoviruses, enteroviruses and herpes viruses (12). Not so rarely, myocarditis can be also caused by other, not specifically cardiotropic viruses, e.g. H1N1 virus (13).

CASE REPORT

A 35-year-old female athlete appealed to her sports physician on new onset of frequent palpitations, just before an important competition. Previous medical history was clear, as well as family history on cardiovascular diseases and SCD. In the months preceding the symptoms, she stated that there were no signs of infection of the respiratory and gastrointestinal system, but she has had occasional palpitations even for more than 6 months, not paying attention to them until the moment.

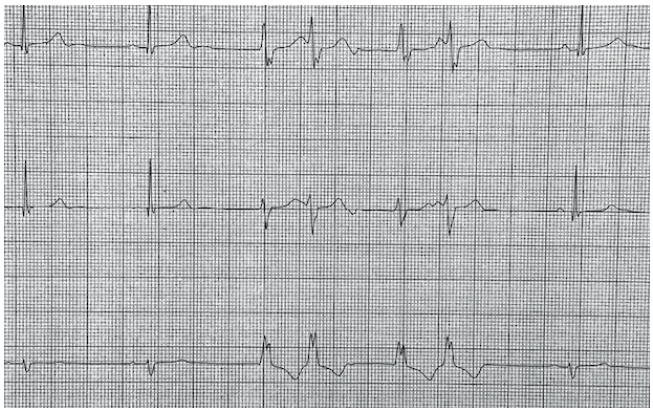

Figure 1. VTns during 24-hour Holter ECG monitoring, indicating origin between anterior and posterior fasciculus of left branch of Hiss bundle. septum and ventricular apex.

Initial electrocardiography revealed unifocal premature ventricular complexes (PVC) in the form of bigeminy and she was immediately referred to the cardiologist. All initial laboratory parameters, markers of inflammation and myocardial necrosis were within normal range. Echocardiography revealed fine-granulated hyperdensic changes in septum, with pericardial adhesions in the region of the posterior wall, without pleural effusion and without segmental and global kinetic disorders, with preserved systolic and diastolic function of the left and right ventricle (LV and RV), with normal flow at all cardiac confluences. Global strain rate was within a range normal, as well as pulsed tissue Doppler ultrasound on the medial and lateral sides of the mitral and tricuspid valves. Right ventricle was without pathological signs of ARVC. Stress test on a treadmill revealed normal coronary reserve, without presence of ischemic heart disease, but in the recovery period, there was the emergence of a large number of uniform PVCs, especially in the form of bigeminy. 24-hour Holter ECG monitoring revealed presence of a large number of isolated PVCs, approximately 200 episodes of ventricular bigeminy and trigeminy and 1 episode of non-sustained ventricular tachycardia (nsVT) with 4 QRS complexes, during sleep. All the above-mentioned PVCs were uniformed, in the term of morphology and pointed out that the origin was from right ventricular outflow tract. However, the episode of nsVT during sleep was not as same morphology as PVCs, but indicated the origin between the anterior and posterior fasciculus of left branch of Hiss bundle (Figure 1.).

After initial clinical examination and standard cardiology treatment, patient was referred for cardiac MRI, which revealed interventricular septum with rougher compounds, but with preserved continuity, with thickness of 10mm, which is in the middle of the LV, in length of 5 mm, thinned to a thickness of 4 mm (Figure 2.). LV had normal morphological characteristics (end-diastolic diameter of 55 mm and end-systolic diameter of 36 mm), without regional contractility disorders and normal systolic function (ejection fraction of left ventricle 62%, end-diastolic volume of left ventricle 198 ml and end-systolic volume of left ventricle 75 ml). RV also had normal morphological characteristics and systolic function.

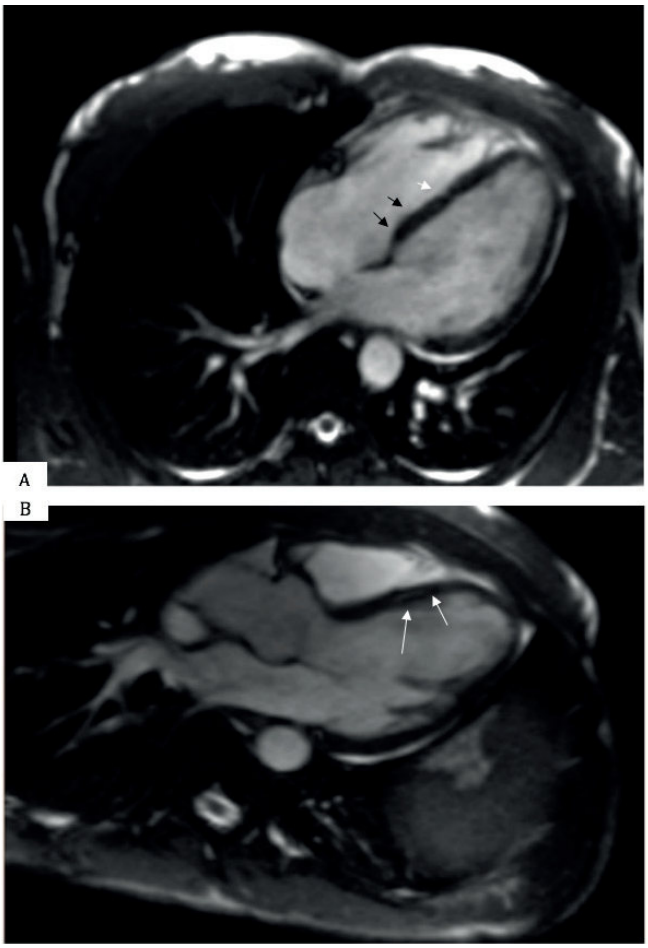

Figure 2. Cardiac MRI. Figure 2A – Four chamber cardiac MRI showing interventricular septum with rough compounds (black arrows), with thickness of 10mm. Thinned interventricular septum is in the middle of LV (white arrows). Figure 2B – Post-contrast T1-weighted delayed midwall septal hyperenhancement (white arrows).

ELISA laboratory test demonstrated an increased titer of IgM antibodies for adenovirus (1.12 with cut-off value of <0.8), confirmed by repeated testing.

Treatment was initiated with low dose of beta-blockers in addition to vitamin and mineral supplementation, as well as coenzyme Q10. She was advised to rest and not to make physical effort in the preceding months. The athlete was in daily contact with her team physician. After 2 weeks she felt better, exercise testing was performed and there was significantly smaller number of PVCs, while inflammatory syndrome was normal.

Six months later, she was subjected to the control echocardiography, which showed bright laminar and grainy echoes in the interventricular septum, while there were no other significant differences in the echo-morphology and functional testing of the heart. 24-hour ECG Holter monitoring showed few PVCs, without episodes of nsVT. Subsequently, the patient was referred for control MRI of the heart, which showed pronounced trabeculation of infero-lateral wall of the left ventricle, but without certain criteria for non-compaction cardiomyopathy. There was T1 oedema component in apical septal segment and apical segment of the left ventricle (Figure 3.). After the delivery of contrast, there was increase of the signal in late gadolinium enhancement (LGE) in the medial parts of the same segments but also in the segment of the basomedial septum, with previous focal myocarditis (Figure 4.). These findings suggest myocardial fibrosis in the segments that were stricken by myocarditis, now without active ongoing myocarditis, but without consequent myocardial fibrosis.

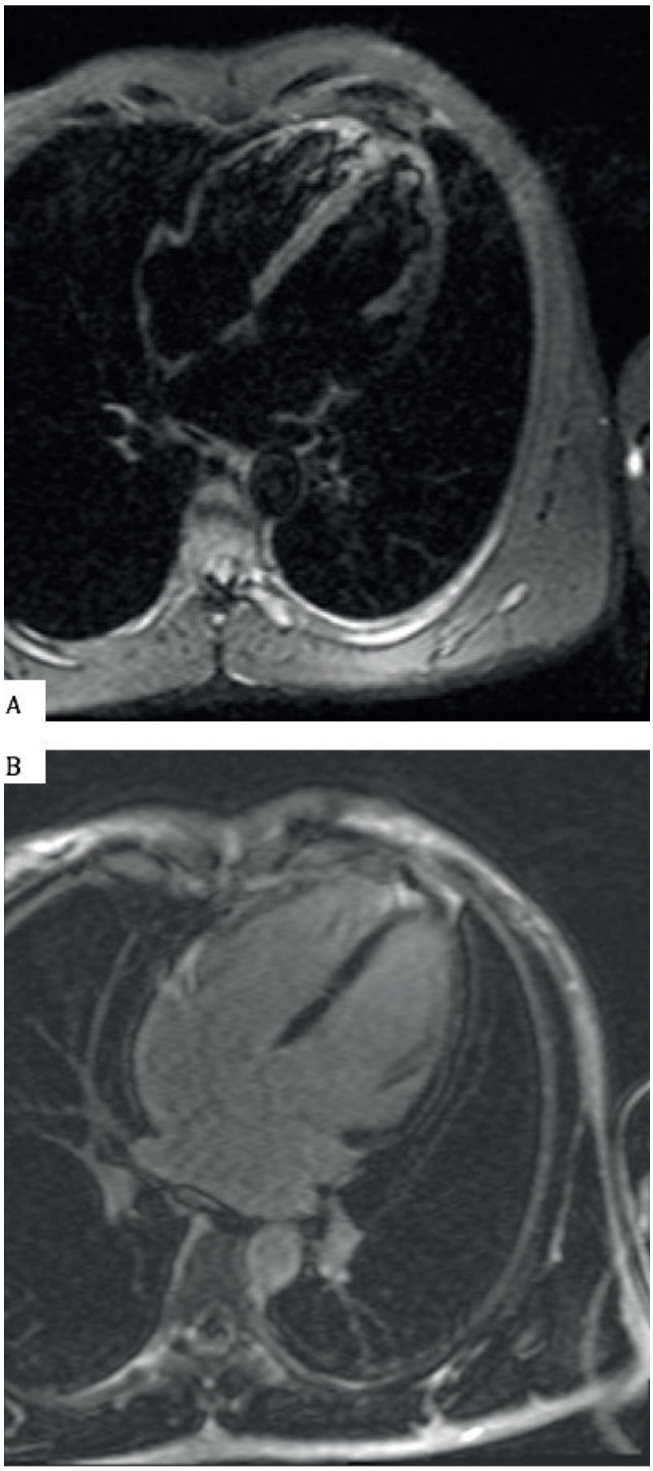

Figure 3. Control cardiac MRI after 6 months. Figure 3A –STIR sequences in septum and the apex. Figure 3B - LGE phenomenon in basomedial

Today, almost a year since the initial event, the patient is feeling good, without any symptoms, she returned to training, but she is under intensive medical supervision because of possible complications in terms of repetitive myocarditis.

DISCUSSION

Current concept of focal myocarditis as an incidental finding is not well established. It is known that small inflammatory foci with necrosis occurs in less than 5% of the autopsied hearts, and is likely a contributing factor in cases od unexplained sudden death (14). However, not all focal myocarditis result in unexplained sudden death. Even though often asymptomatic, a large portion of patients presents with clinical presentation of acute coronary syndrome, new-onset of heart failure and life-threatening arrhythmias (12). Among arrhythmias, most frequent are PVCs, ventricular fibrillation/flutter and sustained ventricular tachycardia.

The significance of focal myocardial inflammation in sudden death is not well established, since there are few studies addressing its frequency in cardiac and non-cardiac arrhythmic death (14). Besides, diagnosis of focal myocarditis can be extremely difficult using conventional techniques such as electrocardiography, echocardiography and standard laboratory tests (15). Nevertheless, detailed anamnesis and clinical examination combined with standard diagnostic techniques (ECG, echocardiography and laboratory tests) may arouse suspicion on focal myocarditis. Endomyocardial biopsy identifies the inflammation markers only in patients with extensive myocarditis (16). Further noninvasive cardiac methods, such as multislice computed tomography (MSCT), single positron emission computed tomography (SPECT) and magnetic resonance imaging (MRI) have been proposed to detect foci of myocarditis (17).

In recent years, MRI has been identified as important diagnostic tool in differential diagnosis of patients with suspected myocarditis, especially in young patients (18). LGE and T2-weighted MRI images are currently crucial diagnostic criteria for defining a focal myocardial injury, however, they are typically compared to the normal appearing myocardium as a reference for diagnosis of myocarditis (19-20). This often leads to errors since intensity and distribution of inflammatory infiltrates are highly variable (21-22). Also, LGE signal differs from one study to another, it is influenced by technical parameters, including the threshold set for differentiation of normal vs. fibrotic myocardium (23). In search for assessment of myocardial injury without need for arbitrarily defined reference tissue, T1 and T2 MRI mapping are emerging (24). Recent studies showed that these mapping techniques can improve diagnostic value of MRI in patients with suspected myocarditis (25). In the study of Radunski et al (18), they even showed that the amount of myocardial injury assessed by LGE imaging was underestimated, as well as that T2 values in normal appearing myocardium were altered to a lesser degree compared to the native T1 and values of extracellular volume fraction. In addition, Ferreira et al (26), demonstrated the value of native T1 mapping to asses' focal myocardial injury in patients with myocarditis.

The significant cost of MRI and logistical limitation is keeping away its integration into the routine pre-participation. Although, there are proofs that ECG screening can reduce the incidence of SCD in athletes (27), it has also been controversial (28). Despite evidences for incorporating ECG in standard pre-participation screening, American Heart Association does not support routine use of ECG and false positive results and cost-effectiveness are main arguments (2). In United States, a standard 12-channel ECG with symptoms, family history and physical examination is recommended, while in Italy, it is mandatory for all competitive athletes (2). One thing is certain, ECG provides information regarding electrical abnormalities of the heart and is also effective in identifying cardiomyopathy, however, some structural abnormalities may pass unnoticed. Therefore, structural assessment of the heart may have its role. Transthoracic echocardiography is the primary modality for cardiomyopathies and myocarditis, but small focal lesions, such as we have seen in our case report, may not necessarily be seen on echocardiography. That is why alternative modalities, such MRI, should be considered in selected individuals.

In general, pre-participation screening with detailed physical examination with family history and symptoms as well as standard, 12-channel ECG can provide sufficient information and arouse suspicion for cardiac abnormalities that could lead to SCD in athletes. In addition, echocardiography and cardiac MRI can readily examine morphology and function and could lead to timely initiation of the treatment which is crucial.

CONCLUSION

Here, we present an interesting case report of a young female athlete with focal myocarditis caused by adenoviral infection. Although echocardiography even with advanced techniques (pulsed Doppler tissue imaging and strain rate) did not point out a possible cause, cardiac MRI revealed focal, inflammatory lesion in the interventricular septum and inflammatory modulation has been confirmed by ELISA test. It is also notable, that prompt diagnosis and treatment in this case lead to the rapid recovery of the patient and not to possible and tragic SCD.

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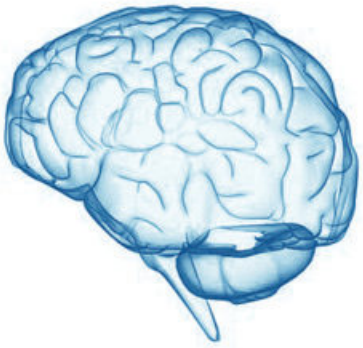

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