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PATOGENETSKI DOKAZ O TERAPEUTSKIM I PREVENTIVNIM MERAMA KOD OZBILJNE INFEKCIJE KORONAVIRUSOM

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ABSTRACT

The basis of coronavirus disease is an infectious process, accompanied by a varying degree of activity of pathological processes. Based on the study of the pathological course of infection, modern approaches to the treatment and prevention of complications of coronavirus infection are presented. The main strategic pathogenetic direction in the creation of effective programs for the treatment of COVID-19, as well as the prevention of fatal complications, should be a set of measures enhancing permissive regulatory influences and events. Endothelium, being a source of inflammatory mediators and a transducer of their regulatory effects on the vascular tone, is involved in the development and alternation of vascular reactions, changing the volume of perfusion. The main mechanism for the development of endothelial dysfunction and damage is associated with an imbalance between the generation of reactive oxygen species and the power of the antioxidant defense system. Any measures to protect the endothelium, reducing the severity of microcirculatory disorders and hypoxia, will have a therapeutic and preventive effect on fatal complications. In this regard, in the treatment of COVID-19, the use of synthetic gas transport preparations based on perfluorocarbon nanodispersed emulsions with a clinical effect directed at once to several pathogenetic links underlying the progression of COVID-19 disease can be quite effective. The necessity of a comprehensive effect on pathogenesis using sanogenetic principles of treatment, allowing influencing the speed and time of onset of resolution of inflammation, which can reduce the number of complications and deaths of the disease, is substantiated.

***Keywords**: COVID-19; inflammatory mediators, endothelium damage, treatment; prevention.*

SAŽETAK

Osnova bolesti izazvane koronavirusom je infektivni process praćen različitim stepenom aktivnosti patoloških procesa. Zasnovan na proučavanju patološkog toka infekcije, savremeni pristupi lečenja i prevencije komplikacija nastalih usled infekcije koronavirusom su predstavljeni. Glavni strateški patogenetski pravac u kreiranju efikasnih programa za lečenje COVID-19, kao i za sprečavanje fatalnih komplikacija, trebalo bi da bude niz mera koje pospešuju dozvoljene regulatorne uticaje i događaje. Endotel, izvor inflamatornih medijatora i transduktor njihovih regulatornih efekata na vaskularni tonus je uključen u razvoj i izmenu vaskularnih reakcija, menjajući volumen perfuzije. Glavni mehanizam za razvoj endotelne disfunkcije i oštećenja je povezan sa neravnotežom između stvaranja reaktivnih vrsta kiseonika i snage antioksidacionog odbrambenog sistema. Bilo koje mere da se zaštiti endotel, smanjujući ozbiljnost mikrocirkulatornih poremećaja i hipoksije, će imati terapeutski i preventivni efekat na fatalne komplikacije. U ovom pogledu, u lečenju COVID-19, upotreba sintetičkih gasnih transportnih preparata, zasnovanih na perfluorokarbonskih nanodisperzovanih emulzija sa kliničkim dejstvom usmerenih neposredno na nekoliko patogenetskih veza koje su u osnovi progresije COVID-19 bolesti, može biti sasvim efikasna. Potreba sveobuhvatnog dejstva na patogenezu korisćenjem sanogenetskih principa lečenja, omogućavajući uticaj na brzinu i vreme početka rezolucije inflamacije, što može da smanji brojne komplikacije i smrt usled bolesti, je potvrđena.

***Ključne reči:** COVID-19, inflamatorni medijatori, oštećenje endotela, lečenje, prevencija.*

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ABBREVIATIONS

ACE - angiotensin-converting enzyme;
AM-1 - endothelial-leukocyte type 1 adhesive molecule;
ARDS - acute respiratory distress syndrome;
CO - carbon monoxide;
COVID-19 - Coronavirus Disease;
DHA - docosahexaenoic acid;
EC - endothelial cells;
ED - endothelial dysfunction;
EL ICAM - integral intercellular adhesive molecules;
EPA - eicosapentaenoic acid;
ESL - endothelial surface plate;
ESR - erythrocyte sedimentation rate;
FAT - platelet activation factor;
H2S - hydrogen sulfide;
IL - interleukin;
LC - leukotrienes;
NO - nitric oxide;
NO3 - peroxynitrite;
NOD - like – nucleotide-binding oligomerization domain-like;
PG - prostaglandins;
PRRs - pattern recognition receptors;
ROS - reactive oxygen species;
SARS-CoV2 – severe acute respiratory syndrome coronavirus-2;
SPMs - specialized pro-resolving mediators;
SPON - multiple organ failure syndrome;
TMPRSS2 - transmembrane serine protease 2;
VC AM-1 - vascular cell adhesive type 1 molecule.

INTRODUCTION

The basis of coronavirus disease is an infectious process, accompanied by a varying degree of activity of pathological processes. Among the main hallmarks of pathogenesis of coronavirus infection, there are inflammation, endothelial dysfunction, hypoxia, metabolic disorders, microcirculatory disorders, as well as impaired function of organs and systems (respiratory, cardiovascular, excretory, liver, digestive, nervous, endocrine, etc.). Inflammation includes a local reaction and a systemic inflammatory response. Endothelial dysfunction, as a component of the inflammatory response, is simultaneously a key link in pathogenesis, and a link between inflammation and other pathological processes (1). These pathological processes unfold and reach their greatest severity during the peak of the disease, determining the main clinical manifestations, consequences and outcome of the infectious process. During this period of time, as a rule, the action of the etiological factor is completed, diagnostics have already been carried out and treatment of patients begins (2).

Modern strategies for treating infections imply killing bacteria and/or inhibiting their growth, and in the case of viral infections, the inhibition of viral replication. The implementation of such strategy during the peak of the disease has prophylactic importance in the prevention of generalization of the infection and the occurrence of complications, but, on the other hand, it is not very effective in the treatment of developed infections. At the same time, therapeutic measures should aim to suppress inflammation and key pathogenetic mechanisms underlying the dysfunction of organs and systems due to this inflammatory process or other pathological processes aggravating the patient's condition (3).

PATHOGENETIC MECHANISMS UNDERLYING SARS-COV2 INFECTION

To date, a consensus has been formed that the penetration of SARS-CoV2 virus (severe acute respiratory syndrome coronavirus-2) into cells is mediated by its interaction with ACE2 receptor protein (angiotensin-converting enzyme 2), as well as with transmembrane serine protease 2 (TMPRSS2) and cathepsin L, which allow the virus to enter the cell and cause it to produce new virions (4-7).

ACE2 is a membrane-bound protein-enzyme, cleaving one amino acid from the C-terminal part of angiotensin II, it turns it into a vasodilator angiotensin 1-7, performing a counter-regulatory function with respect to ACE1, which is involved in the formation of angiotensin II (8). It should be noted that this ACE2 receptor protein is widely represented in the human body, and is found not only in the pulmonary epithelium, but also in endotheliocytes, smooth muscle cells, myocardium, kidneys, gastrointestinal tract, bone marrow, spleen and other organs and tissues. It is believed that such wide representation of ACE2 allows the variety of clinical manifestations of SARS-CoV2 infection and the possibility of developing multiple organ failure syndrome as a complication (5, 9, 10).

It is extremely interesting that the ACE2 gene activity could be stimulated by interferons. This may explain the sudden aggravation of the course of the disease, for example, due to an avalanche-like increase in the lung damage and the occurrence of a cytokine storm (11).

ROLE OF ENDOTHELIAL CELLS IN SARS-COV2 INFECTION

However, this variety of symptoms may also form for another reason. The explanation, built on the basis of the ubiquity of receptor proteins, does not take into account differences in their availability to the action of the virus, the possibility of indirect cell damage, and, consequently, regional characteristics of resistance and reactivity. The number of potential target cells also matters, which undoubtedly affects the likelihood of infection. In this regard, it should be noted that in addition to the pulmonary epithelium, one of the most likely targets for damage are endothelial cells. These are made not only by the high prevalence in the body (occupying the 2nd place after muscle cells), but also by their strategic location (on the border of two media, between the blood and tissue), as well as the importance of functions of the endotheliocyte for the task of ensuring the transport of substances between the blood and tissues (2, 5, 8, 12).

The following list can give some idea of the multiplicity of endothelial functions. The main role of the endothelium is to maintain homeostasis by regulating the following processes in the body:

1. Vascular tone (the balance of vasoconstriction and vasodilation);
2. The anatomical structure of blood vessels (potentiation and inhibition of proliferation factors);
3. Hemostasis (potentiating and inhibition of coagulation, fibrinolysis and platelet aggregation factors);
4. Local inflammation (the development of pro-inflammatory, anti-inflammatory and repairing factors);
5. The development of a systemic inflammatory response (reactions of the acute phase of inflammation), which includes responses of the functional systems of the body at the neuroendocrine, hematopoietic, metabolic and hepatic levels (the examples of such physiological changes are fever, drowsiness and anorexia, hematopoietic shifts, including anemia, leukocytosis and thrombocytosis, while osteoporosis and decreased gluconeogenesis are the examples of metabolic changes). The combination of the above phenomena is included in the prodromal syndrome and is accompanied by other deviations in the plasma concentration of proteins of the acute phase of inflammation, for example, serum amyloid A and C-reactive protein;
6. Vascular support (angiogenesis and angioregression);
7. Formation of stroma of a tissue or organ (endothelial-mesenchymal transition, formation and apoptosis of myofibroblasts, synthesis and destruction of collagen) (13, 14).

Based on this list, one can approximately imagine the entire possible variety of clinical manifestations and consequences associated with the endothelial damage, including the infectious inflammation, for example, with the coronavirus disease (15).

SYSTEMIC MANIFESTATIONS OF SARS-COV2 INFECTION

Given the diversity and importance of the vital functions of endotheliocytes, as well as the systemic nature of disorders that occur when they are massively defeated, it can be confidently stated that the endothelial dysfunction plays a key role in the pathogenesis of developing acute respiratory distress syndrome (ARDS) and multiple organ failure syndrome, and also participates in the development of subsequent complications in the form of fibrotic dysplasia and functional impairment (1, 2, 12).

One of the most formidable complications of a coronavirus pandemic, which WHO called the 2019 Coronavirus Disease (COVID-19), is a severe acute coronavirus respiratory syndrome 2, often fatal (16). Clinicians noted that most patients experienced a sudden deterioration in their health in the later stages of the disease or during the onset of recovery. The acute respiratory distress syndrome (ARDS) and multiple organ failure quickly occurred, the combination of which in many cases was fatal (5, 17).

The delayed occurrence of complications is evidenced by the data on the absence of disorders of the cardiovascular and urinary systems upon admission of patients to the hospital. In this case, acute damage to the kidneys and myocardium usually occurred 8-14 days after the onset of symptoms and was the precursor of an unfavorable prognosis of the course of the disease (5, 18).

Several factors which increase the risk of death have been identified among the adults in Wuhan hospitalized due to COVID-19. Laboratory abnormalities at the admission included lymphopenia, increased levels of C-reactive protein, lactate dehydrogenase, highly sensitive cardiac troponin I, liver transaminases and D-dimer (48). Some studies have reported elevated levels of other inflammatory markers, such as IL-6, ferritin, and ESR (5).

Signs of negative dynamics were also recognized as higher body temperature upon the admission, increased respiratory rate, decreased albumin levels, and increased blood coagulation. In addition to laboratory indicators and basic vital signs, a negative prognosis was evidenced by old age, chronic diseases of the cardiovascular and respiratory systems, obesity, diabetes mellitus, chronic kidney disease, and others. Sepsis was diagnosed in half of these patients (18-21).

As you know, the basis of sepsis is the systemic inflammatory response syndrome (SIRS), which is a hyperergic systemic reaction of the whole organism to an infectious irritant. It is a complex of the systemic phenomena that occurs under the action of cytokines and is also known as the “acute phase” or “preimmune response” response. Despite the fact that this reaction is protective and adaptive in origin, its excessive nature in the case of sepsis, when the so-called unregulated cytokine storm occurs, can cause massive

irreversible dysfunction of vital organs and even lead to death (22-24).

These manifestations of the systemic inflammatory response usually include fever, arthralgia and myalgia, sleep disturbance, decreased appetite, changes in the functioning of physiological systems (respiration, blood circulation, digestion, urination, etc.), as well as changes in the laboratory parameters: increased ESR, leukocytosis, dysproteinemia (C-reactive protein, amyloid A and P, transferrin, ceruloplasmin, immunoglobulins, enzymes, etc.). Considering the mechanisms of the occurrence and development of these manifestations, we can conclude that one of the central roles here is played by ED, which develops under the influence of cytokines. ECs involved in the formation of cytokines also contribute both to the development of general inflammatory reactions and, ultimately, to irreversible dysfunction of vital organs and SPON (multiple organ failure syndrome) (1, 2, 24-27).

To date, the evidence has been obtained that the adverse development of coronavirus infection is due to the development of a cytokine storm (5, 18, 28).

This cytokine disturbance correlates with the severity of endothelial dysfunction, however, the causality of this relationship has not been established, obviously due to the presence of both direct and reverse causal relationships, and also because these phenomena develop in parallel within the framework of the general inflammatory process (2, 15, 22, 28).

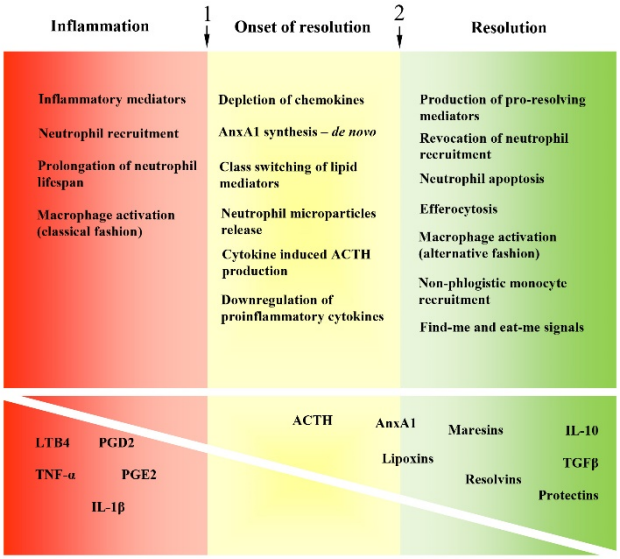
A progress in the study of humoral mediators controlling the inflammation process has led to a change in the outlook on the resolution of inflammation, which, in contrast to previous ideas, is an active rather than a passive process. The main biological activities and functions of pro-resolving mediators are determined, which include:

1. Limitation and termination of penetration of polymorphonuclear neutrophils into tissues;
2. Reducing damage to the surrounding tissues by phagocytes;
3. Reducing the resolution interval;
4. Strengthening of macrophage phagocytosis and efferocytosis;
5. Counteraction to pro-inflammatory chemical mediators (platelet activation factor – FAT, leukotrienes – LC, prostaglandins – PG);
6. Increase in anti-inflammatory mediators (IL-10 and others);
7. Increased destruction of microbes and their clearance by innate immune cells;
8. Improving tissue regeneration.

Thus, “Resolution” is a highly coordinated process at different hierarchical levels (from molecular to tissue, and possibly to systemic), which serves to repair and regenerate damage in organs and tissues, as well as to normalize their functions (29-31).

In the process of acute inflammation, three stages can be distinguished (Figure 1). If we correlate and analyze in chronological sequence all these three stages of the infection process with the adverse development of COVID-19, then we can draw the following conclusions:

Figure 1. A review of the cellular and molecular processes that control inflammation and its resolution.



(Quoted from Michelle A. Sugimoto, Lirlândia P. Sousa, Vanessa Pinho, Mauro Perretti, Mauro M. Teixeira, Resolution of Inflammation: What Controls Its Onset?, Front Immunol. 2016; 7: 160., doi: 10.3389/fimmu.2016.00160 (32)).

1. The period of acute inflammation corresponds to the periods of the height of the disease and recovery. In this case, the onset of inflammation and the onset of resolution correspond to the height of the peak, and the period of recovery corresponds to the resolution and restoration of homeostasis;

2. The signs of unfavorable course of the disease are: higher temperature, leukocytosis, lymphocytopenia, creatinine, lactate dehydrogenase, high sensitivity troponin I, D-dimer and increased markers of inflammation, such as ferritin, IL-6 and procalcitonin, hypercoagulation and myocardial damage, as risk factors for death, they occur at the beginning of the height of the disease, correlate with the severity of the disease, and also progress throughout the disease (Fig. 1 arrow No. 1) (18);

3. The time period during which a change in the course of events can occur (either recovery, or the occurrence of complications and aggravation of the condition) falls at the end of the period of the disease height, before the recovery stage (in Fig. 1 arrow No. 2). This is consistent with the theoretical

model for the progression of COVID-19 disease described previously (5, 28).

Thus, in our opinion, the time-varying balance of pro-inflammatory and pro-resolving mediators that are produced during the inflammation is crucial for determining the fate of an inflammatory reaction. From the review, it becomes clear that one of the possible reasons for the development of a cytokine storm with a hyperergic uncontrolled systemic inflammatory response may be the absolute or relative insufficiency of pro-resolving factors existing or arising in the development process. This insufficiency can occur due to the delay in switching the synthesis of pro-inflammatory mediators to pro-permissive ones. It has traditionally been believed that excessive production of pro-inflammatory mediators underlies chronic inflammation, but a growing body of evidence supports the notion that disturbances in endogenous pro-resolving mechanisms can be an equally important factor (33-35).

We believe that three potential targets should be considered:

1) Cytokines;

2) Specialized pro-allowing mediators;

3) Endotheliocytes and related functional systems, according to which the main directions for the development of therapeutic strategies can be formed.

Attempts to influence the cytokine pathogenesis of COVID-19 have been shown to be effective. SARS-CoV-2 induces excessive and prolonged cytokine/chemokine reactions in some infected people. A cytokine storm causes the acute respiratory distress syndrome or multiple organ dysfunction, which leads to a sharp aggravation of the condition and death. It is commonly believed that timely control of a cytokine storm at its early stage by immunomodulators and cytokine antagonists, as well as reduction of pulmonary infiltration by inflammatory cells, is the key to increasing the treatment efficiency and reducing mortality (28).

But the severity and duration of inflammation depend on competing physiological processes, namely, on pro-inflammatory mechanisms that enhance the inflammation and endogenous inhibition programs, which in turn control the resolution of inflammation (36).

However, the anti-inflammatory effect is not the same as the resolution, which involves specialized pro-resolving mediators (SPMs) in the activation of non-logistic reactions and programs for resolving inflammation, and ends with the restoration of a structure and function with a return to the initial homeostasis and formation of immunological memory.

These pro-inflammatory mediators actively stop the production of pro-inflammatory mediators, but also immediately stimulate phagocytosis by macrophages of both apoptotic cells and bacteria, increase the output of phagocytes from inflammation sites, regulate apoptosis of stab leukocytes, increase the scavenger capture of chemokines, and stimulate tissue repair and regeneration (33).

It must be remembered that cytokines, like other inflammatory mediators, are only a part of the effector link of the launched and implemented genetic program of inflammation, they activate and control protective and adaptive reactions aimed at delimiting and eliminating the phlogogenic factor. Cancellation or blocking of their action reduces the severity or cancels the ongoing protective-adaptive phlogistic reaction, including at the stage of resolution and recovery, which can reduce the positive biological significance of inflammation until complete disappearance (28, 37).

This may be accompanied by a decrease in anti-infectious resistance, generalization of the process, the development of immunosuppression and chronicity of the disease, as well as violation of the repair and regeneration processes, which can manifest itself as the development of fibrosis, pathological angiogenesis, and, as a consequence, violation of the function of an organ or tissue. A number of studies have revealed that excessive acute inflammation and chronic unresolved inflammation were associated with severe and deadly diseases of the human lung, including ARDS, asthma, cystic fibrosis, and chronic obstructive pulmonary disease (33, 38).

The causes of damage and/or delayed response mechanisms may be due to several factors that may contribute to unsuccessful resolution. For example, a decrease in food intake of ω-3 essential fatty acids (EPA eicosapentaenoic acid, DHA docosahexaenoic acid), genetic polymorphisms of enzymes involved in the biosynthesis of specialized resolving mediators or SPM receptors, dysfunctional SPM receptors or a decrease in their expression, abnormal signaling inside as well as toxic drugs (34, 35).

Also, various disorders on the part of cells and their interactions involved in the synthesis of pro-resolving mediators and in the resolution process itself should be considered (39).

Thus, the main strategic pathogenetic direction in the creation of effective programs for the treatment of COVID-19, as well as the prevention of fatal complications, should be a set of measures enhancing permissive regulatory influences and events. To date, the precursors of PSD ω-3 irreplaceable fatty acids (EPA eicosapentaenoic acid, DHA docosahexaenoic acid) in acute and chronic inflammatory processes with a positive result have been studied in experiments and in clinical practice (31).

Synthetic analogues of lipoxins and resolvines already exist, which are undergoing clinical trials (40- 42).

Along with lipid mediators, peptides, proteins and gases also stimulate resolution. For example, endogenous gases such as NO, H2S, and CO play a positive role in resolving inflammation (34).

Carbon monoxide, endogenously produced by the hemoxygenase enzyme system, has become a promising gaseous therapeutic agent that has a protective effect against inflammation, oxidative and mechanical stress and apoptosis, thus potentially limiting the acute lung damage. Numerous studies using in vivo and in vitro models have demonstrated the anti-inflammatory and resolving, anti-apoptotic and antiproliferative properties of carbon monoxide in the lungs when used in low doses before or during ischemia-reperfusion, transplantation, sepsis, hyperoxia or mechanical ventilation, which is called ventilation damage. The molecular mechanisms that are affected by carbon monoxide are still not fully understood (43- 45).

The biological actions of these new mediators in the fight against inflammation are different from modern therapeutic agents. Indeed, while drugs used in clinical practice are designed to inhibit inflammation and block leukocyte recruitment, PSD acts by counteracting the production of pro-inflammatory mediators (cytokines, chemokines and inflammatory eicosanoids), limiting the leukocyte infiltration and facilitating the switching of the leukocyte phenotype. Therefore, these substances do not prevent inflammation, but rather strengthen the regulatory control, preventing its uncontrolled course and, thus, contribute to its cessation. It has been demonstrated that PSDs increase survival and help resolve inflammation, as well as some human diseases associated with dysregulation of the PSD pathways, including PSD generated from DHA and EPA (30, 35, 38).

Another way to improve resolution is to use cyclin-dependent kinase inhibitors (46).

In addition, it can be argued with certainty that transfusion of fresh blood plasma of a healthy person will bring significant benefits. it contains various factors, such as microvesicles containing m-RNA, α-2-macroglobulin, ulinastatin, potentiating the action of pro-resolving factors, limiting hyperergic inflammation and contributing to its resolution (28, 47, 48).

At autopsy of the dead from COVID-19, pulmonary fibrosis is usually present. Those, in some cases, abnormal immune mechanisms initiate and contribute to the appearance of pulmonary fibrosis, possibly due to a cytokine storm (49, 50).

However, diffuse alveolar damage, which is the defining feature of the acute respiratory distress syndrome, was a characteristic histological feature in fatal cases of COVID-19 with the additional observation of microvascular thrombosis (50, 51).

The basis of these structural transformations is a change in the functional activity of many cells: endotheliocytes, pericytes, fibroblasts, epithelial cells, macrophages, lymphocytes and so on. The activation of these cells leads, together with the vascular neoplasm, increased permeability of the wall and edema, to emigration with tissue infiltration by inflammatory cells, an increase in the number and activity of fibroblasts with the development of fibrosis and other degenerative-destructive changes. As a result, a new tissue is formed that does not provide the necessary function of the tissue, which differs from normal in its structural and functional characteristics. We believe that enhancing the action of pro-resolving mediators that act on many key links of inflammation and are involved in restoring the structural and functional homeostasis will be the most effective way to prevent the occurrence of such complications (52, 53).

Finally, the endothelial dysfunction, which is simultaneously one of the key components of inflammation, should be considered the third target for the formation of therapeutic strategies. The enormous role of endothelium in the initiation, development, and completion of inflammation is known. The endothelium, which has many diverse receptors capable of recognizing pathogenicity patterns of Pattern recognition receptors (PRRs): Pathogen-associated molecular pattern (PAMP) and Damage-associated molecular patterns (DAMP), i.e. of all types of toll-like receptors, scavenger receptors, integrins, selectins, nucleotide-binding oligomerization domain-like receptors (NOD-like receptors), etc., is itself the target for phlogogenic factors, it is able to initiate an inflammatory response, and it also recruits immunocompetent cells acting as an antigen-presenting cell secreting pro-inflammatory cytokines and chemokines (54-56).

The endothelium, being a source of inflammatory mediators and, at the same time, a transducer of their regulatory effects on the vascular tone, is involved in the development and alternation of vascular reactions, changing the volume of perfusion (57). As the organizer and regulator of extracorporeal transport, the endothelium affects the volume and composition of exudate (58). Using adhesive molecules and chemoattractants, the endothelium determines the sequence, mass and composition of emigrating cells, and therefore the composition of cell infiltrate (59-61). It participates in resolving inflammation due to the development of anti-inflammatory and pro-resolving factors during interaction with stab leukocytes, macrophages, and other cells (35, 62, 63), as well as in the restoration of structural and functional tissue and organ homeostasis. This significant role of the endothelium has already been partially evaluated in the treatment of COVID-19 (2).

Based on the foregoing, it can be confidently stated that the severity and consequences of COVID-19, as well as the mortal risk of complications, are due to the prevalence and severity of the endothelial dysfunction.

In this regard, it seems important to carry out therapeutic measures aimed at protecting the endothelium and normalizing its functions, as well as activating the phagocytosis of apoptotic endothelial cells and its subsequent regeneration. For example, it is known that with the development of acute inflammation, the glycocalyx of endothelial cells is damaged first, leading to a violation of the integrity of the endothelial surface plate. As a result, the charge of the vascular wall

decreases, its adhesive properties increase, and permeability increases. The experience of clinical oral administration of a mixture of low molecular weight glycosaminoglycans in the form of commercially available sulodexide, which allows restoring the thickness of the endothelial surface plate (ESL) in patients with diabetes mellitus, is described. Sulodexide is a mixture of 82% heparin, 17% dermatan sulfate and <1% chondroitin sulfate and exhibits antithrombotic and profibrinolytic properties, as well as vasculoprotective properties, such as the anti-inflammatory, antioxidant and anti-ischemic effects. The absence of severe side effects, as well as a relatively low price, makes it attractive for use in the treatment and prevention of complications of COVID-19 (64-66).

The main mechanism for the development of the endothelial dysfunction and EC damage is associated with an imbalance between the generation of reactive ROS and the power of the antioxidant defense system. As a result, NO is oxidized with the formation of NO3 (peroxynitrite), the bioavailability of NO decreases, and irreversible nitrosylation of the target proteins occurs, as a result of which a persistent dysfunction of the surrounding cells develops. Suppressing the excessive generation of ROS and restoring balance with the antioxidant defense system will not only protect endothelial cells, but also contribute to normalization of their functioning, restoring antiplatelet, anticoagulant, anti-inflammatory, vasodilator and other functions. Therefore, it is recommended, as a part of the integrated approach to treatment, to use antioxidant defenses (67).

The noticeable and sudden release of many cytokines by the immune system, often called hypercytokinemia or the cytokine storm, has a devastating effect in itself. Cytokines cause type II activation in endotheliocytes, which, in contrast to type I stimulation, does not develop immediately, but after 2-6 hours from the moment of induction by pro-inflammatory factors due to the increased transcription and synthesis of various proteins, cytokines, chemokines and adhesive molecules. During this period, endothelial cells express an endothelial-leukocyte type 1 adhesive molecule (ELAM-1), a larger number of type 1 integral intercellular adhesive molecules (ICAM-1) and Inducible cell adhesion molecule 110 (INCAM-110) with respect to vascular cell adhesive type 1 molecule (VC AM-1), which contributes to the activation, transmigration of neutrophils and tissue infiltration by them, the permeability of the basement membrane increases and plasma leakage sharply increases, stable vasodilation and, as a result, local vascular stasis develops (68-70).

In many infectious diseases, the components of the resulting secondary alteration caused by cytokines may be more pathogenic than the invading microorganisms themselves. Another evidence of the effectiveness of the endotheliopro-tection strategy was the reduction of harmful effects of the cytokine storm upon activation by the soluble Slit ligand of the endothelium-specific, Robo4-dependent signaling pathway, which strengthens the vascular barrier (71). The consequences of ED, which pose a deadly threat to tissues and organs, are microcirculatory and tissue hypoxia, resulting from venous stagnation of blood, sludge and microthrombosis. The reason for their occurrence is: activation of the endothelium with the appearance of adhesive and proaggregate properties, as well as a shift in the balance between potentiation and inhibition of coagulation factors, fibrinolysis to the procoagulant side, followed by the development of thrombophilia (2).

Therefore, any measures to protect the endothelium, reducing the severity of microcirculatory disorders and the severity of hypoxia, will have a therapeutic and preventive effect on fatal complications. Data on the effectiveness of thromboprophylaxis using low molecular weight heparins, the treatment with fibrinolysis activators have already been accumulated, and recommendations for their use have been published (15, 72-75).

Replacement of dead cells by increasing the mobilization and recruitment of endothelial progenitor cells and other stem/progenitor cells from the bone marrow could be considered as another method of the endothelial protection. Clinical studies have shown that in patients with the organ damage, an increase in the number of circulating endothelial progenitor cells correlated with an improvement in prognosis and survival. In animal experiments, it was found that exogenously administered endothelial progenitor cells or other stem/progenitor cells suppress the systemic and organ inflammation, reduce the organ damage, reduce the endothelial permeability, and improve the sepsis survival (39, 76).

In this regard, in the treatment of COVID-19, the use of synthetic gas transport preparations such as Perftoran b Fluoroemulsion III (Russia) based on perfluorocarbon nanodispersed emulsions with a clinical effect directed at once to several pathogenetic links underlying the progression of COVID-19 disease can be quite effective.

The expediency of using gas transportation drugs was caused by the work of Chinese scientists from Sichuan and Yibinsk universities on the effect of COVID-19 on red blood cells, which, as the authors point out, leads to an increase in inflammatory processes and the appearance of frosted glass in the lungs of patients. The authors suggest that COVID-19 can attack human red blood cells by binding to hemoglobin molecules. This leads to a disruption in the transport of oxygen to the organs and tissues of the body and, as a consequence, to hypoxemia. The penetration of viral proteins into red blood cells and binding to hemoglobin leads to “knocking out” of iron ions in blood plasma. In this case, hemoglobin loses its ability to be saturated with oxygen, and the iron ion has a toxic effect, and, as a result, tissue respiration is disturbed with aggravation (aggravation) of oxygen deficiency.

In connection with these studies, it is necessary to cite our work (77) on the elimination of hypoxia in acute autoimmune intravascular hemolysis of erythrocytes caused by a disease such as babesiosis. It is known that in acute intravascular autoimmune hemolysis, both intrinsic and donor erythrocytes are rapidly destroyed with the occurrence of hypoxia and the

release of a large amount of hemoglobin not associated with haptoglobin into the plasma, due to which free hemoglobin has a pathological effect on all organs. Studies have shown that elimination of hypoxia during intravascular hemolysis of erythrocytes is quite effective with perfluorocarbon hemocorrectors such as Perftoran and Fluoroemulsion III, but not with the help of donor blood and red blood cells.

Gas transportation donor blood substitutes based on perfluorocarbon emulsions are multifunctional drugs with a wide spectrum of action, including the ability to carry any gas (oxygen, carbon dioxide and inert gas - helium oxide, etc.). Due to the nano-dispersed size (50-100 nm), particles of perfluorocarbon emulsions with oxygen physically dissolved in them, are able to penetrate into ischemic tissue sites through spasmodic, sclerotic, partially thrombosed and susceptible vessels; improve blood rheology and microcirculation in tissues with disturbed gas exchange and metabolism of various origins; reduce blood viscosity, increase regional blood flow, promote vascular bed recanalization; increase electronegative charges of the membranes of red blood cells, platelets, and endothelial cells, which increases the suspension stability of blood, improves its rheological characteristics, prevents formation of blood aggregates, activates microcirculation in tissues, provides axial current of blood cells in the vessels, causes decay of false aggregates (in the early stages of critical states), which in turn recanalizes the microvascular bed (79). All these factors are clearly manifested in pathological processes caused by the coronavirus disease.

It is necessary to note other positive properties, which we believe are useful in the noted pathological processes of COVID-19. So, the aforementioned perfluorocarbon gas transport hemoproofreaders have the membrane-stabilizing properties: perfluoro-emulsions stabilize and modify biological membranes, activate oxidative phosphorylation, change the functional properties of red blood cell membranes, increase their charge and resistance to the mechanical trauma, cause platelet disaggregation, red blood cells, and platelets increase the fibrinolytic activity, reduce the concentration of fibrinogen and factor XII; antioxidant properties - eliminate the content of primary and intermediate products of lipid peroxidation of blood plasma lipids, while the intensity of free radical oxidation in erythrocytes and blood plasma decreases with the background of perfluoroemulsion, and the activity of natural antioxidant defense systems increases. They also have detoxification properties: they increase the detoxification function of the liver, induce cytochrome P-450, and affect the natural detoxification system in acute poisoning (80).

Thus, in the absence of effective vaccines and sera in the case of COVID-19 disease, among the considered active treatment strategies, the directions with a complex effect on the pathogenesis using sanogenetic treatment principles are the most attractive.

Such pleiotropy is necessary for the action of the above therapeutic agents. This can be well explained in terms of the theory of functional systems. With the complex organization and the multiplicity of various structures and functions performed by them, there is a single ultimate goal: maintaining homeostasis in the whole organism and in each individual part thereof. The common goal forms the key structures and mechanisms of a single pathogenesis, at which the action of drugs should be aimed.

CONCLUSION

Apparently, only the simultaneous corrective effect on the numerous signaling pathways that control the course of the inflammatory reaction can significantly affect the execution of this genetically determined program. Obviously, the rate of development and the time of onset of resolving inflammation can be significantly accelerated using the natural signaling pathways that control these sanogenetic mechanisms. It is also safe to say that such therapeutic effects will be most effective, because they synchronize the work of sanogenetic mechanisms and, interacting with each other, potentiate their action, increasing the degree of biological usefulness of the result. At the same time, the methods and methods proposed for testing should be in addition to the already existing types of treatment that have proved their effectiveness. The start time and duration of treatment with these methods depend on the nature and direction of exposure. For example, it makes sense to start the therapy with the precursors of PSD or synthetic analogues of lipoxins and resolvins from the moment of the onset of clinical manifestations, and breathing with the addition of CO and the infusion of synthetic gas transport preparations from the moment the respiratory failure begins to develop, together with oxygen therapy.

Only correction with multiple regulatory influences that potentiate each other's actions can correct execution of the genetic program, increasing the degree of biological usefulness of the result. At the same time, the methods and methods proposed for testing should be in addition to the already existing types of treatment that have proved their effectiveness.

REFERENCES

1. Bolotov DD, Novikov AA, Bolevich S, Novikova NA, Yakovchenko AV. Influence of systemic inflammatory response to appearance of new foci of chronic inflammation. Ser J Exp Clin Res. 2020; 21 (1): 3-10.
2. Varga Z, Flammer AJ, Steiger P, Haberecker M, Andermatt R, Zinkernagel AS, Mehra MR, Schuepbach RA, Ruschitzka F, Moch H. Endothelial cell infection and endotheliitis in COVID-19. Lancet. 2020; 395(10234): 1417-18.
3. Md Insiat Islam Rabby. Current Drugs with Potential for Treatment of COVID-19: A Literature Review. J Pharm Pharm Sci. 2020; 23(1): 58-64.

4. Zhou P, Yang XL, Wang XG, Hu B, Zhang L, Zhang W, Si HR, Zhu Y, Li B, Huang CL, Chen HD, Chen J, Luo Y, Guo H, Jiang RD, Liu MQ, Chen Y, Shen XR, Wang X, Zheng XS, Zhao K, Chen QJ, Deng F, Liu LL, Yan B, Zhan FX, Wang YY, Xiao GF, Shi ZL. A pneumonia outbreak associated with a new coronavirus of probable bat origin. Nature. 2020; 579(7798): 270-3.
5. Atri D, Siddiqi HK, Lang JP, Nauffal V, Morrow DA, Bohula EA. COVID-19 for the Cardiologist: Basic Virology, Epidemiology, Cardiac Manifestations, and Potential Therapeutic Strategies. JACC Basic Transl Sci. 2020; 5(5): 518-36.
6. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, Schiergens TS, Herrler G, Wu NH, Nitsche A, Müller MA, Drosten C, Pöhlmann S. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. Cell. 2020; 181(2): 271-80.
7. Muus C, et al., Integrated analyses of single-cell atlases reveal age, gender, and smoking status associations with cell type-specific expression of mediators of SARS-CoV-2 viral entry and highlights inflammatory programs in putative target cells. bioRxiv. 2020; doi: 10.1101/2020.04.19.049254.
8. Hamming I, Cooper ME, Haagmans BL, Hooper NM, Korstanje R, Osterhaus AD, Timens W, Turner AJ, Navis G, van Goor H. The emerging role of ACE2 in physiology and disease. J Pathol. 2007; 212(1): 1-11.
9. Hamming I, Timens W, Bulthuis ML, Lely AT, Navis G, van Goor H. Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. J Pathol. 2004; 203: 631-7.
10. Ferrario CM, Jessup J, Chappell MC et al. Effect of angiotensin-converting enzyme inhibition and angiotensin II receptor blockers on cardiac angiotensin-converting enzyme 2. Circulation. 2005; 111: 2605-2610.
11. Carly G, Ziegler K, et al. SARS-CoV-2 receptor ACE2 is an interferon-stimulated gene in human airway epithelial cells and is detected in specific cell subsets across tissues. Cell 2020. doi: 10.1016/j.cell.2020.04.035.
12. Rajendran P, Rengarajan T, Thangavel J, Nishigaki Y, Sakthisekaran D, Sethi G, Nishigaki I. The Vascular Endothelium and Human Diseases. Int J Biol Sci 2013; 9(10): 1057–1069.
13. Kade AK, Zanin SA, Gubareva EA, Turovaya AY, Bogdanova YA, Apsalyamova SO, Merzlyakova SN. Physiological functions of the vascular endothelium. Basic research. 2011; 11(3): 611-617.
14. Chatterjee S. Endothelial Mechanotransduction, Redox Signaling and the Regulation of Vascular Inflammatory Pathways. Front Physiol. 2018; 9: 524.
15. Ciceri F, Beretta L, Scandroglio AM, Colombo S, Landoni G, Ruggeri A, Peccatori J, D'Angelo A, De Cobelli F, Rovere-Querini P, Tresoldi M, Dagna L, Zangrillo A. Microvascular COVID-19 lung vessels obstructive thromboinflammatory syndrome (MicroCLOTS): an atypical acute respiratory distress syndrome working hypothesis. Crit Care Resusc. 2020; 22(2): 95-97.
16. Lai CC, Shih TP, Ko WC, Tang HJ, Hsueh PR. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and coronavirus disease-2019 (COVID-19): The epidemic and the challenges. Int J Antimicrob Agents. 2020; 55(3): 105924.
17. Special Expert Group for Control of the Epidemic of Novel Coronavirus Pneumonia of the Chinese Preventive Medicine Association. An update on the epidemiological characteristics of novel coronavirus pneumonia (COVID-19). Chin J Epidemiol. 2020; 41.
18. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, Xiang J, Wang Y, Song B, Gu X, Guan L, Wei Y, Li H, Wu X, Xu J, Tu S, Zhang Y, Chen H, Cao B. Clinical course and risk factors for mortality of adult in patients with COVID-19 in Wuhan, China: a retrospective cohort study. Lancet. 2020 Mar 28;395(10229):1054-1062.
19. Liu W, Tao Z-W, Wang L, Yuan M-L, Liu K, Zhou L, Wei S, Deng Y, Liu J, Liu H-G, Yang M, Hu Y, Analysis of factors related to the clinical outcome in hospitalized patients with a new type of coronavirus infection. Chin Med J (Engl). 2020; 133(9): 1032-1038.
20. Alhazzani W, Møller MH, Arabi YM, Loeb M, Gong MN, Fan E, Oczkowski S, Levy MM, Derde L, Dzierba A, Du B, Aboodi M, Wunsch H, Cecconi M, Koh Y, Chertow DS, Maitland K, Alshamsi F, Belley-Cote E, Greco M, Laundy M, Morgan JS, Kesecioglu J, McGeer A, Mermel L, Mammen MJ, Alexander PE, Arrington A, Centofanti JE, Citerio G, Baw B, Memish ZA, Hammond N, Hayden FG, Evans L, Rhodes A. Surviving Sepsis Campaign: guidelines on the management of critically ill adults with Coronavirus Disease 2019 (COVID-19). Intensive Care Med. 2020; 46(5): 854-887.
21. Guidelines for the management of critically ill adults with coronavirus disease 2019 (COVID-19), Translated by V.S. Gorokhovsky, M.B. Kutsego, A.A. Naumenko, V.D. Hunter, I.R. Cherkashina, https://rosomed.ru/documents/rukovodstvo-po-vedeniu-kriticheski-bolnyh-vzroslyh-s-koronavirusnoi-boleznu-2019-covid-19-v-perevode-na-russkii-yazyk (date of the application 21.04.2020).
22. Chousterman BG, Swirski FK, Weber GF. Cytokine storm and sepsis disease pathogenesis. Semin Immunopathol. 2017; 39: 517–528.
23. Chakraborty RK, Burns B. Systemic Inflammatory Response Syndrome. [Updated 2020 Apr 28]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2020 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK547669/
24. Gusev EY, Chereshnev VA, Yurchenko LN. Systemic inflammation from the perspective of the theory of a typical pathological process. Cytokines and inflammation. 2007; 6(4): 9-21.
25. Clapp BR, Hingorani AD, Kharbanda RK, Mohamed-Ali V, Stephens JW, Vallance P, MacAllister RJ. Inflammation-induced endothelial dysfunction involves reduced nitric oxide bioavailability and increased oxidant stress. Cardiovasc Res. 2004; 64: 172-8.

26. Moshage H. Cytokines and the hepatic acute phase response. J Pathol. 1997; 181(3): 257-66.
27. Schalkwijk CG, Poland DCW, van Dijk W, Kok A, Emeis JJ, Drager AM, Doni A, van Hinsbergh VWM, Stehouwer CDA. Plasma concentration of c-reactive protein is increased in type i diabetic patients without clinical macroangiopathy and correlates with markers of endothelial dysfunction: evidence for chronic inflammation. Diabetologia. 1999; 42: 351-7.
28. Ye Q, Wang B, Mao J. The pathogenesis and treatment of the `Cytokine Storm' in COVID-19. J Infect. 2020; 80(6): 607-13.
29. Bannenberg GL, Chiang N, Ariel A, Arita M, Tjonahen E, Gotlinger KH, Hong S, Serhan CN. Molecular Circuits of Resolution: Formation and Actions of Resolvins and Protectins. J Immunol. 2005; 174: 4345-55.
30. Norling LV, Dalli LL, Dalli J. Resolving Inflammation by using Nutrition Therapy: Roles for Specialized Pro-Resolving Mediators. Curr Opin Clin Nutr Metab Care. 2017; 20(2): 145-152.
31. Serhan CN, Brain SD, Buckley CD, Gilroy DW, Haslett C, O'Neill LAJ, Perretti M, Rossi AG, Wallace JL. Resolution of inflammation: state of the art, definitions and terms. FASEB J. 2007; 21(2): 325-332.
32. Sugimoto MA, Sousa LP, Pinho V, Perretti M, Teixeira MM. Resolution of Inflammation: What Controls Its Onset? Front Immunol. 2016; 7: 160
33. Sansbury BE, Spite M. Resolution of Acute Inflammation and the Role of Resolvins in Immunity, Thrombosis and Vascular Biology. Circ Res. 2016; 119(1): 113-30.
34. Serhan CN. Treating inflammation and infection in the 21st century: new hints from decoding resolution mediators and mechanisms. FASEB J. 2017; 31(4): 1273-88.
35. Serhan CN, Yacoubian S, Yang R. Anti-Inflammatory and Pro-Resolving Lipid Mediators. Annu Rev Pathol. 2008; 3: 279-312.
36. Serhan CN. Resolution phase of inflammation: novel endogenous anti-inflammatory and proresolving lipid mediators and pathways. Annu Rev Immunol. 2007;25:101–137. DOI:10,1146 / annurev.immunol.25.022106.141647.
37. Molfino A, Amabile MI, Monti M, Muscaritoli M. Omega-3 Polyunsaturated Fatty Acids in Critical Illness: Anti-Inflammatory, Proresolving, or Both? Oxid Med Cell Longev. 2017; 2017: 5987082.
38. Duvall MG, Levy BD. DHA- and EPA-derived resolvins, protectins, and maresins in airway inflammation, Eur J Pharmacol. 2016; 785: 144-155.
39. Papa ND, Pignataro F. The Role of Endothelial Progenitors in the Repair of Vascular Damage in Systemic Sclerosis. Front Immunol. 2018; 9:1383.
40. Maddox JF, Hachicha M, Takano T, Petasis NA, Fokin VV, Serhan CN. Lipoxin A4 stable analogs are potent mimetics that stimulate human monocytes and THP-1 cells via a G-protein-linked lipoxin A4 receptor. J Biol Chem. 1997; 272(11): 6972-8.
41. Guilford WJ, Parkinson JF. Second-generation beta-oxidation resistant 3-oxa-lipoxin A4 analogs. Prostaglandins Leukot Essent Fatty Acids. 2005; 73(3-4): 245-50.
42. Safety and Preliminary Efficacy of Lipoxin Analog BLXA4-ME Oral Rinse for the Treatment of Gingivitis (BLXA4**),** ClinicalTrials.gov Identifier: NCT02342691.
43. Faller S, Hoetzel A. Carbon monoxide in acute lung injury. Curr Pharm Biotechnol. 2012; 13(6): 777-86.
44. Shinohara M, Kibi M, Riley IR, Chiang N, Dalli J, Kraft BD, Piantadosi CA, Choi AM, Serhan CN. Cell-cell interactions and bronchoconstrictor eicosanoid reduction with inhaled carbon monoxide and resolvin D1. Am J Physiol Lung Cell Mol Physiol. 2014; 307(10): L746-57.
45. Dalli J, Kraft BD, Colas RA, Shinohara M, Fredenburgh LE, Hess DR, Chiang N, Welty-Wolf K, Choi AM, Piantadosi CA, Serhan CN. The Regulation of Proresolving Lipid Mediator Profiles in Baboon Pneumonia by Inhaled Carbon Monoxide. Am J Respir Cell Mol Biol. 2015; 53(3): 314-25.
46. Rossi AG, Sawatzky DA, Walker A, Ward C, Sheldrake TA, Riley NA, Caldicott A, Martinez-Losa M, Walker TR, Duffin R, Gray M, Crescenzi E, Martin MC, Brady HJ, Savill JS, Dransfield I, Haslett C. Cyclin-dependent kinase inhibitors enhance the resolution of inflammation by promoting inflammatory cell apoptosis. Nat Med. 2006; 12(9): 1056-64.
47. Dalli J, Norling LV, Montero-Melendez T, Federici Canova D, Lashin H, Pavlov AM, Sukhorukov GB, Hinds CJ, Perretti M. Microparticle alpha-2-macroglobulin enhances pro-resolving responses and promotes survival in sepsis. EMBO Mol Med. 2014; 6(1): 27-42.
48. Njock MS, Cheng HS, Dang LT, Nazari-Jahantigh M, Lau AC, Boudreau E, Roufaiel M, Cybulsky MI, Schober A, Fish JE. Endothelial cells suppress monocyte activation through secretion of extracellular vesicles containing antiinflammatory microRNAs. Blood. 2015; 125(20): 3202-12.
49. Wang J, Wang BJ, Yang JC, Wang MY, Chen C, Luo GX, He WF. Research advances in the mechanism of pulmonary fibrosis induced by coronavirus disease 2019 and the corresponding therapeutic measures. Zhonghua Shao Shang Za Zhi. 2020; 36(8): 691-7.
50. Zhang T, Sun LX, Feng RE. Comparison of clinical and pathological features between severe acute respiratory syndrome and coronavirus disease 2019. Zhonghua Jie He He Hu Xi Za Zhi. 2020; 43(6): 496-502.
51. Barton LM, Duval EJ, Stroberg E, Ghosh S, Mukhopadhyay S. COVID-19 Autopsies, Oklahoma, USA. Am J Clin Pathol. 2020; 153(6): 725-33.
52. Cho JG, Lee A, Chang W, Lee MS, Kim J. Endothelial to Mesenchymal Transition Represents a Key Link in the Interaction between Inflammation and Endothelial Dysfunction. Front Immunol. 2018; 9: 294.
53. Uddin M, Levy BD. Resolvins: natural agonists for resolution of pulmonary inflammation. Prog Lipid Res. 2011; 50(1): 75-88.
54. Al-Soudi A, Kaaij MH, Tas SW. Endothelial cells: From innocent bystanders to active participants in immune responses. Autoimmun Rev. 2017; 16(9): 951-62.
55. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity. 2011; 34(5): 637-50.

56. Salvador B, Arranz A, Francisco S, Córdoba L, Punzón C, Llamas MÁ, Fresno M. Modulation of endothelial function by Toll like receptors. Pharmacol Res. 2016; 108: 46-56.
57. Khakpour S, Wilhelmsen K, Hellman J. Vascular endothelial cell Toll-like receptor pathways in sepsis. Innate Immun. 2015; 21(8): 827-46.
58. Herzog C, Haun RS, Kaushal GP. Role of meprin metalloproteinases in cytokine processing and inflammation. Cytokine. 2019; 114: 18-25.
59. Muller WA. Transendothelial migration: unifying principles from the endothelial perspective. Immunol Rev. 2016; 273(1): 61-75.
60. Reglero-Real N, Colom B, Bodkin JV, Nourshargh S. Endothelial Cell Junctional Adhesion Molecules: Role and Regulation of Expression in Inflammation. Arterioscler Thromb Vasc Biol. 2016; 36(10): 2048-57.
61. Vestweber D. How leukocytes cross the vascular endothelium. Nat Rev Immunol. 2015; 15(11): 692-704.
62. Serhan CN. Novel Pro-Resolving Lipid Mediators in Inflammation Are Leads for Resolution Physiology, Nature. 2014; 510(7503): 92-101.
63. Serhan CN, Chiang N, Dalli J. The resolution code of acute inflammation: Novel pro-resolving lipid mediators in resolution. Semin Immunol. 2015; 27(3): 200-15.
Johansson PI, Stensballe J, Rasmussen LS, Ostrowski SR.
64. A high admission syndecan-1 level, a marker of endothelial glycocalyx degradation, is associated with inflammation, protein C depletion, fibrinolysis, and increased mortality in trauma patients. Ann Surg. 2011; 254(2): 194-200.
65. Neves FM, Meneses GC, Sousa NE, Menezes RR, Parahyba MC, Martins AM, Libório AB. Syndecan-1 in Acute Decompensated Heart Failure--Association With Renal Function and Mortality. Circ J. 2015; 79(7): 1511-9.
66. Sieve I, Münster-Kühnel AK, Hilfiker-Kleiner D. Regulation and function of endothelial glycocalyx layer in vascular diseases. Vascul Pharmacol. 2018; 100: 26-33.
67. Incalza MA, D'Oria R, Natalicchio A, Perrini S, Laviola L, Giorgino F. Oxidative stress and reactive oxygen species in endothelial dysfunction associated with cardiovascular and metabolic diseases. Vascul Pharmacol. 2018; 100: 1-19.
68. Nourshargh S, Alon R. Leukocyte migration into inflamed tissues. Immunity. 2014; 41(5): 694-707.
69. Pober JS, Cotran RS. The role of endothelial cells in inflammation. Transplantation. 1990; 50(4): 537-44.
70. Pober JS, Sessa WC. Evolving functions of endothelial cells in inflammation. Nat Rev Immunol. 2007; 7(10): 803-15.
71. London NR, Zhu W, Bozza FA, Smith MC, Greif DM, Sorensen LK, Chen L, Kaminoh Y, Chan AC, Passi SF, Day CW, Barnard DL, Zimmerman GA, Krasnow MA, Li DY. Targeting Robo4-dependent Slit signaling to survive the cytokine storm in sepsis and influenza. Sci Transl Med. 2010; 2(23): 23ra19.
72. Escher R, Breakey N, Lämmle B. Severe COVID-19 infection associated with endothelial activation. Thromb Res. 2020; 190: 62.
73. Hunt B, Retter A, McClintock C. Practical guidance for the prevention of thrombosis and management of coagulopathy and disseminated intravascular coagulation of patients infected with COVID-19.
74. Kollias A, Kyriakoulis KG, Dimakakos E, Poulakou G, Stergiou GS, Syrigos K. Thromboembolic risk and anticoagulant therapy in COVID-19 patients: emerging evidence and call for action. Br J Haematol. 2020; 189(5): 846-847.
75. Spyropoulos AC, Ageno W, Barnathan ES. Hospital-based use of thromboprophylaxis in patients with COVID-19. Lancet. 2020; 395(10234): e75.
76. Fan H, Goodwin AJ, Chang E, Zingarelli B, Borg K, Guan S, Halushka PV, Cook JA. Endothelial progenitor cells and a stromal cell-derived factor-1α analogue synergistically improve survival in sepsis. Am J Respir Crit Care Med. 2014; 189(12): 1509-19.
77. Halaidych OV, Freund C, van den Hil F, Salvatori DCF, Riminucci M, Mummery CL, Orlova VV. Inflammatory Responses and Barrier Function of Endothelial Cells Derived from Human Induced Pluripotent Stem Cells. Stem Cell Reports. 2018 May 8;10(5):1642-56.
78. Votrin SV, Vorobyev SI, Bolevich SB, Use of perfluorocarbon based blod substitute perftoran in correction of hypoxia dyring acute anemia in animals. Ser J Exp Clin Res. 2019; 20(3): 245-50.
79. Vorobyov SI, Moiseenko OM, Belyaev BL, Srednyakov VA, Luzganov YuV. Colloid-chemical and medico-biological characteristics of the perfluorocarbon Ftoremulsion III. Pharm Chem J. 2009; 43(5): 267-73.
80. Vorobyov SI. Perfluorocarbon blood-replacing emulsions of the 1st and 2nd generation. Pharm Chem J. 2009; 43(4): 30-40.

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THE INFLUENCE OF CONTINUOUS AND INTERVAL AEROBIC TRAINING ON THE OXIDATIVE STATUS OF WOMAN BASKETBALL PLAYERS

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UTICAJ KONTINUIRANOG I INTERVALNOG AEROBNOG TRENINGA NA OKSIDATINI STATUS KOD KOŠARKAŠICA

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ABSTRACT

Oxidative stress is a state of disturbed balance between reactive oxygen species and reactive nitrogen species on the one hand and on the other antioxidant protection. Increased oxygen consumption during exercise could be the cause of oxidative stress. The aim of this study was to monitoring the parameters of oxidative stress and components of antioxidative defense during the training process, establish oxidative status basketball players in standby mode after the load caused by two types of aerobic training - continuous aerobic and interval (HIIT) training.

As part of a longitudinal experimental study selected a sample of 12 basketball players during the training process. All respondents were female, age 14 to 27 years. The study was conducted in preparatory stage. Oxidative status was determined through the index of lipid peroxidation (measured as TBARS), nitric oxide (NO) in the form of nitrite (NO_2) levels of superoxide anion radicals (O_2) and hydrogen peroxide (H2O2), while the activity of the enzyme protection from oxidative damage was determined through superoxide dismutase (SOD), catalase (CAT) and reduction glutathione (GSH).

The group analyzed in relation to the type of the training intervention was significantly different from the results in the test in the parameters of NO and TBARS. When the enzyme activity of protection against oxidative damage statistically significant differences between groups arise for CAT and GSH.

The emergence of oxidative stress is not necessary phenomenon of high aerobic training load, training leads to the maintenance of physiological balance in the body.

Keywords: *oxidative stress, antioxidant protection, continuous training, HIIT training.*

SAŽETAK

Oksidativni stres je stanje poremećene ravnoteže između reaktivnih vrsta kiseonika i reaktivnih vrsta azota sa jedne strane i antioksidativne zaštite sa druge. Povećana potrošnja kiseonika u toku treninga može biti uzrok oksidativnog stresa. Cilj ove studije bio je da se praćenjem parametara oksidativnog stresa i komponenti antioksidativne zaštite u toku trenažnog procesa, utvrdi oksidativni status košarkašica u stanju mirovanja i nakon opterećenja izazvanog dvema vrstama aerobnog treninga – kontinuirani aerobni i intervalni (HIIT) trening.

U sklopu longitudinalne, eksperimentalne studije izabran je uzorak od 12 košarkašica u toku trenažnog procesa. Svi ispitanici bili su ženskog pola, životnog doba od 14 do 27 godina. Ispitivanje je sprovedeno u pretakmičarskoj fazi. Određivan je oksidativni status preko indeksa lipidne peroksidacije (meren kao TBARS), azot monoksida (NO), u formi nitrita (NO_2^-), nivoa superoksid-anjon radikala (O_2^-) i vodonik-peroksida (H_2O_2), dok je aktivnost enzima zaštite od oksidacionih oštećenja određivana preko superoksid-dismutaze (SOD), katalaze (CAT) i redukovaniog glutationa (GSH).

Analizirane grupe u odnosu na vrstu trenažne intervencije statistički se značajno razlikuju u odnosu na rezultate u testu u parametrima NO i TBARS. Kod aktivnosti enzima zaštite od oksidativnih oštećenja statistički značajne razlike između grupa se javljaju za CAT i GSH.

Nastanak oksidativnog stresa nije nužna pojava treninga viskog aerobnog opterećenja, odnosno sam trening dovodi do održavanja fiziološkog balansa u organizmu.

Ključne reči: *oksidativni stres, antioksidativna zaštita, kontinuirani trening, HIIT trening.*

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INTRODUCTION

In order to track and obtain best possible performance results and health condition, it is inevitable to keep track of occurring oxidative stress during everyday physical strains. In the past several years there was an increase in discussing oxidative stress in terms of sport, which indicates a phenomenon that occurs in healthy individuals, therefore a natural phenomenon which occurs during normal physiological functions. Continuous tracking and blood test analysis contributes to prevention of latent disorders, and improved quality control of training processes (1).

During the oxidation process our bodies generate energy we need to survive. Furthermore, the process creates free radicals which have positive physiological functions. However, when our health status indicates there is an increase in free radicals production next to the decreased ability for their removal and neutralization, it leads to oxidative stress which causes unwanted changes in human bodies. Free radicals stand for unstable molecules with highly chemically reactive unpaired electrons, and their harmful effects come from the need to achieve electron stability, causing them to react with the first stable molecule, taking their electron and creating new free radicals (1), therefore causing biochemical, morphological, and functional disorders.

Oxidative stress is defined by the imbalance between reactive oxygen species (ROS) and reactive nitrogen species (RNS) on the one hand, and antioxidative stress (AOS) on the other hand. Excessive production of ROS and RNS, and their disproportional production (in particular superoxide anion radical (O_2^-, and NO), or the lack of antioxidative protection, can cause stress on cells and tissue (2). The main method of protection is to preserve the natural antioxidative protection of the organism, but also to reduce risk factors such as weight loss, smoking, and consuming unsaturated fatty acids. The best source of antioxidants are vegetables and fruits, as plant produce antioxidants for their own protection against free radicals.

People who don't exercise, unlike people who do, are more susceptible to greater changes in their bodies due to oxidative stress during physical activities. It is well known that both active and inactive skeletal musculature produce reactive oxygen and nitrogen species, although it is still unknown where are they being produced during physical activity (3). Increased production of reactive oxygen and nitrogen species, as well as oxidative stress, occur even in top athletes during maximum strains, regardless of the sport type and its energy demand (1). Recent studies indicate that the higher intensity of exercise positively affects the improvement of VO_2max, in comparison to medium intensity exercises (4), as well as that the body response in terms of muscle inflamation is significantly lower in this type of training, when comparing to continuous aerobic exercises (5).

On the other hand, impact of aerobic/anaerobic exercise on free radical production is still poorly understood with controversial results. In addition, there are lack of data referring to gender differences in regard to this issue. Therefore, the main goal of the research was to determine how different types of aerobic training affect the oxidative status of female basketball players.

MATERIAL AND METHODS

As part of the longitudinal and experimental study 12 female basketball players in the training process were selected. All of the respondents were 14 to 27 aged old, with at least 5 years of sports training experience (the respondents were also part of the national team for at least a year). The research was conducted in the precompetitive stage. The study was planned in accordance with the ethical standards of the Helsinki declaration. The respondents were informed in detail about the procedures of the study, potential risks, as well as their obligations and possibilities to withdraw from the study at any time. The informed consent from all participants were obtained prior to beginning of the study.

The homogeneous group of female basketball players was used to examine the effects of different types of aerobic training on their oxidative status two times with 14 days apart. The first training that was used in the process of oxidative status evaluation was a continuous training method which includes applying low, middle and high strains, mostly in equal rates, during which the heart rate works on a submaximal level in a longer period of time, with moderate or high consumption of oxygen (60-80% VO_2 max). The other type of training that was used in the research was HIIT aerobic training (high-intensity interval training), i.e. training in which the periods of highly intensive activity are alternated with periods of active rest, which aim to improve performance in short-term high-intensity trainings. HIIT includes exercise repetition 30 seconds to one minute that are separated with 1-5 minutes of rest (6). This type of training significantly improves the aerobic strength and durability, and is adaptable to all levels of training. The end effect of the interval training, besides the amount of strain and rest duration, depends on the total number of repetead intervals during training. In our study, as high intensity intermittent (HIIT) training, we used 3 minute high-speed runs at 90-95% VO_2max, with 3 minutes of low-speed runs at 50% VO_2max in 3 series. Basic anthropometric measures were taken prior to treatment using the OMRON BF511 body composition monitor. The measures taken were: body height (BH), body mass (BM), body mass index (BMI), body fat percentage (BFP), and muscle mass (MM). Blood samples were taken before and after every training from the cubital vein, in the following way: before starting the training treatment first blood samples were taken in the morning between 7 and 8 am, shortly before the training, and 2 hours after their last meal. Following samples were taken right after training.

Biochemical assays

The analyzed parameters of oxidative stress were:

- Lipid peroxidation index (TBARS),
- nitric oxide (NO), as nitrite (NO_2^-),
- superoxide anion radical (O_2^-),
- hydrogen peroxide (H_2O_2),

Activity of protection enzymes against oxidative damage:

- superoxide dismutase (SOD),
- catalase (CAT),
- reduced glutathione (GSH).

Blood samples were taken from an antecubital vein into Vacutainer test tube containing sodium citrate anticoagulant. Blood samples were analyzed immediately. Blood was centrifuged to separate plasma and red blood cells (RBCs). Biochemical parameters were measured spectrophotometrically.

Superoxide anion radical determination

The level of superoxide anion radical (O_2^-) was measured using nitro blue tetrazolium (NBT) reaction in TRIS-buffer combined with plasma samples and read at 530 nm (7).

Hydrogen peroxide determination

The protocol for measurement of hydrogen peroxide (H_2O_2) is based on oxidation of phenol red in the presence of horseradish peroxidase (8). 200 µl sample with 800 µl PRS (phenol red solution) and 10 µl POD (Horseradish Peroxidase) were combined (1:20). The level of H_2O_2 was measured at 610 nm.

Nitric oxide determination

Nitric oxide (NO) decomposes rapidly to form stable metabolite nitrite/nitrate products. Nitrite (NO_2^-) was determined as an index of nitric oxide production with Griess reagent (9). 0.1 ml 3 N PCA (Perchloride acid), 0.4 ml 20 mM ethylenediaminetetraacetic acid (EDTA), and 0.2 ml plasma were put on ice for 15 min, then centrifuged 15 min at 6,000 rpm. After pouring off the supernatant, 220 µl K_2CO_3 was added. Nitrites were measured at 550 nm. Distilled water was used as a blank probe.

Index of lipid peroxidation (thiobarbituric acid reactive substances, TBARS)

The degree of lipid peroxidation in plasma was estimated by measuring of TBARS using 1 % TBA (thiobarbituric Acid) in 0.05 NaOH, incubated with plasma at 100 °C for 15 min and read at 530 nm. Distilled water was used as a blank probe. TBA extract was obtained by combining 0.8 ml plasma and 0.4 ml trichloro acetic acid (TCA), then samples were put on ice for 10 min, and centrifuged for 15 min at 6,000 rpm. This method was described previously (10).

Determination of antioxidant enzymes

Isolated RBCs were washed three times with three volumes of ice-cold 0.9 mmol/l NaCl, and hemolysates containing about 50 g Hb/l (11) were used for the determination of CAT activity. CAT activity was determined according to Beutler (12). Then 50 µl CAT buffer, 100 µl sample, and 1 ml 10 mM H_2O_2 were added to the samples. Detection was performed at 360 nm. Distilled water was used as a blank probe. SOD activity was determined by the epinephrine method of Misra and Fridovich (13). A hundred µl lysate and 1 ml carbonate buffer were mixed, and then 100 µl of epinephrine was added. Detection was performed at 470 nm. The level of reduced glutathione (GSH) was determined based on GSH oxidation with 5.5-dithio-bis-6.2-nitrobenzoic acid, using Beutler method (14); the concentration is expressed as nanomoles per milliliter of RBCs.

Statistical analyses

Data was processed in the statistical program package - Statistical Package for Social Science (SPSS). Among the descriptive statistical parameters for the levels of analyzed markers, the arithmetic mean (r) was calculated with dispersion measures (standard deviation – SD and standard error – SE), 95% confidence interval, median, as well as percentile distribution, and relative frequencies. The differences between the results of samples were calculated using the univariate variance analysis (ANOVA). Minimal condition for statistical significance is p (level of significance) lower or equal to 0.05.

RESULTS

The anthropometric characteristics of the respondents (Table 1) show that these are young athletes, with an average life span of 17.75 ± 4,070. Distribution of body height and body weight varies precisely because of the diversity of the structure and the constitution according to the position of players in the team. Therefore an average height was177.89 ± 7.001 while an average weight was 67.32 ± 10.438. The average BMI of the examined athletes was 21.68 ± 2.598, with a body fat percentage of 27.43 ± 8.072 and a body muscle ratio of 31.74 ± 4.322. with the note that the measurement was done at the beginning of the preparation period after a pause.

Table 1. Antropometric characteristics of examinees

	Min	Max	AS	SD
Life time (years)	14	27	17,75	4,070
Body height (cm)	165	189	177,96	7,001
Body weight (kg)	49	80	67,32	10,438
Body mass index (kg/m²)	18	25	21,68	2,598
Fat (in percent)	13	42	27,43	8,072
Muscles (in percent)	22	38	31,74	4,322

Legend: Min – minimum value; Max – maximal value; AS – arithmetic mean; SD – standard deviation.

The results of the univariate analysis of variance (Figure 1-4) for the analyzed parameters of oxidative stress show that the F-ratio is above the limit of 1 for NO (F = 5.634; p <0.005) and TBARS (F = 5.220; p <0.005). Because of this, it can be noticed that the analyzed groups, in relation to the type of training interventions, statistically significantly differ from the results in the test. Differences in the average values of O_2^- and H_2O_2 before and after both training intervals have not been confirmed as statistically significant, although the F ratio is greater than one for both parameters.

Post Hoc test results (Table 2) refer to differences in analyzed parameters before and after both training interventions. In continuous aerobic training, the results showed that statistically significant differences were recorded before and after training in the values of NO, TBARS and CAT at the level of p = 0.05, while in interval training, statistically significant differences were recorded only in the values of NO and CAT at the level of p = 0.05. Accordingly, NO value after both types of training have lower values than those before training (3.13 versus 2.51 after continu-

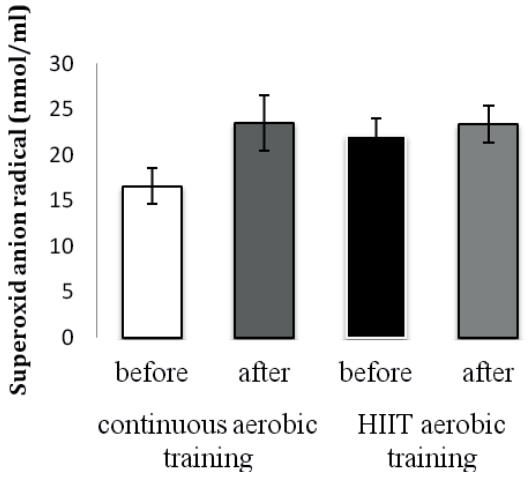

Figure 1. Superoxide anion radical concentrations (O_2^-), in blood before and after continuous and interval (HIIT) training. Results are expressed as mean. Results were compared using univariate analysis of variance (ANOVA), F values are given in the figure, and p<0.05 was considered as significant.

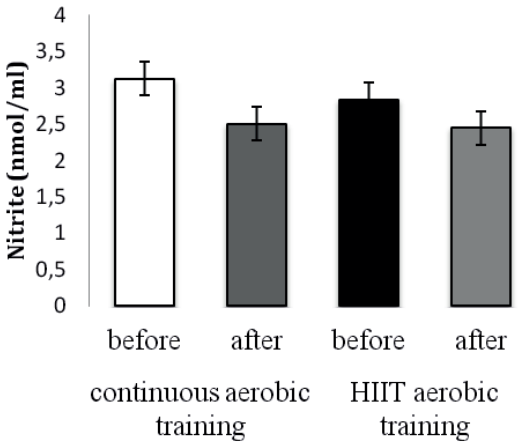

Figure 2. Nitrite (NO_2^-) concentrations in blood before and after continuous and interval (HIIT) training. Results are expressed as mean. Results were compared using univariate analysis of variance (ANOVA), F values are given in the figure, and p<0.05 was considered as significant.

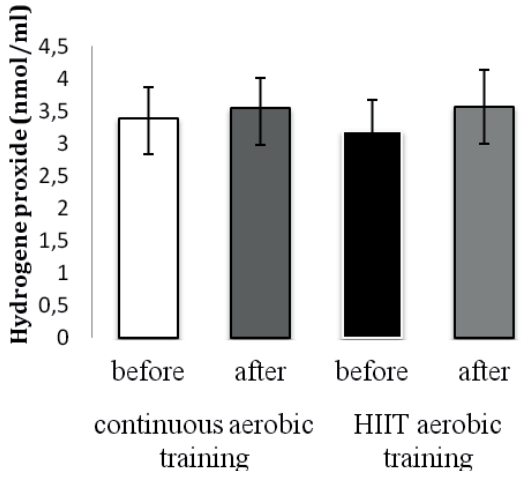

Figure 3. Hydrogen peroxide (H_2O_2) concentrations in blood before and after continuous and interval (HIIT) training. Results are expressed as mean. Results were compared using univariate analysis of variance (ANOVA), F values are given in the figure, and p<0.05 was considered as significant.

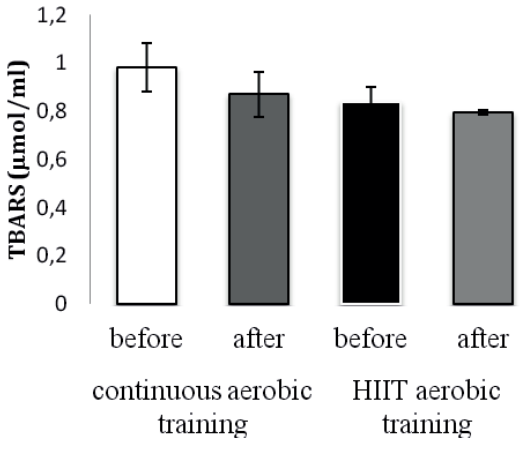

Figure 4. Index of lipid peroxidation (TBARS) concentrations in blood before and after continuous and interval (HIIT) training. Results are expressed as mean. Results were compared using univariate analysis of variance (ANOVA), F values are given in the figure, and p<0.05 was considered as significant.

When we analyze the activities of the enzyme protection against oxidative damage (Figure 5-7), the F value for CAT (F = 3.004; p <0.05) and GSH (F = 2.831; p <0.05) show differences between groups versus training intervention, while differences in the average values of SOD activity before and after both training interventions were not confirmed as statistically significant.

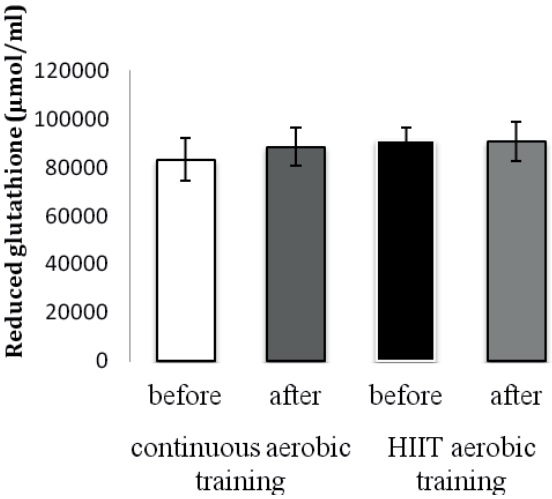

Figure 5. Reduced glutatione (GSH) concentrations in blood before and after continuous and interval (HIIT) training. Results are expressed as mean. Results were compared using univariate analysis of variance (ANOVA), F values are given in the figure, and p<0.05 was considered as significant.

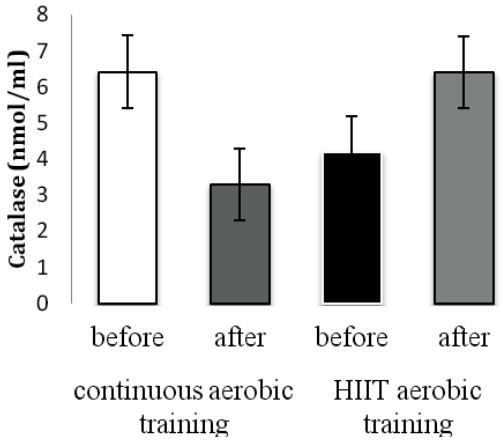

Figure 6. Catalase (CAT) concentrations in blood before and after continuous and interval (HIIT) training. Results are expressed as mean. Results were compared using univariate analysis of variance (ANOVA), F values are given in the figure, and p<0.05 was considered as significant.

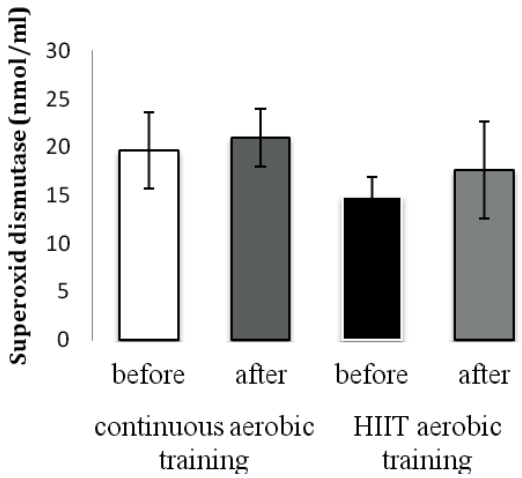

Figure 7. Superoxid dismutase (SOD) concentrations in blood before and after continuous and interval (HIIT) training. Results are expressed as mean. Results were compared using univariate analysis of variance (ANOVA), F values are given in the figure, and p<0.05 was considered as significant.

ous training and 2.84 versus 2.5 after interval training). TBARS value after continuous training records a reduction compared to those prior to training (0.98 versus 0.87). There is no statistically significant difference before and after interval training in the variable TBARS. CAT values after continuous training record a reduction compared to those prior to training (6.42 versus 3.29), while in interval training, CAT marks an increase in value after training compared to pre-values (4.19 versus 6.40). Other markers show no statistically significant difference before and after both types of training.

Tabela 2. Results of the LSD Post Hoc test between trainings

Varijable	Trainings	Difference AS	p
NO	Initial vs. final continuous	0,623	0,002
	Initial vs. final HIIT	0,391	0,044
TBARS	Initial vs. final continuous	0,111	0,030
CAT	Initial vs. final continuous	3,125	0,020
	Final continuous vs. HIIT	-3,104	0,020

Legend: Difference AS – Difference arithmetic mean between trainings; p – statistical significance differences AS.

DISCUSSION

Anthropometric data indicate that this is a heterogeneous group of female basketball players that are at the beginning of the preparation period, and the high percentage of fat in the muscles is evident. There is a difference in height in relation to the position in the team and therefore also the difference in body mass.

Influence of training on redox status depend from characteristics of training protocol such us frequency, volume, intensity, aerobic/anaerobic components, and other elements (2). Therefore, literature data are inconsistent and show both positive or negative effects of training on oxidative stress in athletes (2). In addition, gender in another important factor and strongly affects obtained results, whereby there are lack of information regarding oxidative status of females in different exercise types and sports. In that sense this study was aimed to help in better understanding of all these issues through assessment of two types of training regimes (continuous aerobic and interval (HIIT) training) on the oxidative status of female basketball players. Oxidative stress is the accompanying occurrence of any physical activity that requires effort, and thus the accompanying appearance of a dynamic basketball game.

The analyzed parameters of oxidative stress show different results. Index of lipid peroxidation (TBARS) concentrations were decreased after continuous training and also as the only significant change recorded in high inten-

sity interval training. Similar results showed Marin at al. (15) where TBARS levels were gradually decreased until they reached levels below baseline showing an inverse relationship to the erythrocyte enzyme activities. They suggest that regular aerobic training enhanced enzymatic antioxidant defenses and thus reduced the exercise-induced lipid peroxidation in erythrocytes.

The changes that occurred in nitric oxide (NO), in the form of nitrite (NO_2^-) were recorded after continuous aerobic training, as well as high intensity interval training. The change was reflected in a decrease in concentration of NO in the blood. On the other hand, there was no significant difference between the two types of training. According to Djordevic at al. (16) in athletes, structural changes of blood vessels, minimize or even eliminate the need for increased NO production after exercise.

Although it was detected an slight increase in superoxide anion radical concentrations (O_2^-) in the blood plasma after both training, there was no significant difference between the two types of training as well as in other study (17). There was no significant changes in superoxide before and after continuous as well as after HIIT training session.

Analyzing concentration of hydrogen peroxide (H_2O_2) in the blood results showed slight increase after both type of training, although not statistically significant. According to Djordevic at al. (16) athletes who were better aerobically prepared had significantly lower levels of $H_2O_{2.,}$ which was not the case in our study. Since our athletes are at the beginning of the preparation period and with poor aerobic power these results are expected. On the other hand, the differences between both types of training are evident and statistically significant. After HIIT session they experience higher exercise-induced oxidative stress when compared with continuous aerobic training. This may suggest that improving aerobic capacity may be important factor in inducing positive changes in redox state (16).

When we analyze the activity of the protection enzymes against oxidative damage, the antioxidant enzymes SOD, CAT and GPH are the primary defense against ROS generated during exercise and increase in response to exercise (18).

In our study we found an irreversible change in activity of catalase (CAT) when comparing the first and second type of training, which was the case in the study of Fisher at al. (19). After the first training, the values of CAT are reduced, according to the not aerobically prepared athletes, while after the second session, along with improvement in training, CAT increases.

Reduced glutatione (GSH) after continuous training records increasing in concentrations in the blood, as well as lightly decreasing after high intensity interval training. Both of changes are not statistically significant, but they are present.

Small differences in superoxid dismutase SOD activities are consistent with the findings of other studies (20,21), that is, SOD activity is significantly changed only after high production of reactive oxygen species (22), which was not the case in our study.

CONCLUSION

The results of present study may help in better understanding of the effects of the two types of physical load on redox state in female atheletes. Long-term, continuous physical exercise does not necessarily produce oxidative stress, while intermittent (HIIT) training emphasizes its formation and activation of antioxidant enzymes. Oxidative stress and antioxidant biomarkers can change during the season, reflecting physical stress and muscle damage that occur as a result of exercise. If the training is properly dosed, the occurrence of oxidative stress is not necessarily a consequence of a training of a high aerobic load. The training itself leads to the maintenance of the physiological balance in the organism.

REFERENCES

1. Stanković, M., & Radovanović, D. (2012). Oxidative stress and physical activity. SportLogia, 8(1), 1-10.
2. Pešić, S., Jakovljević, V., Čubrilo, D., Živković, V., Jorga, V., Mujović, V., & Stojimirović, B. (2009). Evaluation of the oxidative status of top athletes-karateists in the training process. Vojnosanitetski Pregled, 66(7), 551-555.
3. Powers, S. K., & Jackson, M. J. (2008). Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. Physiological reviews, 88(4), 1243-1276.
4. Gormley, S. E., Swain, D. P., High, R., Spina, R. J., Dowling, E. A., Kotipalli, U. S., & Gandrakota, R. A. M. Y. A. (2008). Effect of intensity of aerobic training on V˙ O2max. Medicine & Science in Sports & Exercise, 40(7), 1336-1343.
5. Zwetsloot, K. A., John, C. S., Lawrence, M. M., Battista, R. A., & Shanely, R. A. (2014). High-intensity interval training induces a modest systemic inflammatory response in active, young men. J Inflamm Res, 7, 9-17.
6. Shiraev, T., & Barclay, G. (2012). Evidence based exercise: Clinical benefits of high intensity interval training. Australian family physician, 41(12), 960.
7. Auclair C, Voisin E (1985) Nitroblue tetrazolium reduction. In: Greenvvald RA (ed) Handbook of methods for oxygen radical research. CRC Press, Boka Raton, pp 123–132.
8. Pick E, Keisari Y (1980) A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture. J Immunol Methods 38:161–170.
9. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR (1982) Analysis of nitrate, nitrite and [15N] nitrate in biological fluids. Anal Biochem 126:131–138.
10. Ohkawa H, Ohishi N, Yagi K (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95:351–358.

11. McCord JM, Fridovich I (1969) The utility of superoxide dismutase in studying free radical reactions. I. Radicals generated by the interaction of sulfite, dimethyl sulfoxide, and oxygen. J Biol Chem 244(22):6056–6063.
12. Beutler E (1982) Catalase. In: Beutler E (ed) Red cell metabolism, a manual of biochemical methods. Grune and Stratton, New York, pp 105–106.
13. Misra HP, Fridovich I (1972) The role of superoxide-anion in the autooxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem 247:3170–3175.
14. Beutler E (1975) Reduced glutathione—GSH. In: Beutler E (ed) Red cell metabolism: a manual of biochemical methods. Grane and Straton, New York, pp 112–114.
15. Marin, D. P., Bolin, A. P., Campoio, T. R., Guerra, B. A., & Otton, R. (2013). Oxidative stress and antioxidant status response of handball athletes: implications for sport training monitoring. International immunopharmacology, 17(2), 462-470.
16. Djordjevic, D., Cubrilo, D., Macura, M., Barudzic, N., Djuric, D., & Jakovljevic, V. (2011). The influence of training status on oxidative stress in young male handball players. Molecular and cellular biochemistry, 351(1-2), 251-259.
17. Cubrilo, D., Djordjevic, D., Zivkovic, V., Djuric, D., Blagojevic, D., Spasic, M., & Jakovljevic, V. (2011). Oxidative stress and nitrite dynamics under maximal load in elite athletes: relation to sport type. Molecular and cellular biochemistry, 355(1-2), 273-279.
18. Steinbacher, P., & Eckl, P. (2015). Impact of oxidative stress on exercising skeletal muscle. Biomolecules, 5(2), 356-377.
19. Fisher, G., Schwartz, D. D., Quindry, J., Barberio, M. D., Foster, E. B., Jones, K. W., & Pascoe, D. D. (2010). Lymphocyte enzymatic antioxidant responses to oxidative stress following high-intensity interval exercise. Journal of Applied Physiology, 110(3), 730-737.
20. Tauler, P., Gimeno, I., Aguilo, A., Guix, M. P., & Pons, A. (1999). Regulation of erythrocyte antioxidant enzyme activities in athletes during competition and short-term recovery. Pflügers Archiv, 438(6), 782-787.
21. Vider, J., Lehtmaa, J., Kullisaar, T., Vihalemm, T., Zilmer, K., Kairane, Č., & Zilmer, M. (2001). Acute immune response in respect to exercise-induced oxidative stress. Pathophysiology, 7(4), 263-270.
22. Spasić, M. B., Saičić, S., Buzadžić, B., Korać, B., Blagojević, D., & Petrović, V. M. (1993). Effect of long-term exposure to cold on the antioxidant defense system in the rat. Free Radical Biology and Medicine, 15(3), 291-299.

THE EDUCATION OF EMPLOYEES AS A MOTIVATION FACTOR IN THE MANAGEMENT OF CLINICAL CENTER OF SERBIA

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EDUKACIJA ZAPOSLENIH KAO MOTIVACIJA U ORGANIZACIJI KLINIČKOG CENTRA SRBIJE

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ABSTRACT

With more than seven thousand employees, KCS is the largest provider of health services in Serbia and one of the largest in Europe, which has more than one million patients per year. The aim of the research is to determine the assessment of employee satisfaction by various factors of motivation, various education programs at the Clinical Center of Serbia, which to some extent contribute to motivation in the workplace. This cross-sectional randomized descriptive study was performed from April 1st to 30th 2016, and included 151 health care professionals in Clinical Center of Serbia. Study included employees both gender and various years old, with different qualificantion and formal education. The anonymous survey about motivation of emplyoeers covered a total of 15 questions with responses (questions of closed type). Based on our results, we can conclude that despite subjectivism attributed to the survey as a method of research, in this case, we got a result that fairly objectively shows the situation in the Clinical Center of Serbia. From all of the above, we can conclude that the organizational culture is not weak in the Clinical Center of Serbia, but certainly it is necessary to work on strengthening its strength, which can be connected with the insufficient development of organizational culture as a scientific discipline in the academic community in Serbia.

Keywords: *motivation, continous medical education, health care professionals, Clinical Center Serbia, survey.*

SAŽETAK

Sa više od sedam hiljada zaposlenih, KCS je najveći pružalac zdravstvenih usluga u Srbiji i jedan od najvećih u Evropi, koji godišnje ima više od milion pacijenata. Cilj istraživanja je da se utvrdi procena zadovoljstva zaposlenih korišćenjem različitih faktora motivacije i različitih obrazovnih programa u Kliničkom centru Srbije, koji delimično doprinose motivaciji na radnom mjestu. Ova unakrsna dijagnostička slučajna deskriptivna studija odrađena je od 1. do 30. aprila 2016. godine, a obuhvaćen je 151 zdravstveni radnik u Kliničkom centru Srbije. Studija je uključivala oba pola i različita godišta, sa različitim kvalifikacijama i formalnim obrazovanjem. Anonimno istraživanje o motivaciji zaposlenih pokrivalo je ukupno 15 pitanja sa odgovorima (pitanja zatvorenog tipa). Na osnovu naših rezultata možemo zaključiti da uprkos subjektivizmu koji se pripisuje upitniku kao metodu istraživanja, u ovom slučaju, smo dobili rezultat koji objektivno pokazuje situaciju u Kliničkom centru Srbije. Iz svega navedenog možemo zaključiti da organizaciona kultura u Kliničkom centru Srbije nije slaba, ali svakako je potrebno raditi na povećanju njene snage, koja se može povezati sa nedovoljnim razvojem organizacione kulture kao naučne discipline u akademskoj zajednici Srbije.

Ključne reči: *motivacija, kontinuirana medicinska edukacija, zdravstveni radnici, Klinički centar Srbije, anketa*

ABBREVIATIONS

KCS - Clinical Center Serbia

sciendo
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INTRODUCTION

With more than seven thousand employees, KCS is the largest provider of health services in Serbia and one of the largest in Europe, which has more than one million patients per year (1). In the stationary activity of KCS, more than 90 000 patients are treated annually, more than 950 000 patient days are treated, performed over 50 000 operations and more than 7 000 births. In day-care hospitals 25 000 patients are treated annually and perform over 5,000 operations (2).

Education for healthcare workers and healthcare associates at the Clinical Center of Serbia is organized in order to continuously improve professional knowledge in order to maintain and improve skills and competencies in providing health services to patients (3). The goal of organizing education of employees is the continuous monitoring of new scientific and professional knowledge and skills, which in synergy with generally recognized and already applied doctrines and methods, enable the development and improvement of professional potentials on an individual and institutional level, within the Clinical Center of Serbia as a high specialized medical institutions of tertiary health care, and teaching bases of the Medical Faculty of the University of Belgrade (3, 4).

Education within the Clinical Center of Serbia is carried out in the form of professional meetings organized for healthcare workers and healthcare associates. Lecturers at professional meetings are healthcare workers and medical associates employed at the Clinical Center of Serbia, then lecturers by invitation from other institutions. A very developed and significant form of education is the organization of professional meetings within the association of the Association of Physicians of Serbia, the Serbian Medical Society, where the regular and successful participation of doctors of the Clinical Center of Serbia, all specialties, within the work of individual sections was noticed.

The reports about continous medical education for health care professionals bring to us the next information: for nurses were organized 6 courses and for technicians were organizied 123 expert meetings with lecturers from 23 Organizational units of the Clinical Center of Serbia (5, 6). In 2014, a total of 18225 participants attended the meetings. During 2015, 22.254 certificates were awarded, of which 7302 for doctors and 14952 for nurses of the Clinical Center of Serbia, who attended educational programs organized at the Clinical Center of Serbia (7-9).

In that sense, the aim of the research is to determine how certain methods, as well as continuous education of employee motivation, become the driving force of a certain, desired human behavior, and how they lead to the effective realization of the goals of the organization. Also, the aim of the research is to determine the assessment of employee satisfaction by various factors of motivation, various education programs at the Clinical Center of Serbia, which to some extent contribute to motivation in the workplace.

PARTICIPANTS AND METHODS

Study population

This cross-sectional randomized descriptive study was performed from April 1st to 30th 2016, and included 151 health care professionals in Clinical Center of Serbia. Study included employees both gender and various years old, with different qualificantion and formal education.

Instrument

The anonymous survey about motivation of emplyoeers covered a total of 15 questions with responses (questions of closed type). Questions in the survey were closed type, i.e. the answer is chosen from the offered answers, according to the principle of the highest level of matching of the offered answer with the opinion of the participants in the survey (Table 1). The results of the survey were analyzed for specific issues with accent on those on which it is possible to draw a reliable conclusion from the answers (1, 8).

RESULTS

Demographic characteristcs of study population

This study included 151 participants. Of the total number of female respondents, 119 are female, while 32 are male respondents. The research involved doctors (25 doctors) and nurses (126 nurses). Also, the most of participants were between 20-30 years old (48.43%), than between 31-40 years old (31.13%) (Figure 1).

According to duration of working experience, all participants were divided into groups: the most of participants were with 5-10 years working experience (37.09%), than with less than 5 years experience (34.44%), 10-20 years working experience (20.53%) and with 20-30 years working experience were in the smallest percent (7.95%) (Figure 2).

The attitude of the participants about motivation at work (Questions 1-6)

Question 1: A fifth of respondents (20.5%) consider their work only as a means of securing material conditions for life, 7.9% of respondents consider their work a way of achieving professional preferences and interests. The largest number of respondents opted for one and the other, i.e. believes that through their call, financial security and professional satisfaction can be achieved (71.5% of respondents). Observing the relationship between the age of the respondent and the attitude towards the work they perform, it can be observed that patients aged between 21-30 years ($p = 0.000$) and persons who have up to 10 years of working experience ($p = 0.023$) only perceive their work as a means of securing material conditions (37% of the 20-30-year-olds have this attitude, as well as a quarter of respondents who have up to 10 years of working experience).

Table 1. Structured Survey for analyses of motivation, educationa and communication skill of healthcare professionals

General information: Position: a) Physician b)Technician Gender: a) male b)female Years: a) 20-30 b)31-40 c) 41-50 d) 51-60 e)>60 Working experience: a) 0-5 b) 5-10 c) 10-20 d)20-30 e) 30-40	
Question 1: Is the job you are currently doing at the Clinical Center of Serbia for you?	a)the only way of securing material living conditions b) achieving professional tendencies and interests c) both
Question 2: Are you familiar with what is expected of you at work?	a) completely b) partially c) no d) I have no definite position on this
Question 3: Are you satisfied with the amount of total remuneration for your work?	a) I am very satisfied b) I am mostly c) I'm not satisfied at all d) I have no definite position on this
Question 4: What kind of personal stimulation, in your opinion, has the most effect on employees?	a) monthly stimulation b) quarterly, semi-annual or annual bonus c) Commendation or public recognition d) I have no definite position on this
Question 5: Do you think that your work, performance and commitment are permanently praised and evaluated objectively and fairly?	a) yes b) partially c) no d) I have no definite position on this
Question 6: In your opinion, does the reward system in the Clinical Center stimulate the quality of work, dedication, creativity and innovation?	a) completely b) partially c) no d) I have no definite position on this
Question 7: A grade of 1 (not at all important) to 5 (very important) assess the importance of education and professional development for the work you do on your workplace.	**1 2 3 4 5**
Question 8: Is, in your opinion, the level of investment in the professional development of employees (professional meetings, seminars, medical education, postgraduate studies...) such that it enables successful work?	a) completely b) partially c) no d) I have no definite position on this
Question 9: Do you think that in the Clinical Center of Serbia you have all the conditions for advancement in the profession?	a) completely b) partially c) no d) I have no definite position on this
Question 10: Are professional competencies, responsibility and ethical values, in your opinion, dominant for advancement within the hierarchical structure of the Clinical Center of Serbia?	a) completely b) partially c) no d) I have no definite position on this
Question 11: Evaluate your general opinion on the relationships between associates in the Clinical Center of Serbia by a full grade of 1 (unsatisfactory) to 5 (excellent).	**1 2 3 4 5**
Question 12: Assess the degree to which you are satisfied with the attitude of your immediate manager in everyday business communication with an integer score of 1 (non-honest) to 5 (very correct)	**1 2 3 4 5**
Question 13: Do you perceive yourself as a member of a team that participates in the achievement of common results?	a) completely b) partially c) no d) I have no definite position on this
Question 14: Assess the degree to which, in your opinion, the views and suggestions of employees are taken into consideration by an integer score of 1 (ignored) to 5 (extremely important)?	**1 2 3 4 5**
Question 15: Do you feel good, safe and accepted in your work environment?	a) completely b) partially c) no d) I have no definite position on this

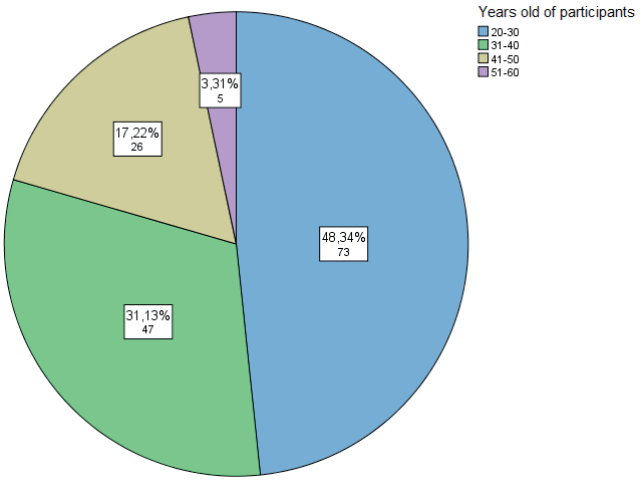

Figure 1. Distribution of participants by age

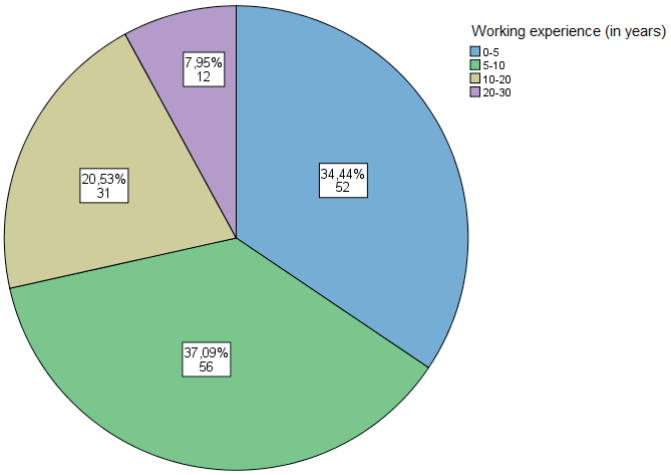

Figure 2. Distribution of participants by working experience in years

Question 2: The largest number of participants, 51.7% of them stated that they were fully acquainted with what is expected of them at work, 40% are partially familiar, while 7.1% are not familiar.

Question 3: 64.2% of respondents are not satisfied with the amount of material income, and a 19.2% are mostly satisfied, 12.6% expressed their satisfaction and 4% there is no definite position on this.

Question 4: The largest percentage, 58.3% of them consider monthly personal stimulation 27.2% believe that the quarterly, half-yearly or annual bonus is the best form of stimulation, while 12.6% of employees are praised and publicly acknowledged, while 2% do not have a defined attitude about it.

Question 5: Participants who believe that their work is evaluated objectively and fairly in the majority, 47.7%, a large number of respondents thinks that they are partly 36.4%, while 15% of those who think that their work is not valued in the right way.

Question 6: With the reward system in KCS stimulating the quality of the work, almost all participants consider that it is completely compatible with this (47.7%) or partly (49.7%). Only 4 respondents disagree or do not have an attitude related to this topic.

The attitude of the participants about education at work (Questions 7-10)

Question 7: 97.3% of participants consider education very important (Likert scale-grade 5) and important (Likert scale-grade 4). No respondent stated that education is not important at all (Likert scale-grade 1) and does not matter (Likert scale-grade 2).

Question 8: With 95.3% of participants agreeing that investment in professional development of employees enables successful work, 57.6% of them are completely satisfied, while 37.7% agree in part.

Question 9: The majority of participants fully believe that the CCS has conditions for promotion of 68.9%, partly 22.5%. There is no statistically significant difference in responses among participants with different length of service and age (p>0.05).

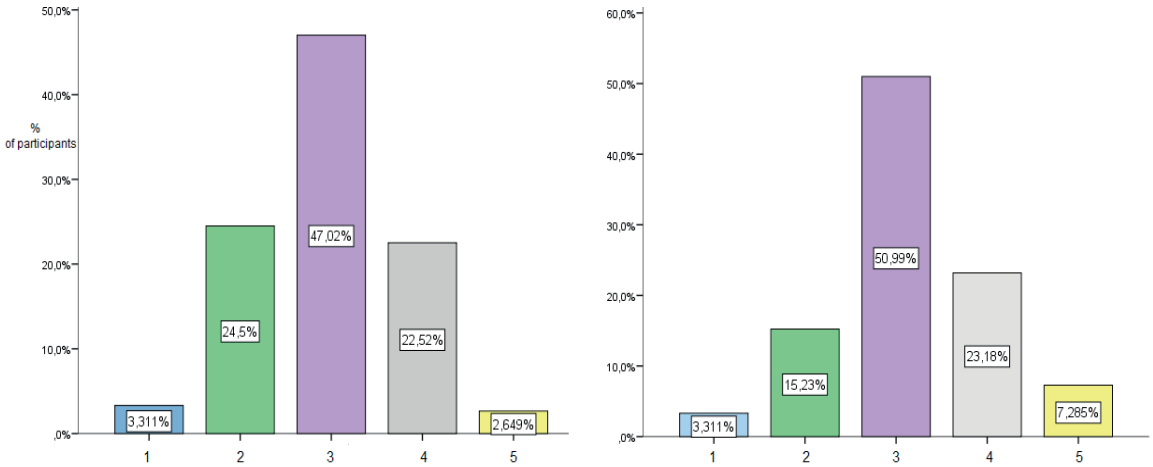

Figure 3.

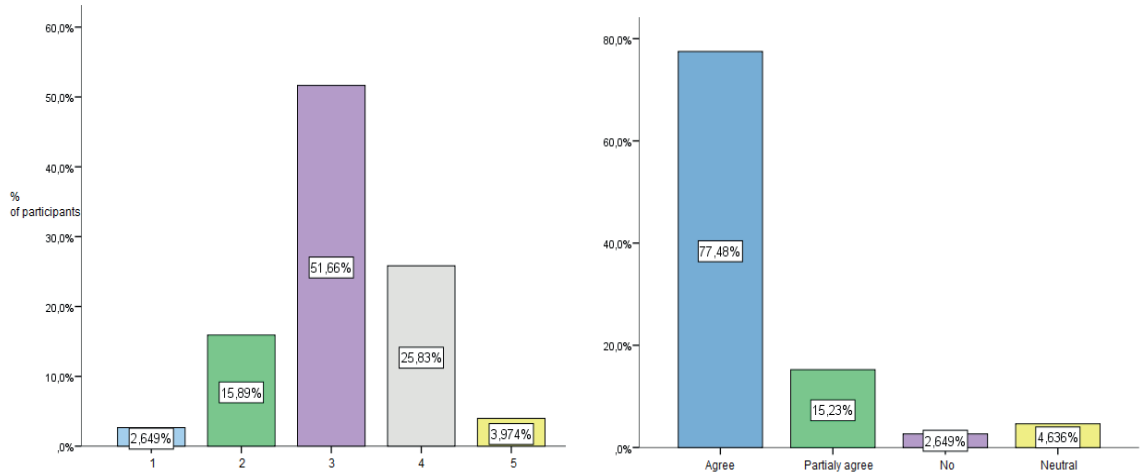

Figure 4. Graphic distribution of the answer to Question 14: Assess the degree to which, in your opinion, the views and suggestions of employees are taken into consideration by an integer score of 1 (ignored) to 5 (is extremely respected)? and Question 15: Do you feel good, safe, and accepted in your work environment?

Question 10: Professional abilities, responsibility and ethical values are the dominant ones for progress within the hierarchical structure of the Clinical Center of Serbia in full, according to opinion, close to 50.3% of participants, and partly according to 44.44%. 2.6% does not exist such compliance, and also 2.6% have no defined position on this.

The attitude of the participants about communication skills and team work in organization (Questions 10-15)

Question 11: The highest number of participants is the assessment of the relationships between associates in KCS grade 3, close to half of respondents (47.02%). Points 2 and 4 rated 24.5% and 22.52%, while rating 1 and 5 rated 3.3% and 2.6% respectively (Figure 3).

Figure 3. Graphic distribution of the answer to Question 11: Evaluate your general opinion on relationships among associates at the Clinical Center of Serbia by a full grade of 1 (unsatisfactory) to 5 (excellent) and Question 12: Assess the degree to which you are satisfied with the attitude of your immediate manager in everyday business communication with a full score of 1 (incorrect) to 5 (very correct).

Question 12: The participants assessed the satisfaction with the attitude of their immediate manager in their everyday business com- munication with a score of 3 (51% of subjects). About a fifth of participants rated the relationship as correct (4) 23.2%. Points 2, 5 and 1 rated it (15.2%, 7.3% and 3.3% respectively). There is no statistically significant relationship between age or work experience and satisfaction with the attitude of your immediate manager in everyday business communication (p>0.05) (Figure 3).

Question 13: The participants perceive themselves as members of the team participating in the achievement of the common results, a total of 94% of the respondents, of which 60.9% are completely, 33.1% partially. The small share of participants, 4%, does not perceive themselves as a team member, while 2% of the participants have no opinion about it. There is no statistically significant correlation between age or years of service and experiencing oneself as a team member (p>0.05).

Question 14: Recognizing the attitudes and suggestions of employees can greatly motivate to work and give new proposals in order to improve the functioning of the institution in which they work. A large share of the respondents considered that consideration of attitudes and suggestions of employees could be estimated with a score of 3 (51.66%). A quarter of the respondents said they believed that the grade for appreciation was 4 (25.83%), while 15.89% were rated as 1, 3.97% by grade 5 and 2, 65% by grade 1. There is no statistically significant correlation between age or years of service and an opinion on the degree to which the views and suggestions of employees are respected (p>0.05) (Figure 4).

Question 15: The atmosphere in the collective is very important for the productivity of an individual. The majority of the respondents stated that 77.5% were fully accepted, 15.2% were partially accepted, while those who do not have the opinion about this were 2.6% and 4.6%. There is no statistically significant correlation between age or years of service and the attitude of acceptance and security in the collective (p>0.05) (Figure 4).

DISCUSSION

The aim of the this research is to determine how certain methods, as well as continuous education of employee motivation, become the driving force of a certain, desired human behavior, and how they lead to the effective realization of the goals of the organization. Workforce motivation as an important function within the human resources management process requires careful and detailed research, therefore special attention is devoted to the study of this phenomenon, its methods and factors (5-8). Also, the aim

of the research is to determine the assessment of employee satisfaction by various motivation factors, various education programs at the Clinical Center of Serbia, which to some extent contribute to motivation in the workplace.

The fact that the majority of respondents (71%) think that the job is something that will equally provide them with material conditions for living and achievement of professional affinity and interest, proves that the employees of the Clinical Center of Serbia are assigned to positions that suit them to a large extent, both in terms of income, and according to the possibilities for achieving professional preferences and interests.

The issue of material income is always a sensitive topic. A very high percentage of employees who are not satisfied with material income, which can be explained by the recent salary reduction, both in the Clinical Center of Serbia and in all health institutions in Serbia. The fact that work is not compensated for by just the amount of earnings can be troubling, because the insufficiency of employees in health institutions can negatively affect the quality of the services they provide, which would be very bad for citizens, users of health services (9).

The fact that 58.33% of respondents indicated monthly stimulation as the type of personal stimulation that has the greatest effect on employees, can be supported by the logical assumption that the employees most of the price for this type of stimulation is the price for the simple reason that it is short-term, that is, its effects are quickly noticed in the monthly level. Namely, in the case of quarterly, semi-annual or annual bonuses, for which 28.70% of respondents answered, it is necessary to wait for the reward for the invested effort much longer, which employees do not really like.

Furthermore, permanent monitoring of the work, performance and commitment of employees for the objective and fair evaluation is one of the basic tasks of management in the application of the employee reward system (11, 12). In that sense, most respondents think that their work is evaluated objectively and fairly, encourages and points to the existence of an extremely qualitative system of rewarding (10). Also, the reward system applied at the Clinical Center is contemporary, universal, motivating and focused on stimulating the quality of work, advocating employees, creativity and innovation. With this conclusion, close to 44% of the respondents agreed. In this respect, we can say that the reward system is such that it exclusively motivates employees to work more valuable, with more commitment and interest.

Regarding to education in healthcare systems and organizations, our results indicate that education and professional training are very important for all medical staff, both for doctors and for medical technicians (Figs. 3 and 4). A small percentage of those who think that education is only partially important are employees who carry out desk services and do not provide any medical services. Also, high attention is paid to continuing education, with special emphasis on the education of doctors. In this sense, as a confirmation of this, as many as 53.70% of respondents consider that the level of investment in the professional development of employees is such that it enables successful work. Finally, the most of respondents believe that the company has all the conditions for promotion.

Finally, in this study we evaluated communication skills and general communication entire the organization (Fig. 4). If relationships between associates are disturbed for any reason, this will have a negative impact on the results. Most respondents, giving grade 3, confirm that only with good employee relationships can work become a pleasure. Since most of the time is spent at work, it is extremely important that the employees feel good and that a positive atmosphere is in place (2, 14). A good relationship with the direct management is very important, and can greatly affect the fact that employees do not do their job in the right way or within the stipulated deadlines (12, 13). Of great importance is the so-called two-direction communication that should be everyday, exhaustive and comprehensive, with clear and precise tasks, but also with the space for employees to be creative, innovative and free to propose, give suggestions and their opinions. Even 45.37% of respondents believe that the relations of employees and their direct managers are good, with the tendency to further develop further cooperation.

Forming teams or working groups is another way for employees to contribute to the overall success of the Clinical Center of Serbia. Team spirit and feeling that they are part of one successful team is a special pleasure and a strong motive (15-17). All employees are different, but about these differences, they are talking, harmonizing and working perfectly (18, 19). In the Clinical Center of Serbia, mutual help, team decision-making, and consensus are cultivated. Team spirit and community that employees feel at work in the team, if judging by these 58% of the respondents who fully perceive themselves as members of the team involved in achieving common results, evidence is that there is a high percentage of employees who are extremely motivated to give their maximum in achieving common results. Recognizing attitudes and suggestions can be a great motivation for employees. According to the results of the survey, 47.22% of the respondents believe that at the Clinical Center of Serbia, the attitudes of employees are appreciated to a considerable extent, which indicates that employees are respected and appreciated their contribution to the overall success of this health institution.

Over 75% of participants think that they feel fully, well and accepted in their work environment, and 15.74% in part. Undoubtedly it feels 3.70%, and 5.55% does not have a defined attitude about it. Acceptance of employees, especially young people, of newly employed workers is of great importance for their overall satisfaction and motivation. Employees at work should not feel insecure, unadjusted, and dismissed, both from their associates and from direct managers. In the Clinical Center of Serbia, this is paid great attention, with a special emphasis on newly employed workers, as well as those who move from one position to another. That 75% of the participants testify that this is largely successful.

CONCLUSION

Well, based on our results, we can conclude that despite subjectivism attributed to the survey as a method of research, in this case, we got a result that fairly objectively shows the situation in the Clinical Center of Serbia. From all of the above, we can conclude that the organizational culture is not weak in the Clinical Center of Serbia, but certainly it is necessary to work on strengthening its strength, which can be connected with the insufficient development of organizational culture as a scientific discipline in the academic community in Serbia. Only by encouraging internal factors such as education and upgrading it can be increased employee motivation in order to encourage creativity and innovation. Also, the management of the organization should plan and develop a model of organizational culture, which will enable the professional potential and the overall intelligence of the collective to be used in the best way. Generally, it is important that the system being applied maintains the culture of an organization and is trained to achieve goals and, if that is not the case, it should be changed.

CONFLICT OF INTERSTS

None.

REFERENCES

1. Kanfer R, Chen G, Pritchard RD. The Three C's of Work Motivation: Content, Context and Change, In: Kanfer R., Chen G., Pritchard RD (Eds.), Work Motivation: Past, Present and Future, New York, London, Routledge, 2008.
2. Kelly, K. Motorola: Training for the Millennium, Business Week, Mar. 28: 158-162., 1994.
3. Arena, R. Organization and Knowledge in Alfred Marshall's Economics, in Arena & Quere, Eds, The Economics of Alfred Marshall: Revisi_ng Marshall's Legacy, Palgrave MacMillan, New York, 2003
4. Orlić R. Personnel Management, Zoran Damnjanović and Sons, Belgrade, 2005.
5. Hofer J., Bond MH. Do Implicit Motives. Add to Our Understanding of Psychological and Behavioral Outcomes, Winthin and Cultures, In: Sorrentino R., Yamaguchi S. (Eds.), Handbook of Motivation and Cognition across Cultures, San Diego : Elsevier, Academic Press, 2008.
6. Stefanović Ž. Human Resources Management, Faculty of Economics, Kragujevac, 2007.
7. Kuzmanović S.Theory of Motivation, Scientific Meeting Management and Pro-Men's Management, YUPMA, Zlatibor, 2003.
8. Legetić, B. et al., Approaches and Methods of Management in Health Institutions, European Center for Peace and Development, United Nations Peace Institute, Scientific Paper, Belgrade, 2008.
9. Robbins, S., Essential Elements of Organizational Behavior, MATE, Zagreb, 1996.
10. Beck R. C. Motivation: Theory and Principles, Naklada Slap, Zagreb, 2009.
11. Mihajlović, D., M. Paunović. Employee Motivation by Maslov's Hierarchy Needs, XXXV Symposium on Operational Research, Sokobanja, 2008.
12. Mihajlović, D. Management of behavior of managers and problems of securing the glacier, Business Policy, Belgrade, p. 45-46, 2006.
13. Bernardin, H. J. Human resource management: an experiential approach, McGraw-Hill Irwin, Boston, 2003.
14. Boxall P., Purcell J. Strategy and Human Resource Management, Palgrave Macmillan, Great Britain, 2003.
15. Drucker, P. My Look at Management, Adigees, Novi Sad, 2006.
16. Vujić D., Human Resource Management and Quality, Center for Applied Psychology of the Society of Psychologists of Serbia, Belgrade, 2009.
17. Gringberg, J., Baron, R. A. Behavior in Organizations, Želind, Belgrade, 1998.
18. Dryden, G., Vos, J. The Revolution in Learning - how to change the way the world teaches, Eduka, Belgrade, 2004.
19. Balkin D. B., Logan J. W. Reward Policies That Support Entrepreneurship, Compensation and Benefits Review, Vol. 20, Iss. 1, Jan / Feb, 1998.

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THE MATERNAL LEUCOCYTES IN THROMBOPHILIA AND HYPOTHYROIDISM AND THEIR INFLUENCE ON FETAL CELLS

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LEUKOCITI MAJKE U TROMBOFILIJI I HIPOTIREOIDIZMU I NJIHOV UTICAJ NA FETALNE ĆELIJE

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ABSTRACT

The literature data show that thrombophilia and maternal dysfunction of thyroid gland during pregnancy are associated with an increased risk of miscarriage, placental abruption, hypertensive disorders and fetal growth retardation. It was shown that thyroid hormones and hypercoagulable states influence onto a leucocyte activity. The aim of this study has been to investigate maternal leucocytes changes and their correlation with frequency of fetal cells micronuclei in pregnant women with thrombophilia and hypothyroidism. The samples of blood and amniotic fluid were collected from healthy pregnant women and pregnant women with inherited thrombophilia and hypothyroidism (16 - 18 weeks of gestation). Hematological characteristics were determined by using standard hematological methods. The frequency of micronuclei was determined in fetal cells after amniocentesis by using standard cytogenetic methods. The results of this study showed significant higher levels of β-hCG, number of monocytes and eosinophils in blood of pregnant women with thrombophilia. A large number of eosinophils was documented in blood of pregnant women with hypothyroidism. Increased percentage distribution of eosinophils and basophils is shown in both investigated groups of pregnant women. The increased fetal cells micronuclei frequency and their correlation with percentage distribution of eosinophils and basophils were indicated in pregnant women with hypothyroidism. The obtained results suggest that an increased percentage of eosinophils and basophils in pregnant women with hypothyroidism contribute to a formation of micronuclei in fetal cells.

Keywords: *thrombophilia, hypothyroidism, leukocytes, fetal cells, micronuclei*

SAŽETAK

Podaci iz literature pokazuju da su trombofilija i disfunkcija štitaste žlezde majke tokom trudnoće povezane sa povećanim rizikom od pobačaja, odlubljivanjem placente, hipertenzivnim poremećajima i zastojem rasta fetusa. Pokazano je da tireoidni hormoni i stanje hiperkoagulacije utiču na aktivnost leukocita. Cilj ove studije je bio da se ispitaju promene leukocita majke i njihova veza sa učestalošću mikronukleusa u fetalnim ćelijama kod trudnica sa trombofilijom i hipotireoidizmom. Uzorci krvi i amnionske tečnosti sakupljeni su od zdravih trudnica i trudnica sa naslednom trombofilijom i hipotireoidizmom (16 - 18 nedelja gestacije). Hematološke karakteristike su određene korišćenjem standardnih hematoloških postupaka. Učestalost mikronukleusa je određena u fetalnim ćelijama nakon amniocenteze upotrebom standardnih citogenetičkih metoda. Rezultati ove studije pokazuju da je u krvi trudnica sa trombofilijom značajno veća koncentracija β-hCG, broja monocita i eozinofila. U krvi trudnica sa hipotireoidizmom pokazan je veći broj eozinofila. Kod obe grupe ispitivanih trudnica povećan je procenat zastupljenosti eozinofila i bazofila. Kod trudnica sa hipotireoidizmom pokazana je povećana učestalost mikronukleusa u fetalnim ćelijama i njihova korelacija sa procentom zastupljenosti eozinofila i bazofila u krvi majke. Dobijeni rezultati pokazuju da povećani procenat eozinofila i bazofila kod trudnica sa hipotireoidizmom doprinosi formiranju mikronukleusa u fetalnim ćelijama.

Ključne reči: *trombofilija, hipotireoidizam, leukociti, fetalne ćelije, mikronukleus*

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INTRODUCTION

Thrombophilia is inherited or acquired condition that predisposes patients to venous and/or arterial thrombosis and thromboembolic events. In pregnancy and postpartum period, the risk of thromboembolic events increased at a 4- to 5-fold compared to non-pregnant women (1). The main reason for the increased risk of thromboembolism in pregnancy is hypercoagulability, which is a result of hormonal changes and increase of pressure of pelvic veins (2). The literature data show that thrombophilia in pregnancy is associated with many complications such as severe preeclampsia, intrauterine growth retardation, abruption placentae, still birth and recurrent miscarriage (3). Thrombophilia in pregnancy is characterized by microthrombi generation, which induces mechanical damage of endothelium and leukocytes activation. On the other hand, inflammatory stimuli activate the coagulation (4). Wirstlein and coworkers (5) showed that the concentrations of pro-inflammatory factors are increased in the new-borns cord blood of mothers with thrombophilia.

The optimal function of thyroid gland during pregnancy is very important for a mother and a fetus development. The most common thyroid disorder in pregnancy is hypothyroidism, which occurs with a frequency of 3-5% of all pregnant women (6). Maternal dysfunction of thyroid gland during pregnancy is associated with an increased risk of miscarriage, placental abruption, hypertensive disorders and growth restriction (7). Thyroid hormones have a significant role in regulation of activation of leucocytes, which are important sources of reactive oxygen and nitrogen species (8). In hyperthyroid patients have been found increase number of eosinophils and mononuclear cells as well as the reduction in the number of neutrophils (9). Activated leucocytes produce large amount of reactive oxygen and nitrogen species that can damage DNA, lead to genomic instability and consequence to a formation of micronuclei (10).

Micronuclei frequency in cells cytoplasm is the sensitive biomarker of DNA damage by endogenous and exogenous toxins. Micronuclei are the structures formed as a result of DNA fragmentation or lagging of acentric chromosome or chromatid during mitosis. The increased frequency of micronuclei as biomarkers of genetic instability is shown in cancer, diabetes, autoimmune, neurodegenerative and cardiovascular diseases (11-13).

The aim of this study was to investigate maternal hematological changes and their correlation with frequency of fetal cells micronuclei in pregnant women with thrombophilia and hypothyroidism.

MATERIAL AND METHODS

Patients

The study protocol was approved by the Ethics Committee of Clinical Centre "Kragujevac" and all patients had given an informal consent. The subjects were selected from pregnant women who attended Obstetrics and Gynecology Department of Clinical Centre "Kragujevac", from June 2014 to June 2015. The indications for amniocentesis were the subject inclusion criterion. Three groups of pregnant women, gestational age from 16 to 18 weeks, were recruited to the study: (i) 32 healthy pregnant women (control group) who recommended age (> 35 years) for amniocentesis, (ii) 23 pregnant women with inherited thrombophilia and (iii) 23 pregnant women with hypothyroidism. The maternal age, thrombophilia and hypothyroidism in pregnancy provide justification for amniocentesis, since in these conditions; there is an increase of the incidence of perinatal morbidity. The inherited thrombophilia was defined by following mutations: FV Leiden (G1691A), FII (G20210A), MTHFR (C677T) and PAI-1 mutation. All mutations were confirmed by molecular diagnostic methods. Pregnant women with inherited thrombophilia received proper doses of Fraxiparine or Clexane, depending on the type of mutation, and it was present in a homozygous or heterozygous form. Hypothyroidism was defined by serum concentrations of thyrotropin (TSH) and free thyroxine (FT4). Pregnant women with hypothyroidism received different doses of Eutyrox, depending on the serum hormone concentrations. The study included pregnant women who were non-smokers and free from cardiovascular, hepatic and renal disorders. Red blood cells and platelet count, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, hematocrit value, prothrombin time, concentrations of C-reactive protein, hemoglobin and chorionic gonadotropin were determined in hospital laboratories. The clinical and hematological characteristics of pregnant women are shown in Table 1.

Isolation and cultivation of fetal cells from amniotic fluid

The samples, in the amount about 15 to 20 ml, were obtained by amniocentesis from amniotic fluid from pregnant women at gestation weeks from 16 to 18. The samples were centrifuged at 1800 rpm for 20 minute and supernatant removed. After centrifugation, the cell pellet was washed and resuspended in growth medium AmnioMax (Gibco, Life Technologies, USA) containing fetal bovine serum and transferred to the cell culture flask. The cells were incubated at 37 ^{0}C in 5% CO_2 atmosphere to achieving confluence.

Micronucleus in vitro assay in fetal cells

For *in vitro* micronucleus assay, it was used stock of Cytochalasin-B disolved in DMSO and diluted in medium before use. After achieving needed confluence of cell culture, 4μg/ml Cytochalasin-B was added to cell culture flask to block cytokinesis and incubated for 16 h at 37 ^{0}C in 5% CO_2 atmosphere. Wen incubation was finished and the cells were detached from the flask by 0.1% trypsin, which was inactivated

Table 1. Clinical and hematological characteristics of healthy pregnant women and pregnant women with thrombophilia and hypothyroidism.

Characteristic	Control	Thrombophilia	Hypothyroidism
Maternal age (years)	37.57 ± 0.34	37.72 ± 0.50	37.50 ± 0.84
Gestational age (weeks)	17.53 ± 0.07	17.41 ± 0.09	17.44 ± 0.60
Number of deliveries	0.94 ± 0.13	0.44 ± 0.14*	0.80 ± 0.20
Number of spontaneous abortion	0.42 ± 0.15	0.88 ± 0.19*	0.30 ± 0.10
C-reactive protein (CRP) (nmol/L)	3.65 ± 0.52	4.65 ± 0.80	4.80 ± 0.71
Red blood cells count (x 10^{12}/L)	4.11 ± 0.45	3.98 ± 0.90	3.91 ± 0.06*
Hematocrit (%)	0.36 ± 0.004	0.36 ± 0.03	0.35 ± 0.06
Hemoglobin (g/L)	121.74 ± 1.27	120.77 ± 1.67	117.10 ± 1.99*
Mean corpuscular volume (MCV) (μm^3)	87.18 ± 0.78	91.25 ± 0.84*	89.43 ± 1.00
Mean corpuscular hemoglobin (MCH) (pg/cell)	36.63 ± 6.95	45.48 ± 14.92	30.17 ± 0.37
Mean corpuscular hemoglobin concentration (MCHC) (g/L)	339.77 ± 2.55	315.07 ± 18.06	337.79 ± 3.28
Platelet count (x 10^9/L)	230.57 ± 9.41	230.39 ± 13.41	258.70 ± 14.49
Prothrombin time (PT) (s)	0.96 ± 0.013	0.98 ± 0.017	0.96 ± 0.01
Human chorionic gonadotropin (β-hCG) (U/L)	1.29 ± 0.80	2.13 ± 0.93*	1.60 ± 0.37

Values are means ± S.E.M. * $p < 0.05$, thrombophilia and hypothyroidism versus control.

after 2 to 3 minute by adding 1 ml DMEM, containing fetal bovine serum. The cell suspension was centrifuged at 1000 rpm for 10 minute, washed by 0,9% NaCl and subjected to a mild hypotonic treatment by 0.75% KCl in 1 minute. Then, the cells were centrifuged 1000 rpm for 10 minute and fixed in Carnoy's fixative (3:1, methanol : acetic acid) for 30 minutes. Finally, the cells were resuspended in a small volume of fixative (100-200 µl), dropped onto cleaned slides and dried in air. The slides stained by 4% Giemsa stain in buffer (pH 6.8), for a few seconds. The slides were covered with a coverslip and observed under a microscope. The minimum 1000 binuclear cells were scored from each slide for the presence of micronuclei. The identified and scored micronuclei were expressed as percentage micronuclei, at the 1000 binuclear cells (12).

Statistical analysis

The data is expressed as mean ± S.E.M. The statistical analysis was performed by using SPSS (version 20.0) for Windows. The independent *t*-test was used for comparison of data between the control group and the examined groups. The correlation coefficient (*r*) was determined by using Pearson Correlation. The statistical significance was accepted at the $p \leq 0.05$ for all comparisons and in all correlations. Only significant correlation coefficients are reported.

RESULTS

Clinical and hematological characteristics of healthy pregnant women and pregnant women with thrombophilia and hypothyroidism

Table 1 shows clinical characteristics of healthy pregnant women and pregnant women with thrombophilia and hypothyroidism. The maternal and gestational ages were not significantly different between the groups. Based on the results, significantly a low level of successful completion of pregnancy and significantly a higher incidence of spontaneous abortions were shown in women with thrombophilia compared to healthy pregnant women. In addition, pregnant women with thrombophilia showed significantly higher values of mean corpuscular volume (MCV) and chorionic gonadotropin (β-hCG) in their blood. Red blood cells count and concentration of hemoglobin were significantly lower in the blood of women with hypothyroidism in comparison to healthy pregnant women. Concentrations of C-reactive protein (CRP) and hemoglobin, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), prothrombin time (PT), platelet count and hematocrit were not significantly different between groups.

White blood cells count, the number and percentage of different types of leukocytes in the blood of healthy pregnant women and pregnant women with thrombophilia and hypothyroidism

The white blood cells count in the blood of healthy pregnant women and pregnant women with thrombophilia and hypothyroidism is presented in Figure 1. The obtained results show there is no difference in the number of white blood cells between the examined groups. Figure 2 shows results of the number of certain types of leukocytes in blood of healthy and pregnant women with thrombophilia and hypothyroidism. The results show no difference in neutrophils, lymphocytes and basophiles count between these groups. Pregnant women with thrombophilia have a significantly higher number of monocytes and basophil granulocytes in their blood

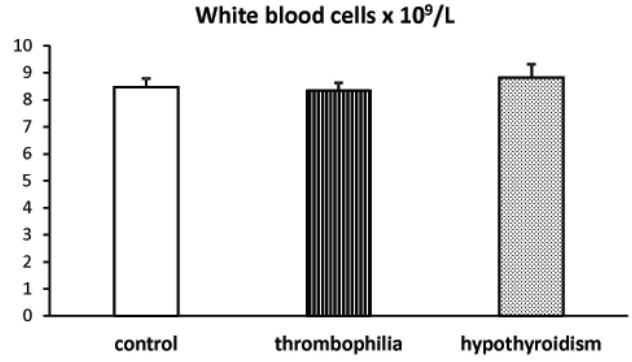

Figure 1. Total number of white blood cells in healthy pregnant women and pregnant women with thrombophilia and hypothyroidism.
Values are means ± S.E.M. * p < 0.05, thrombophilia and hypothyroidism versus control.

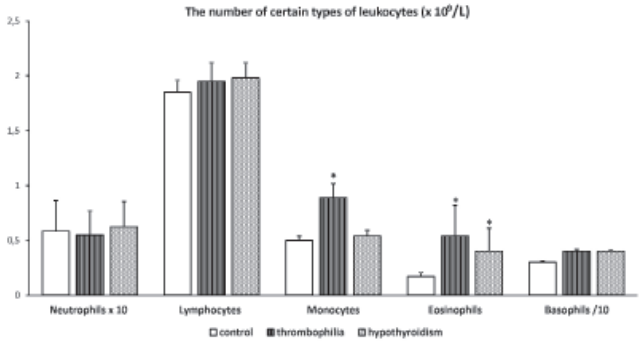

Figure 2. The number certain type of leucocytes in healthy pregnant women and pregnant women with thrombophilia and hypothyroidism.
Values are means ± S.E.M. * p < 0.05, thrombophilia and hypothyroidism versus control.

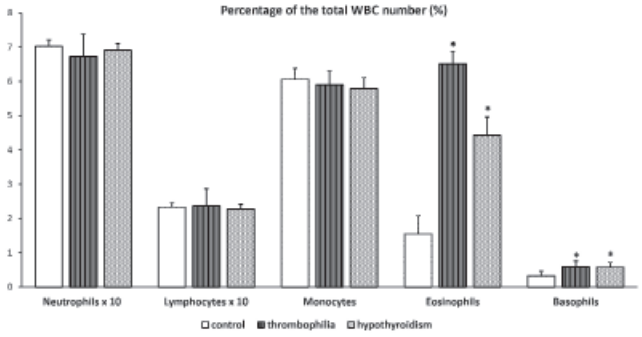

Figure 3. Percentage of distribution certain type of leucocytes of the total white blood cells number (WBC) in healthy pregnant women and pregnant women with thrombophilia and hypothyroidism.
Values are means ± S.E.M. * p < 0.05, thrombophilia and hypothyroidism versus control.

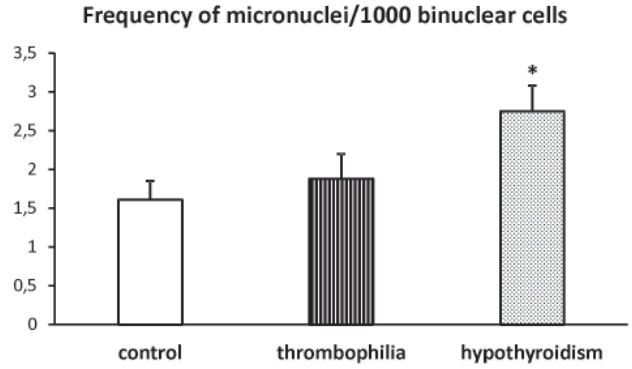

Figure 4. Frequency of fetal cells micronuclei in healthy pregnant women and pregnant women with thrombophilia and hypothyroidism.
Values are means ± S.E.M. * p < 0.05, thrombophilia and hypothyroidism versus control.

than healthy pregnant women. The number of eosinophil granulocytes is significantly higher in the blood of women with hypothyroidism in comparison to healthy pregnant women. The results of percentage different type of leucocytes are presented in Figure 3. The percentage of eosinophil and basophil granulocytes is significantly higher in the blood of women with thrombophilia and hypothyroidism compared to healthy pregnant women. The percentage of other type leukocytes showed no significant difference in blood between the examined groups.

Frequency of micronuclei in fetal cells

The frequency of micronuclei in fetal cells is presented in Figure 4. Our results show significantly higher frequencies of micronuclei in fetal cells of pregnant women with hypothyroidism than in healthy pregnant women. The difference in the frequency of micronuclei in fetal cells of healthy pregnancy and pregnant women with thrombophilia is not indicated.

Correlations

The results of this study show that the frequency of micronuclei in fetal cells has a significant positive correlation with percentage of eosinophil (r = 0.915, p < 0.011) and basophil (r = 0.767, p < 0.044) granulocytes in maternal blood in pregnant women with hypothyroidism. The positive correlations of eosinophil and basophil granulocytes percentage in maternal blood with frequency of micronuclei in fetal cells were not significant in healthy and women with thrombophilia.

DISCUSSION

Hormonal and metabolic changes during normal pregnancy predispose mother to thrombosis and higher hormone output by thyroid gland (3,14). Inherited thrombophilia in pregnancy additionally increases predispose to thrombosis and thromboembolic events (1) leading to fetal

morbidity and mortality (3). The results of this work show that pregnant women with inherited thrombophilia in comparison to healthy pregnant women have a significantly lower incidence of life birth and a significantly higher incidence of miscarriages of the total number of pregnancy. In pregnancy with thrombophilia increased clotting in the placental vasculature resulting in placental infraction and hypoxic condition (15), leading to spontaneous abortion. The increased concentration of β-hCG in maternal serum indicates ischemic condition of the placenta leading to placental infraction (16-17). According to our results, the concentration of β-hCG is significantly higher in maternal serum with thrombophilia than in healthy pregnancy. Reports in the literature are in contradiction and indicate no significant difference (18) and significantly lower (19) concentration of β-hCG in pregnancy with inherited thrombophilia compared to healthy pregnant women.

In normal pregnancy increases the total number of red blood cells, but relative less than increases the plasma amount, which results in a dip in hemoglobin concentration. This dilutional anemia is physiological condition in normal pregnancy (20). Our results indicate that the total number of red blood cells and concentration of hemoglobin are significantly lower in pregnant women with hypothyroidism in comparison to healthy pregnancy. The data in the literature suggests that anemia is often the first sign of hypothyroidism (21), but based on our results, the reduction in the total number of red blood cells and concentration of hemoglobin in women with hypothyroidism are in the level of reference values for pregnancy (20). A higher level of MCV, which observed in pregnant women with thrombophilia, is within reference range (80 - 100 μm^3).

Numerous reports in the literature suggest that hemostasis is intimately linked to inflammation, where in each process propagate and intensifies the other, creating the potential for a vicious cycle of thrombosis and inflammation (22,23). The results obtained in our study show that the total number of white blood cells is not significantly different in pregnancy with thrombophilia and hypothyroidism compared to the control one. Interestingly, we found a significant higher number of monocytes and eosinophils in the whole blood of pregnant women with thrombophilia than in healthy pregnancy. In addition, we found a significant difference in leukocyte formula between thrombophilia and control, and our results show that the blood of pregnant women with thrombophilia presence of eosinophils and basophils is significantly higher. Hypercoagulable state in pregnancy with inherited thrombophilia is accompanied with increasing in platelets activity and aggregability (3). Activated platelets degranulate and express of P-selection on surface, which binds to ligands located on monocytes (24). The result of this interaction is the formation of platelet-leukocyte aggregates that produce proinflammatory, procoagulant, oxidative and mitogen substances that can cause arterial thrombosis (25). Lukanov and coworkers (26) showed that levels of platelet-leukocyte aggregates are significantly higher in pregnant women with thrombophilia. The increased number of eosinophils is associated with a high risk of thromboembolism. During degranulation, eosinophils release tissue factor, which initiates of clotting cascade, and major basic protein that inhibits potent anticoagulant thrombomodulin and activates of platelets (27). Released major basic protein from eosinophils induces activation and number increase of basophils, which have granules that contain histamine, platelet-activating factor, several cytokines, proteolytic enzymes and bioactive proteoglycans (28).

Thyroid hormone showed to influence the immune system and disturbance in thyroid hormone secretion may disturb the immune response (29). In the present study, a higher number of eosinophils were observed in pregnant women with hypothyroidism than in healthy pregnant women. Eosinophils percentage distribution of the total WBC number is also significantly higher in pregnant women with hypothyroidism. Jafarzadeh and co-workers (30) suggest that hypothyroidism is a condition characterized by an increase in eosinophils. It is known that thyroid hormones regulate human hematopoietic system and that eosinophils differentiation from bone marrow is stimulated in hypothyroidism condition (9). Thyroid hormones exert a depressive effect on the differentiation and the number of basophils. Literature data show that number of basophils is not statistically different in hypothyroidism comparison to healthy subjects (31). Our results also show that there is no difference in the number of basophils between pregnant women with hypothyroidism and healthy ones. However, on the basis of these results, the basophils percentage distribution of the total WBC number was significantly higher in pregnant women with hypothyroidism.

Eosinophils and basophils are capable for oxidative brust by production of reactive oxygen species (32,33). Reactive oxygen species produced in large amount from maternal eosinophils and basophils can cross the placenta and induce genomic instability and consequence to formation of micronuclei (10) in fetal cells. Our results indicate a significantly higher frequency of micronuclei in fetal cells in pregnant women with hypothyroidism than in healthy pregnant women. Based on the present results, the frequency of fetal cells micronuclei is significantly positive correlated with the percentage of maternal eosinophil and basophil in pregnant women with hypothyroidism. These results suggest that an increased percentage of the distribution and activities of eosinophils and basophils in hypothyroidism contribute to the formation of micronuclei in fetal cells and can affect to a normal fetus development and successful completion of pregnancy.

CONCLUSION

In summary, the results of the present study demonstrate significant hematological changes in pregnant women with thrombophilia including higher levels of β-hCG, number of monocytes and eosinophils. Hematological

changes in pregnancy with hypothyroidism were reflected in the increase of eosinophil number. Eosinophil and basophil percentage distribution were documented in thrombophilia and hypothyroidism. The increased fetal cells micronuclei frequency and their correlation with eosinophils and basophils were indicated in hypothyroidism. Future studies require investigating a concentration of products activities of maternal eosinophil and basophil in amniotic fluid and their correlation with the frequency of micronuclei in fetal cells.

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Conflict of interest

The authors have no conflicts of interest to declare.

REFERENCES

1. James AH, Jamison MG, Brancazio LR, Myers ER. Venous thromboembolism during pregnancy and the postpartum period: incidence, risk factors, and mortality. Am J Obstet Gynecol. 2006; 194:1311-15.
2. Bremme KA. Haemostatic changes in pregnancy. Best Pract Res Clin Haematol. 2003; 16: 153-68.
3. Kupferminc MJ. Thrombophilia and pregnancy. Curr Pharm Des. 2005;11:735-48.
4. Esmon CT. Molecular circuits in thrombosis and inflammation. Thromb Haemost.2013; 109: 416-20.
5. Wirstlein PK, Mikołajczyk M, Jasiński P, Skrzypczak J. Evaluation of the markers of inflammation in the umbilical cord blood of newborns of mothers with thrombophilia. Am J Reprod Immunol. 2014;72 :561-70.
6. Teng W, Shan Z, Patil-Sisodia K, Cooper DS. Hypothyroidism in pregnancy. Lancet Diabetes Endocrinol. 2013;1: 228-37.
7. Carney LA, Quinlan JD, West JM. Thyroid disease in pregnancy. Am Fam Physician. 2014; 89:273-8.
8. Mezosi E, Szabo J, Nagy EV, Borbely A, Varga E, et al. Nongenomic effect of thyroid hormone on free-radical production in human polymorphonuclear leukocytes. J Endocrinol. 2005;185: 121-9.
9. Axelrod AR, Bergman L. The bone marrow in hyperthyroidism and hypothyroidism. Blood. 1951; 6: 436-53.
10. Sprung CN, Ivashkevich A, Forrester HB, Redon CE, Georgakilas A, et al. Oxidative DNA damage caused by inflammation may link to stress-induced non-targeted effects. Cancer Lett. 2015;356: 72-81.
11. De Bont R, van Larebeke N. Endogenous DNA damage in humans: a review of quantitative data. Mutagenesis. 2004;19: 169-85.
12. Fenech M. The in vitro micronucleus technique. Mutat Res. 2000; 455: 81-95.
13. Torres-Bugarín O, Macriz Romero N, Ramos Ibarra ML, Flores-García A, Valdez Aburto P, et al. Genotoxic Effect in Autoimmune Diseases Evaluated by the Micronucleus Test Assay: Our Experience and Literature Review. Biomed Res Int. 2015; 2015: 194031.
14. Glinoer D. What happens to the normal thyroid during pregnancy? Thyroid. 1999; 9: 631-5.
15. Kinzler WL, Prasad V, Ananth CV. New Jersey-Placental Abruption Study Investigators. The effect of maternal thrombophilia on placental abruption: Histologic correlates. J Matern Fetal Neonatal Med. 2009; 22: 243-8.
16. Fox H. Effect of hypoxia on trophoblast in organ culture. A morphologic and autoradiographic study. Am J Obstet Gynecol. 1970;107: 1058-64.
17. Lieppman RE, Williams MA, Cheng EY, Resta R, Zingheim R, et al. An association between elevated levels of human chorionic gonadotropin in the midtrimester and adverse pregnancy outcome. Am J Obstet Gynecol. 1993; 168: 1852-6.
18. Cikman MS, Seckin KD, Karsli MF, Baser E, Cikman DI, et al. The effect of inherited thrombophilia on second trimester combined aneuploidy screening test markers. Arch Gynecol Obstet. 2015;291: 787-90.
19. Karsli MF, Baser E, Seckin KD, Yeral Mİ, Togrul C, et al. The impact of inherited thrombophilia on first trimester combined aneuploidy screening test parameters. J Matern Fetal Neonatal Med. 2014;27: 346-9.
20. Chandra S, Tripathi AK, Mishram S, Amzarul M, Vaish AK. Physiological changes in hematological parameters during pregnancy. Indian J Hematol Blood Transfus. 2012;28: 144-6.
21. Erdogan M, Kösenli A, Ganidagli S, Kulaksizoglu M. Characteristics of anemia in subclinical and overt hypothyroid patients. Endocr J. 2012;59: 213-20.
22. Esmon CT. The interactions between inflammation and coagulation. Br J Haematol. 2005;131:417-30.
23. Granger DN, Senchenkova E. Inflammation and the Microcirculation. First Edition. San Rafael (CA): Morgan & Claypool Life Sciences. 2010.
24. Larsen E, Celi A, Gilbert GE, Furie BC, Erban JK, et al. PADGEM protein: a receptor that mediates the interaction of activated platelets with neutrophils and monocytes. Cell. 1989;59: 305-12.
25. McGregor ., Martin J, McGregor JL. Platelet-leukocyte aggregates and derived microparticles in inflammation, vascular remodelling and thrombosis. Front Biosci. 2006;11: 830-37.
26. Lukanov TH, Veleva GL, Konova EI, Ivanov PD, Kovacheva KS, et al. Levels of platelet-leukocyte aggregates in women with both thrombophilia and recurrent pregnancy loss. Clin Appl Thromb Hemost. 2011;17:181-7.
27. Ames PR, Margaglione M, Mackie S, Alves JD. Eosinophilia and thrombophilia in churg strauss syndrome: a clinical and pathogenetic overview. Clin Appl Thromb Hemost. 2010;16: 628-36.

28. Janeway AC Jr, Travers P, Walport M, Shlomchik JM. Immunobiology: The Immune System in Health and Disease. Fifth Edition. Garland Science: New York. 2001.
29. Armstrong MD, Klein JR. Immune-endocrine interactions of the hypothalamus-pituitary–thyroid axis:interaction, communication and homeostasis. Arch Immunol Ther Exp. 2001; 49: 231-237.
30. Jafarzadeh A, Poorgholami M, Izadi N, Nemati M, Rezayati M. Immunological and hematological changes in patients with hyperthyroidism or hypothyroidism. Clin Invest Med. 2010; 33: 271-9.
31. Petrasch SG, Mlynek-Kersjes ML, Haase R, Benker G, Olbricht T, et al. Basophilic leukocytes in hypothyroidism. Clin Investig. 1993;71: 27-30.
32. DeChatelet LR, Shirley PS, McPhail LC, Huntley CC, Muss HB, et al. Oxidative metabolism of the human eosinophil. Blood. 1977;50: 525-35.
33. Strenzke N, Grabbe J, Gibbs BF. Pharmacological studies on the role of reactive oxygen species in IgE-dependent histamine secretion from human basophils. Inflamm Res. 2005; 54: 11-2.

CAN A PRESEPSIN (SCD14-ST) OBTAINED FROM TRACHEAL ASPIRATE BE A BIOMARKER FOR EARLY- ONSET NEONATAL SEPSIS

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MOŽE LI PRESEPSIN (SCD14-ST) DOBIJEN IZ TRAHEALNOG ASPIRATA BITI BIOMARKER ZA RANO OTKRIVANJE SEPSE KOD NOVOROĐENE DECE

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ABSTRACT

In absence of clear clinical signs and clear definition, neonatal sepsis is still one of the major cause of morbidity and mortality. Most researchs in past time was directional on finding new biomarkers with greater sensitivity and specificity in detection of neonatal sepsis. The aim of our study was to investigate if presepsin obtained from tracheal asprate in intubated newborns, can be a novel biomarker of systemic bacterial infection. Our "case control" study included 60 newborns, 11 with suspected neonatal sepsis. Tracheal aspirate for examination was taken in the usual toilets, in aseptic conditions, by lavage with 2 ml of 0.9% NaCl in Mucus suction set. In the same day were mesured presepsin (blood), CRP, PCT, leukocytes and neutrophyls, as well as blood culture. Our research showed higher levels for PCT and presepsin (blood) in septic newborns, as well as in newborns with clinical signs of SIRS. Presepsin obtained from a tracheal aspirate had high score for septic newborns. As the coefficients of simple linear correlation showed, there was quantitative agreement between presepsin (blood) with presepsin (tracheal aspirate)- increase in the value of one leads to an increase in other. In conjunction with an already validated markers of infection, presepsin obtained from tracheal aspirate cam be turned on in diagnostic procedures.

Key words: *newborn, presepsin, tracheal aspirate, sepsis.*

SAŽETAK

U odsustvu jasnih kliničkih znakova i definicije, neonatalna sepsa još uvek ostaje jedan od vodećih uzroka neonatalnog morbiditeta i mortaliteta. Većina istraživanja, u poslednje vreme, bila su usmerena na pronalaženje novih biomarkera sa što većom senzitivnošću i specifičnošću u potvrdi neonatalne sepse. Cilj naše studije bio je da ispitamo da li presepsin dobijen iz trahealnog aspirata novorođenčeta može biti novi marker sistemske infekcije novorođene dece. U studiji "slučaj-kontrola" učestvovalo je 60 novorođenčadi, njih 11 sa suspektnom sepsom. Trahealni aspirat je uziman pri uobičajenoj toaleti tubusa, u sterilnim uslovima, sa 2ml 0,9%NaCl pomoću Mucus suction seta. U istom danu su merene vrednosti presepsina (iz krvi), CRP, PCT, leukociti, uz bakteriološku obradu (hemokultura). Istraživanje je pokazalo više vrednosti PCT-a i presepsina (iz krvi) kod novorođenčadi sa neonatalnom sepsom i sa znacima SIRSa. Presepsin iz trahealnog aspirata je imao više vrednosti kod pacijenata obolelih od neonatalne sepse. Kako je pokazao koeficijent proste linearne korelacije, postoji kvantitativno slaganje između presepsina (iz krvi) i presepsina (iz trahealnog aspirata)- visoke vrednosti jednog uzrokuju povećanje vrednosti drugog. Udružen sa drugim potvrđenim markerima infekcije, presepsin dobijen iz trahealnog aspirata može biti uključen u dijagnostičke procedure.

Ključne reči: *novorođenče,presepsin, trahealni aspirat, sepsa.*

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INTRODUCTION

Neonatal sepsis is still one of the major causes of morbidity and mortality worldwide in newborns. Clinically, it can show a diverse clinical signs and there is no clear consensus in neonatal sepsis definition (1). The highest frequency of early neonatal sepsis was noticed in infants with maternal perinatal risk factors such as premature rupture of membrane (PROM), mothers fever, positive vaginal culture etc (2).

In response to bacterial infection phagocytosis, most probably, plays a major role in genererating presepsin (3-6). Monocytes / macrophages are normally present in the circulation and activation of these cells physiologically exist. Early increase in its concentration indicates a bacterial infection and an increase in its intensity depends on the innate immune response (3,7). The sCD14 is significantly elevated in patients with sepsis and septic shock in comparison with healthy people, which is substantially relateed to the severity and prognosis (3-6).

Most researches in recent years were determined to find new biomarkers with greater sensitivity and specificity in detection of early-onset neonatal sepsis. Presepsin, soluble CD14 subtype (sDC14-ST), is well known biomarker of systemic bacterial infections. Isolated from blood, it proved to be more accurate biomarker of early-onset sepsis compared to procalcitonin (PCT) and C-reactive protein (CRP) (8, 9). Obtained from tracheal aspiration presepsin proved to be significant in the diagnosis of early neonatal pneumonia (9). Variables that are affecting C-reactive protein and procalcitonin values do not affect presepsin levels. Accordingly, presepsin independently, can be an effective sepsis marker (10). However , the efficacy of presepsin for the detection of disease severity or prognosis is still being investigated (11).

The aim of our study was to investigate significance of determination and interconnectedness between presepsin (blood) and presepsin (obtained from tracheal aspirate) in intubated newborns in the presence of maternal risk factors associated with clinical signs for early onset neonatal sepsis.

METHODS

In this study we included 60 newborns hospitalized in Department of neonatology, in Pediatric Clinic, Clinical Center Kragujevac. According to the following criteria, we selected examined group (with 11 newborns):

- Two of the following signs including fiver or deviation of the number of leukocytes determine the systemic inflammatory response (SIRS): fever (>38°C), hypothermia (<38°C), tachycardia (>180/min), bradycardia (<100/min), tachypnea (>40/min), systolic TA (<65/8.7 mmHg/kPa), leukocytosis (>30x10^9/L) and leukopenia (<7.5x10^9/L) .
- Presence of one or more of the following clinical signs determines possible signs of neonatal sepsis: respiratory distress, apnea, cyanosis, compromised circulation, enteral feeding intolerance, unexplained jaundice, lethargy, hypotonia, seizures and irritability .
- Presence of one or more of the following risk factors (PROM ≥18 hours), maternal infections, maternal fever, chorioamnionitis .

In control group we included 49 newborns, without mentioned signs.

All blood analyzes were conducted on the first day of the life. Blood and tracheal samples were collected by standard techniques and used for leukocyte count (NIHON KOHDEN Celltae E), CRP (by immuno- turbidimetric), PCT (by hemi- luminescent immunoassay) , presepsin level in blood and plasma (by immunoanalyzer- PATHFAST) and blood culture assessment.

Tracheal aspirate was obtained under sterile conditions by lavage with 2 ml of 0.9% NaCl at the time of the usual toilet tube using the "Mucus suction set" (FG 6, or 8 depending on the size of the intratraheal tube) - Protos Company medical.

According to the Declaration of Helsinki, the study protocol and prior to initiation written informed consent was obtained from The regional Ethics Committee of University Clinic Center in Kragujevac,

Statistical analysis

Using G-power software, we calculated the sample size for our study. Calculated on the basis of the differences in the presepsin level from previous investigations in 41 patients (25male- 16 female) with 294,2 ± 121,4/817,9 ± 572,7 presepsin level ratio (14), with the effect size of 0.849 and with α error probe of 0.05 and study power of 0.9, we calculated that we needed the minimum of 9 patients with pneumonia in the experimental group and the minimum of 18 patients in the control group.

Information on basic characteristics of the sample were processed and presented by descriptive statistics, with the mean values ± SD for continuous variables and frequency (percentages) for categorical variables. We used parametric (Student's) t (t) test, nonparametric Mann-Whitney test (Z) test and $\chi 2$-squared test ($\chi 2$). The linear correlation coefficient, was used to measure the interactive relationship between variables

RESULTS

This "cross-sectional" clinical study included 60 newborn children, divided in 2 groups: 11 in the examined group and 49 in the control group. Whole study included 37 male and 23 female newborns. The data stratification according to gender (p=0,221), gestational age (p=0,335), birth body weight (p=0,275) and Apgar score (p=0,354) did

Table 1. Demographic characteristics of patients

variable	I	II	The value and significance of the tests
gender (m/f) * %	45.5 / 54.5	65.3 / 34.7	χ 2 = 1.498 p = 0.221
gestational age ($\bar{x}$, weeks)	32.91±4.16	34.31±4.34	t = 0.972 p = 0.335
birth body weight ($\bar{x}$, grams)	2080±858.7	2454.69±1050.57	t = 1.101 p = 0.275
Apgar score (1stmin)	6.45±2.58	5.78±2.76	Z= - 0.926 p = 0.354

I group- examined; II group- control
* m- male, f- female
Source: own research made in SPSS 20.0

Table 2. Risk factors for infection

variable	I	II	The value and significance of the tests
delivery way (SC/ N) % *	45.5 / 54.5	65.3 / 34.7	χ 2 = 1.498 p = 0.221
amniotic fluid (b/o) % **	72.7 / 27.3	71.4 / 28.6	χ 2 = 0.007 p = 0.931
maternal infection (yes/no) %	36.4 / 63.6	2 / 98	χ 2 = 13.854 **p = 0.000**
maternal fever (yes/ no) %	18.2 / 81.8	2 / 98	χ 2 = 4.927 **p = 0.026**
positive maternal culture (yes/no) %	0 / 100	4.1 / 95.9	χ 2 = 0.464 p = 0.496
PROM* > 18 h (yes/no)**	36.4 / 63.6	2 / 98	χ 2 = 13.854 **p = 0.000**
Transport (yes/no)	54.5 / 45.5	46.9 / 53.1	χ 2 = 0.208 p = 0.931

I group- examined; II group- control
*SC- Sectio cesarea; N- natural conception
**b- brighnt; o (other)- milky, cloudy, green or meconial
***PROM- premature rupture of membranes
Source: own research made in SPSS 20.0

not show significant differences in regard to frequency between examined and control groups (Table 1).

Statistical analyzis showed that maternal infections in the last trimester of pregnancy and premature rupture of membranes were highly significant risk factors for the occurrence of infections in early neonatal period ($p<0,01$). Maternal fever has also proved to be a significant factor ($p<0,05$), while positive maternal swabs to bacterial infections didn't show statistical significance ($p>0,05$) (Table 2). We investigated if there was a statistically significant difference between factors of inflammation: leukocyte, neutrophils, CRP, PCT, presepsin (blood) and presepsin (tracheal aspirate). None of these factors has proved to be a valuable for the diagnosis of early neonatal infection ($p_1 = 0.354$, $p_2 = 0.257$, $p_3 = 0.276$, $p_4 = 0.256$, $p_5 = 0.115$ and $p_6 = 0.219$) (Table 3).

Correlation and regression: Coefficients of simple linear correlation showed statistically significant connection between presepsin (plasma) and the type of delivery, sort of amniotic fluide, leucocyte count, CRP, PCT and presepsin (tracheal aspirate).

Extremely low value of the correlation coefficient r, $r_{sepsis} = 0.032$; $r_{sirs} = 0.0162$ and their statistical significance $p_{sepsis} = 0.808 > \alpha = 0.05$; $p_{sirs} = 0.221 > \alpha = 0.05$ showed that there wasn't a statistically significant quantitative agreement between: presepsin (blood) and sepsis as well as presepsin (blood) and SIRS (Table 4).

Contrary, low statistical significance paf= $0.003<\alpha = 0.05$ indicated the presence of statistically significant correlation between variables: presepsin (blood)- sort of amniotic fluid. For the CRP, PCT and presepsin (tracheal aspirate), correlation coefficient also showed high statistical significance between those variables and presepsin (blood). Also, it was detected low statistical significance ($p=0.025$, $<\alpha = 0.05$) for variables type of delivery- presepsin (plasma). Type of delivery was measured for caesarean section, which means that there will be higher values

Table 3. Values of laboratory parameters of inflammation in both groups

variable ($\bar{x}$)	I	II	The value and significance of the tests
leukocytes x10^9	23.127±9.673	16.726±13.887	Z = -0.926 $p_1 = 0.354$
neutrophils %	61.527±9.672	56.535±13.599	t = 1.145 $p_2 = 0.257$
CRP mg/l	3.45±1.445	2.90±1.418	Z = -1.090 $p_3 = 0.276$
PCT ng/l	25.111±36.968	9.721±11.964	Z = -1.137 $p_4 = 0.256$
Presepsin (blood) pg/ml	828.545±348.618	681.495±460.612	Z = - 1.576 $p_5 = 0.115$
Presepsin (traheal aspirate) pg/ml	695.100±652.1	429.393±387.3	t = 1.300 $p_6 = 0.219$

I group- examined; II group- control
Source: own research made in SPSS 20.0

Table 4. Coefficients of simple linear correlation Y-presepsin (blood) in sepsis

variable (X)	correlation coefficient	significance p
	I	II
type of delivery	**,255**	**,025**
amniotic fluid (af)	**,347**	**,003**
gestational age (ga)	,067	,305
birth body weight (bbw)	,079	,273
apgar score	,016	,450
leukocytes (l)	**,270**	**,019**
neutrophils (n)	-,133	,156
CRP	**,231**	**,038**
PCT	**,329**	**,005**
Sepsis	,032	,808
SIRS	0,162	,221
presepsin (blood) (ppl)	1,000	
presepsin (tracheal aspirate) (pta)	**,317**	**,007**
mathernal infection (mi)	,053	,342

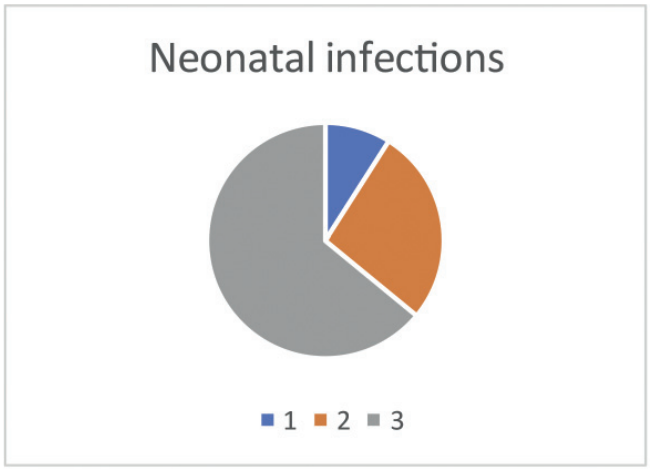
Source: own research made in SPSS 20.0

for presepsin (blood) in the caesarean section contrary to spontaneous type of delivery. All data are in table 4.

Graphic 1, present neonatal infection distribution.

DISCUSION

In this "cross-sectional" study we have evaluated the use of presepsin obtained from tracheal aspirate in intubated newborns, hospitalized in neonatal intensive care units (NICU). According to our knowledge, there is no literature data showing the association of presepsin from tracheal aspirate and neonatal sepsis onset. Patients from our examined group weredetected and included in investigation as the patients with high chances of neonatal systemic infections. Beside high perinatal risk factors, they were intubated on the first day of the hospitalization. It was interesting that only 9% of patients in this group had positive blood culture and 27% of them had elevated inflammatory parameters associated with signs of SIRS.



1- Sepsis; 2- SIRS; 3- other

Graphic 1. Distribution of neonatal infections

The main challenge for our research is that no measurements of presepsin from tracheal aspirate have ever been performed. In the absence of other experiences, we conducted our independent research. Some authors have investigated the use of presepsin obtained from cerebrospinal fluid (CSF) in the case of the meningitis and ventriculitis in children. Measurements of presepsin levels in CSF, showed that it can be added to the diagnostic process in conjunction with biochemical analysis of CSF (12). Only a minor increase of presepsin level in serum was detected in patients with intracerebral infection. In contrast to this knowledge, the same authors stated that dramatically elevated concentrations of presepsin were detected in CSF in experimental murine model study (13). Similar findings were reported in the study of presepsin levels measured in synovial fluid in patients with various stages of Lyme disease (14). Synovial fluid presepsin level was elevated compared to the serum level from healthy individuals, which may play a role in the pathogenesis of arthritis (13,14).

It was sugested that presepsin (blood) is significantly lower in patients without an infection (294.2 pg/ml) as a complication, versus those with local infection (721 pg/ml), sepsis (817.9 pg/ml) and severe sepsis (1,992.9 pg/ml) (15). Possible role of presepsin in discriminating bacterial and nonbacterial infections (including systemic inflammatory response syndrome) have also been under discussion. In multicenter prospective study, authors compared sCD14-ST with procalcitonin (PCT), interleukin-6 (IL-6) and blood cultures. In comparision to conventional inflammatory blood markers and blood culture, presepsin was superior marker for the diagnosis of sepsis (16).

Our research also showed higher levels for PCT and presepsin (blood) in septic newborns, as well as in newborns with clinical signs of SIRS. Presepsin obtained from a tracheal aspirate was elevated in septic newborns compared to the control, but without significance. It was shown to be more precise in neonatal pneumonia than in neonatal sepsis (9).

Although PCT was higher in septic newborns, there were no statistically significant differences between the groups in our investigation. Results revealed similar finding for CRP level. As described earlier, CRP and PCT can have high blood levels after multiorgan failure in the presence of autoimmune diseases (14). Some studies, reported that serum levels of CRP could also increase in non-infected neonates with perinatal asphyxia, intracranial hemorrhage, pneumothorax, or after resuscitation, and these conditions had negatively affected the specificity of PCT (17). These findings could be an explanation for our findins of higher levels of PCT in both groups without statistical significance, without increased levels CRP increase.

In general, procalcitonin is more sensitive for earlier detection of sepsis than CRP (18,19). CRP levels rise within 6 to 8 h of infection, with a peak at 24 h. It has its best predictive value if measured within 24 to 48 h of onset of infection (18). As we measured CRP levels within first 6 to 10 hours, it was expected that CRP levels were approximately the same in both groups.

It was shown that PCT level is significantly higher in septic and infants with suspected infection in comparison with healthy group (20), which is in alignment with our results.

As the coefficients of simple linear correlation showed, there was quantitative agreement between presepsin (blood) with presepsin (tracheal aspirate). This leads us to the conclusion that presepsin (blood) and presepsin (tracheal aspirate) can be a significant marker of early neonatal infection. As value of presepsin (tracheal aspirate) are in positively correlatoion with presepsin (blood), increase in the value of one leads to an increase in other. Newborns in NICU are mostly hemodynamically unstable and taking blood for analysis additionally compromises them. In intubated newborns in NICU, tracheal aspirate in usual toilet of tube can be simply taken as uninvasive procedure. That makes presepsin from the tracheal aspirate one of the first markers to recognize neonatal sepsis, in conjuction with already used markers.

CONCLUSION

Presepsin obtained from tracheal aspirate can be used in diagnostic procedures in conjunction with already validated markers of infection. However, we ere aware that the sample size in our study could be interpreted as modest. This limitation discouraged us to make a final conclusions about possibility of the use of presepsin (tracheal aspirate) as an early marker in neonatal sepsis. Considering quantitative agreement with presepsin (blood), it would be justifiable to carry out additional tests on a larger group of patients to confirm our hypothesis.

CONFLICT OF INTEREST

Authors report no conflict of interest.
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REFERENCES

1. Raymond SL, Stortz JA, Mira JC, Larson SD, Wynn JL, Moldawer LL. Immunological Defects in Neonatal Sepsis and Potential Therapeutic Approaches. Front Pediatr 2017;5(14);DOI:10.3389/fped.2017.00014.
2. Mukhopadhyay S, Puopolo KM. Risk Assessment in Neonatal Early-Onset Sepsis. Seminars in perinatology. 2012;36(6):408-15; DOI:10.1053/j.semperi.2012.06.002
3. Liu B, Chen Y.X., Yin Q, Zhao Y.Z., Li C.S. Diagnostic value and prognostic evaluation of Presepsin for sepsis in an emergency department. Critical Care 2013, 17:R244;DOI:10.1186/cc13070.
4. Ishikura H, Nishida T, Murai A, Nakamura Y, Irie Y, Tanaka J et al. New diagnostic strategy for sepsis-induced disseminated intravascular coagulation: a prospective single-center observational study. Critical Care 2014,18:R19;DOI:10.1186/cc13700.
5. Spanuth E, Ebelt H, Ivandic B, Werdan K. Diagnostic and prognostic value of presepsin (soluble CD14 subtype) in emergency patients with early sepsis using the new assay PATHFAST Presepsin. 21st International Congress of Clinical Chemistry and Laboratory Medicine, IFCC-WorldLab – EuroMedLab, Berlin, 15 -19 May 2011. Poster 0333.
6. Wu J, Hu L, Zhang G, Wu F, He T. Accuracy of Presepsin in Sepsis Diagnosis: A Systematic Review and Meta-Analysis. PLoS ONE 10(7):e0133057.DOI: 10.1371/journal.pone.0133057.
7. Faix JD. Presepsin — The new kid on the sepsis block. Clin Biochem. 2014 May;47(7-8):503-4;DOI:10.1016/j.
8. Montaldo P, Rosso R, Santantonio A, Chello G, Giliberti P. Presepsin for the detection of early-onset sepsis in preterm newborns.. Pediatr Res. 2017 Feb;81(2):329-34;DOI:10.1038/pr.2016.217.
9. Savić D, Simović A, Marković S, Kostić G, Vuletić B, Radivojević S, Lišanin M, Igrutinović Z, Pavlović R. The Role of Presepsin Obtained from Tracheal Aspirates in the Diagnosis of Early Onset Pneumonia in Intubated Newborns. Indian J Pediatr. 2018 Apr 14;DOI:10.1007/s12098-018-2676-2. [Epub ahead of print]
10. Pugni L, Pietrasanta C, Milani S, Vener C, Ronchi A, Falbo M et al. Presepsin (Soluble CD14 Subtype): Reference Ranges of a New Sepsis Marker in Term and Preterm Neonates. PLoS One 2015;10(12):e0146020;DOI:10.1371.
11. Topcuoglu S, Arslanbuga C, Gursoy T, Aktas A, Karatekin G, Uluhan R, Ovali F. Role of presepsin in the diagnosis of late-onset neonatal sepsis in preterm infants. J Matern Fetal Neonatal Med. 2016;29(11):1834-9;DOI:10.3109/14767058.
12. Stubljar D, Kopitar AN, Groselj-Grenc M, Suhadolc K, Fabjan T, Skvarc M. Diagnostic Accuracy of Presepsin (sCD14-ST) for Prediction of Bacterial Infection in Cerebrospinal Fluid Samples from Children with Suspected Bacterial Meningitis or Ventriculitis. J Clin Microbiol 2015;53(4):1239-44;DOI: 10.1128/JCM.03052-14.
13. Sansano S, Fearns C, Ulevitch R, Zimmerli W, Landmann R. The origin and function of soluble CD14 in experimental bacterial meningitis. J Immunol 1999;162:4762–72.
14. Lin B, Noring R, Steere AC, Klempner MS, Hu LT. Soluble CD14 levels in the serum, synovial fluid, and cerebrospinal fluid of patients with various stages of Lyme disease. J Infect Dis 2000;181(3):1185-8;DOI:10.1086/315357.

15. Shozushima T, Takahashi G, Matsumoto N, Kojika M, Okamura Y, Endo S. Usefulness of presepsin (sCD14-ST) measurements as a marker for the diagnosis and severity of sepsis that satisfied diagnostic criteria of systemic inflammatory response syndrome. J Infect Chemother 2011;17(6):764-9;DOI:10.1007/s10156-011-0254-x.
16. Endo S, Suzuki Y, Takahashi G, Shozushima T, Ishikura H, Murai A et al. Usefulness of presepsin in the diagnosis of sepsis in a multicenter prospective study. J Infect Chemother 2012;18(6):891-7;DOI:10.1007/s10156-012-0435-2.
17. Janota J, Stranák Z, Bělohlávková S, Mudra K, Simák J. Postnatal increase of procalcitonin in premature newborns is enhanced by chorioamnionitis and neonatal sepsis. Eur J Clin Invest. 2001 Nov; 31(11):978-83;DOI:org/10.1046/j.1365-2362.2001.00912.x
18. Simonsen KA, Anderson-Berry AL, Delair SF, Davies HD. Early-Onset Neonatal Sepsis. Clinical Microbiology Reviews 2014;27(1):21-47; DOI:10.1128/CMR.00031-13.
19. Wellinghausen N, Kochem AJ, Disqué C, Mühl H, Gebert S, Winter J et al. Diagnosis of bacteremia in whole-blood samples by use of a commercial universal 16S rRNA gene-based PCR and sequence analysis. J Clin Microbiol 2009;47(9):2759-65.;DOI: 10.1128/JCM.00567-09.
20. Adib M, Bakhshiani Z, Navaei F, Saheb Fosoul F, Fouladi S, Kazemzadeh H. Procalcitonin: A Reliable Marker for the Diagnosis of Neonatal Sepsis. Iranian Journal of Basic Medical Sciences. 2012;15(2):777-782.

INFLUENCE OF DIALYSIS MODALITY ON THE TREATMENT OF ANEMIA IN PATIENTS WITH END-STAGE KIDNEY DISEASE

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UTICAJ MODALITETA DIJALIZE NA LEČENJE ANEMIJE KOD BOLESNIKA SA ZAVRŠNIM STADIJUMOM BOLESTI BUBREGA

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ABSTRACT

Anemia is a common complication among the patients with end-stage kidney disease. Management of anemia is influenced by several factors: iron deficiency, subtherapeutic dosage of erythropoietin, microinflammation, vitamin D deficiency, increased iPTH levels and inadequate hemodialysis.

The aim of the study was to examine impact of dialysis modality on blood hemoglobin level as well as status of iron, status of vitamin D, hemodialysis adequacy and erythropoietin dose.

The study included 120 patients which were divided into two groups: the group of patients treated with hemodiafiltration and the group of patients treated with standard hemodialysis. For statistical analysis Kolmogorov-Smirnov test, Student's t-test and Mann-Whitney U-test were used.

Blood hemoglobin level and parameters of hemodialysis adequacy (Kt/V index, spKt/V index, URR index), hematocrit ad protein catabolic rate (nPCR) were statisticaly significant lower in patients treated with regular hemodialysis compared to patients treated with regular hemodiafiltration. Serum ferritin level, C-reactive protein level and average monthly dose of intravenous iron were higher in the patients treated with regular hemodialysis compared to patients treated with hemodiafiltration.

Patients treated with hemodiafiltration have lower grade of microinflammation, better iron status and better control of anemia compared to the patients treated with regular hemodialysis. Dialysis modality is an important factor that influences management of anemia in the patients with end-stage kidney disease.

Keywords: *hemodialysis, hemodiafiltration, erythropoietin, anemia.*

SAŽETAK

Anemija je česta komplikacija kod bolesnika sa završnim stadijumom bolesti bubrega. Na lečenje anemije utiču: nedostatak gvožđa, nedovoljna doza eritropoetina, mikroinflamacija, nedostatak vitamina D, povećana koncentracija iPTH, neadekvatna hemodijaliza.

Rad je imao za cilj da ispita uticaj modaliteta dijalize na koncentraciju hemoglobina u krvi, status gvožđa, vitamin D, adekvatnost hemodijalize i dozu eritropoetina.

Ispitivanje je uključilo 120 bolesnika. Bolesnici su podeljeni u dve grupe: lečeni hemodijafiltracijom i lečeni standardnom hemodijalizom. Za statističku analizu korišćeni su: Kolmogorov Smirnov test, Student-ov T test, Mann-Whitney U test.

Bolesnici koji se leče redovnom hemodijalizom imaju visoko statistički značajno ($p < 0.01$) manju: koncentraciju hemoglobina u krvi, vrednost parametara adekvatnosti hemodijalize (Kt/V indeks spKt/V indeks, URR indeks), statistički značajno ($p < 0.05$) manju vrednost hematokrita i brzinu razgradnje proteina (nPCR), kao i statistički značajno ($p < 0.05$) veću: koncentraciju feritina u serumu, C-reaktivnog proteina i prosečnu mesečnu dozu i.v. gvožđa, u odnosu na bolesnike koji se leče redovnom hemodijafiltracijom.

Bolesnici koji se leče redovnom hemodijafiltracijom imaju manji stepen mikroinflamacije, bolji status gvožđa u organizmu i optimalnu kontrolu anemije, u odnosu na bolesnike koji se leče standardnom hemodijalizom. Modalitet dijalize je značajan faktor za lečenje anemije kod bolesnika sa završnim stadijumom bolesti bubrega.

Ključne reči: *hemodijaliza, hemodijafiltracija, eritropoetin, anemija.*

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INTRODUCTION

Anemia is present in about ninety percent of patients starting treatment with regular hemodialysis. The main factor that causes anemia among these patients is the lack of endogene erythropoietin which stimulates proliferation and differentiation of erythroid precursors in the bone marrow [1, 2]. Another common cause is a blood loss due to occult gastrointestinal hemorrhage related to uremic gastritis, extracorporeal thrombosis, frequent blood sampling [1, 2]. In order to diagnose anemia on time in hemodialysis patients it is necessary to check hemoglobin concentration, hematocrit, red blood cell indices (MCV, MCH, MCHC), serum iron (Fe^{2+}) and ferritin (FER) concentration, transferrin saturation (TSAT) and serum concentration of C-reactive protein (CRP) [1, 2].

Anemia is independent risk factor for cardiovascular diseases in hemodialysis patients. When hemoglobin concentration is lower than 100 g/L hemodynamic mechanisms of adaptation are activated. Left ventricle is overloaded with volume which leads to development of excentric left ventricle hypertrophy and ischemic heart disease [3-7]. Other clinical consequences of anemia are: deterioration of renal residual function, cognitive disorders, reduced working ability as well as reduced quality of life of hemodialysis patients [8, 9].

Treatment of anemia with erythropoietin is recommended when hemoglobin level is lower than 100 g/L, while target Hb concentration is within range 100-120 g/L [10]. Optimal iron status (TSAT = 20-40%, FER = 100-500 ng/mL) should be achieved prior to treatment with erythropoietin [11-14]. Target Hb concentration is not achieved among about 10-20% of patients [15, 16]. Risk factors that affect treatment of anemia in hemodialysis patients are: iron deficiency, insufficient dose of EPO, microinflammation, malnutrition, vitamin D deficiency, secondary hyperparathyroidism, inadequate hemodialysis, and the existence of antibodies on EPO [15-18].

Hemodiafiltration is dialysis modality that combines diffusion and convection that provide better clearance of uremic toxins of small and medium molecular weight compared to standard "low-flux" hemodialysis [19-23]. Online hemodiafiltration requires ultrapure dialysis solution and polysulphonate "high-flux" membranes with Kuf > 50 ml/h x mmHg [19-23]. This dialysis modality provides hemodynamic stability of patients, delays reduction of residual renal function, improves nutritional and cognitive status, while reduces: microinflammation, resistence on erythropoietin, amount of erythropoietin needed for achievement of target hemoglobin level and cardiovascular morbidity and mortality rate [19-23]. During hemodiafiltration course levels of serum proinflamatore mediators as well as level of hepcidine are reduced. Decreased hepcidine level promotes iron releasing from its depot, increases its availability for erythropoiesis in bone marrow and decreases resistance on erythropoietin treatment [19-23]. Individualization and optimization of dialysis treatment, online hemofiltration, polysulphonate "high-flux" membrane with Kuf > 50 ml/h x mmHg and ultrapure dialysis solution altogether can decrease proinflamatore mediators levels (interleukin-1, interleukin-6, tumor necrosis factor-α), CRP and hepcidine in serum, provide optimal control of anemia and survival of patients on regular hemodialysis [19-23].

AIM OF THE STUDY

The aim of the study was to determine influence of dialysis modality on the treatment anemia in patients with end-stage kidney disease.

PATIENTS AND METHODS

One hundred and twenty patients of Center for nephrology and dialysis, Clinic for Urology, Nephrology and Dialysis of Clinical Center Kragujevac, Kragujevac, Serbia participated in the study. The study protocol was in accordance with the principles of the Declaration of Helsinki and was approved by The Ethics Comitee of Clinical Center Kragujevac. All patients signed informed consent prior to enrollment. All participants were treated with regular bicarbonate hemodialysis, 12 hours per week for period longer than three months on hemodialysis maschines type Fresenius 4008S, 5008S and type Gambro AKA200US and Gambro Artis. Ultrapure dialysis fluid and "high-flux" as well as low-flux polysulfone dialysis membrane were used while for hemodiafiltration "high-flux" polysulphone dialysators with Kuf > 50 ml/h x mmHg were used. Exclusion criteria were active bleeding and active infection.

In order to evaluate impact of dialysis modalityon management of anemia in hemodialysis patients the following parameters were measured: hemoglobin (Hb), hematocrit (Hct), FER, TIBC, unsaturated iron binding capacity (UIBC), transferrin saturation (TSAT), serum calcium (Ca^{2+}), inorganic phosphate (PO_4^{3-}), alkaline phosphatase (ALP), vitamin D and intact parathyroid hormone (iPTH). Parameters of hemodialysis adequacy were also considered.

Blood sampling for laboratory examination was performed prior to starting with hemodialysis and hemodiafiltration and prior to heparin administration. Every laboratory parameter was assigned with the value that was the average of two measuring in two succesive months.

Total hemoglobin was measured using colorimetric method. The target hemoglobin level in patients on dialysis was 100-120 g/L.

The normalized protein catabolic rate (nPCR) was calculated using formula of National Cooperative Dialysis Study: nPCR = (PCR x 0.58)/Vd. Formula for calculating PCR is PCR = 9.35G + 0.29Vd, where G - urea production rate, Vd - volume of body fluid (Vd = 0.58 x BW). Urea production rate was calculated by formula G = [(C1-C2)/Id] x Vd, where C1 is serum urea concentration prior to dialysis (mmol/L), C2 - serum urea concentration after di-

alysis (mmol/L), Id - time (hours) between two successive dialysis. Normal range for nPCR is 1.1 ± 0.3 g/kg/day.

Serum concentration of iron, ferritin, total iron binding capacity, calcium, inorganic phosphorus and CRP were measured using Beckman Coulter AU680 analyzer. Serum iron was determined by photometric method using TPTZ [2,4,6-Tri-(2-pyridyl)-5-triazine] as the chromogen. Serum iron reference range is 6.6 - 26.0 μmol/L. TIBC was done indirectly by the Unsaturated Iron Binding Capacity (UIBC) method. TIBC reference range is 48 - 56 μmol/L. Transferrin saturation - TSAT was calculated using formula TSAT = (Fe/TIBC) x 100%. Reference range for TSAT in hemodialysis patients is 20-40%. UIBC was measured using spectrophotometric method. Reference range for UIBC is 28 -54 μmol/L. Method for ferritin was turbidimetric. Ferritin reference range in the patients underwent regular hemodialysis is 100 - 500 pg/mL.

CRP level in the serum was determined by turbidimetric method. Normal CRP level in the serum is ≤ 5 mg/L. Microinflammation is defined as level of CRP in serum higher than 5 mg/L.

Calcium concentration in serum was determined by a photometric test. Normal calcium level in serum is 2.20 - 2.65 mmol/L. Phosphate level in serum was determined by a photometric test. The normal phosphate level in the serum is 0.80 - 1.60 mmol/L.

Level of vitamin D in the serum was determined by the method of electrochemiluminiscence using Cobas e 411 analyser. Normal level of vitamin D in serum is 20 - 40 ng/mL. In hemodialysis patients, normal vitamin D level is ≥ 30 ng/mL (30 - 80 ng/mL). A severe deficit is defined as the level of vitamin D < 10 ng/mL, vitamin D deficiency exists if level is 10 - 20 ng/mL, and the insufficiency is defined as the level of vitamin D in the serum of 20-30 ng/mL.

Level of intact parathormone in serum was determined by immunoradiometric method (IRMA) using gamma counter WALLAC WIZARD 1470. Normal concentration of intact parathormone in serum is 11.8-64.5 pg/mL. Patients on regular hemodialysis had iPTH up to 300 pg/mL.

The adequacy of hemodialysis was assessed on the basis of the single-pool Kt/Vsp index calculated according to the Daugridas second-generation formula:

$$Kt/Vsp = -\ln(C_2/C_1 - 0.008 \times T) + (4 - 3.5 \times C_2/C_1) \times UF/W,$$

with: C1 - the value of urea before dialysis, C2 - the value of urea after dialysis (mmol/L), T - duration of hemodialysis (h), UF - interdialysis yield (L), W - body weight after hemodialysis (kg). According to K/DOQI guidelines, hemodialysis is adequate if Kt/Vsp ≥ 1.2.

The degree of reducing urea - URR index is calculated using following formula: URR = (1-R) × 100%, where: R is the ratio of the urea concentration in serum after and before the treatment with hemodialysis. Hemodialysis is adequate if the URR index = 65-70%

Vascular access blood flow - Qavf was determined by Color-Doppler ultrasound by Logic P5 machine 7.5 MHz, where blood flow were estimated by equation: $Qavf = r^2\pi/4 \times Vmean \times 60$ (mL/min), r - radis of vascular access, Vmean - mean flow rate through vascular access. Blood flow is estimated as average value of three measurements, 2-4 cm on vain that serves as vascular access, proximally of the anasthomosis site. Blood flow rate that provides adequacy of hemodialysis is 500-1000 mL/min.

Depending on dialysis modality patients were divided into two groups. The first group included patients treated with standard hemodialysis, and the second group included patients treated with hemodiafiltration.

The statistical analysis was performed using the Kolmogorov-Smirnov test, Student's t-test and Mann-Whitney U test. The threshold of significance was the probability of 0.05 and 0.01.

RESULTS

At the Clinic for Urology, Nephrology and Dialysis of the KC Kragujevac a cross section study was conducted including patients who were treated with regular hemodialysis and hemodiafiltration over a period of more than three months. 120 patients (75 men, 45 women) were examined, mean age 63.15 ± 10.39 years, average duration of dialysis treatment 6.18 ± 5.95 years and average index of hemodialysis adequacy Kt/Vsp 1.01 ± 0.27. General patient data are shown in Table 1.

The treatment of anemia of examined patients included short-acting and long-acting erythropoietins, iron (i.v.), folic acid (p.o.), vitamin B complex (i.v.). The average monthly dose of short-acting erythropoietin was 18517.24 ± 9361.04 IU, long-acting erythropoietin 121.07 ± 75.98 mg, intravenous iron 155.83 ± 180.76 mg, folic acid 153.75 ± 23.52 mg, and the average monthly number of Beviplex ampules was 11.37 ± 1.47 (vitamin B12 45.48 ± 5.88 g).

In order to evaluate the influence of dialysis modality on the treatment of anemia the following parameters were examined: the concentration of hemoglobin in blood (Hb), hematocrit (Hct), the concentration of iron in the serum (Fe^{2+}), total iron binding capacity (TIBC), free iron binding capacity (UIBC), saturation of transferin with iron (TSAT), the concentration of ferritin in the serum (FER), the concentration of calcium (Ca^{2+}) and magnesium (Mg^{2+}) in the serum, the concentration of vitamin D, intact parathormone (iPTH), nutritive status parameters, the concentration of total proteins (TP) and albumin (Alb) in the serum, the concentration of uric acid in the serum (UA), the rate of decomposition of proteins (nPCR), the parametres of hemodialysis adequacy (Kt/V index, spKt/V index, URR index), as well as the average monthly dose of short-acting (KDE-M) and long-acting erythropoietin (DDE-M), the index of resistance of short-acting (KDE/Hb) and long-acting erythropoietin (DDE/Hb), and the average monthly dose of i.v. iron (PMDG). Depending on the type of dialysis, the patients were divided into two groups. The first group consisted of patients treated with regular hemodialysis (HD),

Table 1. General patient data

GENERAL DATA Xsr ± SD		Statistical parameters
Number (N)		120
Pol (m/f, %)		70/45 (62.5/37.5%)
Age (yr.)		63.15 ± 10.43
Length of Treatment with Hemodialysis (yr.)		6.18 ± 5.98
Body Mass Index - BMI (kg/m^2)		24.68 ± 4.59
Systolic Arterial Blood Pressure - STA (mmHg)		121.83 ± 14.61
Diastolic Arterial Blood Pressure - DTA (mmHg)		72.82 ± 10.33
Medium Arterial Blood Pressure - SAP (mmHg)		89.16 ± 10.80
Dry Body Mass of the Patient - W (kg)		71.46 ± 15.55
Ultrafiltration - UF (L)		2475.00 ± 992.30
Residual Diuresis - RD (mL/24h)		594.17 ± 710.08
Blood Flow through a Vascular Approach - Qavf (mL/min)		841.33 ± 433.48
Hemodialysis Adequacy Index - Kt/V		1.01 ± 0.27
Single-pool Index of Hemodialysis Adequacy - spKt/V		0.01 ± 0.25
Urea Reduction Ratio - URR (%)		61.91 ± 8.80
Primary Kidney Disease	Glomerulonephritis Chronica	12 (10.00%)
	Nephropathia Hypertensiva	39 (32.50%)
	Nephropathia Diabetica	16 (13.33%)
	Nephropathia Obstructiva	8 (6.67%)
	Nephropathia Endemica	1 (0.83%)
	Nephropathia Chronica	18 (15.00%)
	Pyelonephritis Chronica	3 (2.50%)
	Renes Polycystici	21 (17.50%)
	Nephritis Tubulointerstitialis	2 (1.67%)
Comorbidities		
Hypertension		69 (57.50%)
Hypotension		3 (2.50%)
Diabetes Mellitus		18 (15.00%)
Cardiovascular Disease		30 (25.00%)

whereas the second group consisted of patients treated with regular hemodiafiltration (HDF). The average values of examined parameters are shown in Table 2.

Based on the Kolmogorov-Smirnov test, the Student's T test for two independent samples was used to examine the significance of the difference between the examined groups (hemodialysis:hemodiafiltration) for the following parameters: hemoglobin (Hb), hematocrit (Hct), mean erythrocyte volume (MCV), mean hemoglobin concentration in erythrocyte (MCHC), the number of leukocyte (Le), the concentration of iron in the serum (Fe^{2+}), total iron binding capacity (TIBC), free iron binding capacity (UIBC), the saturation of iron with transferin (TSAT) the concentration of uric acid (UA), total serum protein (TP) and serum albumin (Alb), the rate of decomposition of proteins (nPCR), the concentration of aspartate aminotransferase in serum (AST), the concentration of calcium (Ca^{2+}), phosphate (PO_4^{3-}) and vitamin D in serum, the parametres of hemodialysis adequacy (Kt/V index, spKt/V index, URR index), the average monthly dose of long-acting erythropoietin (DDE-M), the index of resistance of short-acting erythropoietin (KDE/Hb), the index of resistance of long-acting erythropoietin (DDE/Hb), Table 2. To determine the statistical significance of the difference between the examined groups for the average amount of hemoglobin in the erythrocyte (MCH), ferritin (FER), C-reactive protein (CRP), alanine aminotransferase (ALT), gamma glutamyl transferase (GGT), alkaline phosphatase (ALP), intact parathormone (iPTH) and the average monthly dose of i.v. iron (PMDG) Mann-Withney-U-test was used, Table 3.

Patients treated with regular hemodialysis have a high statistically significant ($p < 0.01$) lower: concentration of hemoglobin in blood (Hb), values of adequacy parameters of hemodialysis (Kt/V index, spKt/V index, URR index), statistically significant ($p < 0.05$) lower value of hematocrit (Hct) and protein decomposition rate (nPCR), as well

as statistically significant ($p < 0.05$) larger: concentration of ferritin in serum (FER), C-reactive protein (CRP), and the average monthly dose of i.v. iron (PMDG) compared to the patients treated with regular hemodiafiltration, Table 2 and Table 3. There is no statistically significant difference ($p > 0.05$) between patients treated with hemodialysis and hemodiafiltration in other parameters of the study.

DISCUSSION

Anemia is present in 90% of patients in the end-stage of chronic kidney disease who begin their treatment with regular hemodialysis. Its main clinical consequences are: progressive decline in residual kidney function, development of cardiovascular complications, cognitive function disorders, and reduced quality of life [24, 25].

Regardless of the appropriate treatment of anemia, which involves parenteral application of iron and erythropoietin, anemia is still a common complication in the population of patients treated with regular hemodialysis. The prevalence of anemia, defined as a hemoglobin concentration in the serum lower than 100 g/L, is high - 50% of examined patients. The most significant risk factors that affect the treatment of anemia in patients with hemodialysis are: iron deficiency, insufficient dose of erythropoietin, inflammation, secondary hyperparathyroidism, lack of vitamin D, increased concentrations of parathormone in the

Table 2. The influence of the type of dialysis modality on the treatment of anemia in patients treated with regular dialysis (Student Test)

Test parameters	Type of dialysis		Significance differences (p)
	Hemodialysis	Hemodiafiltration	
	Xsr ± SD	Xsr ± SD	
Hb (g/L)	100.93 ± 11.06	106.88 ± 7.32	t = -2.667, p = 0.009
Hct (%)	29.46 ± 3.22	32.63 ± 2.93	t = -2.607, p = 0.040
MCV (fL)	94.91 ± 4.23	94.25 ± 4.87	t = 0.696, p = 0.488
MCHC (g/L)	331.78 ± 6.13	331.57 ± 6.11	t = 0.160, p = 0.873
Le (x 10^9/L)	7.09 ± 1.87	6.42 ± 1.96	t = 1.639, p = 0.104
Fe^{2+} (mmol/L)	10.44 ± 3.45	9.64 ± 3.14	t = 1.093, p = 0.277
TIBC (mmol/L)	33.91 ± 6.18	35.25 ± 7.11	t = -0.974, p = 0.277
UIBC (mmol/L)	23.46 ± 6.26	25.55 ± 7.38	t = -1.484, p = 0.140
TSAT (%)	31.58 ± 11.24	29.16 ± 8.62	t = 1.049, p = 0.296
UA (mmol/L)	373.63 ± 73.26	372.00 ± 66.29	t = 0.105, p = 0.916
TP (g/L)	61.47 ± 5.20	61.48 ± 4.09	t = -0.014, p =0.989
Alb (g/L)	36.54 ± 3.76	36.16 ± 2.56	t = 0.479, p = 0.620
nPCR (g/kg/day)	1.61 ± 0.65	1.95 ± 0.46	t = -2.605, p = 0.010
AST (IU/L)	16.45 ± 5.97	16.39 ± 4.29	t = 0.048, p = 0.962
Ca^{2+} (mmol/L)	2.23 ± 0.18	2.28 ± 0.19	t = -1.370, p = 0.173
PO_4^{3-} (mmol/L)	1.49 ± 0.38	1.50 ± 0.35	t = -0.187, p = 0.852
$Ca^{2+}xPO_4^{3-}$ ($mmol^2/L^2$)	3.30 ± 0.86	3.44 ± 0.90	t = -0.723, p = 0.471
Mg^{2+} (mmol/L)	1.17 ± 0.25	1.25 ± 0.24	t = -1.569, p = 0.119
Vitamin D (ng/mL)	16.32 ± 10.47	16.68 ± 4.44	t = -0.174, p = 0.862
Kt/Vindex	0.95 ± 0.24	1.20 ± 0.30	t = -4.381, p = 0.0001
spKt/Vindex	0.97 ± 0.26	1.13 ± 0.19	t = -2.862, p = 0.0050
URR (%)	60.18 ± 8.84	67.58 ± 5.89	t = -4.152, p = 0.0001
KDE-M (IU)	18.883.72±9971.89	18133.33±7614.52	t = 0.265, p = 0.792
DDE-M (mg)	115.44 ± 73.40	149.38 ± 89.38	t = -1.130, p = 0.265
KDE/Hb (IU/g)	201.03 ± 118.94	174.38 ± 74.35	t = 0.812, p = 0.420
DDE/Hb (mg/g)	1.15 ± 0.83	1.28 ± 0.97	t = -0.419, p = 0.678

Abbreviations: Hb - hemoglobin, Hct - hematocrit, MCV - average erythrocyte volume, MCHC - mean hemoglobin concentration in erythrocyte, Le - number of leucocytes, Fe^{2+} - iron, TIBC - total iron binding capacity, UIBC - free iron binding capacity, TSAT - saturation of transferrin with iron, UA - uric acid, TP - total proteins, Alb - albumin, nPCR - rate of protein decomposition, Ca^{2+} - calcium, PO_4^{3-} - phosphate, Ca^{2+} x PO_4^{3-} - product of solubility, Kt/V - index of hemodialysis adequacy, spKt/V - index of hemodialysis adequacy, URR - index of hemodialysis adequacy, KDE-M - average monthly dose of short-acting erythropoietin, DDE-M - average monthly dose of long-acting erythropoietin, KDE/Hb - index of short-acting erythropoietin, DDE/Hb - index of long-acting erythropoietin

Table 3. The influence of the type of dialysis on the treatment of anemia in patients on regular dialysis (Mann-Whitney U test): I - hemodialysis, II - hemodiafiltration

Examined parameters	Statistical parameters								Significance-p-value
	Med-I	Med-II	Min-I	Min-II	Max-I	Max-II	IQR-I	IQR-II	
MCH (pg)	31.70	31.15	27.90	27.60	65.10	35.15	2.13	2.56	Z = -0.760 p = 0.447
FER (ng/mL)	836.00	716.50	102.00	19.50	2325.00	1062.00	402.80	310.80	Z = -1.970 p = 0.049
CRP (mg/L)	5.58	4.28	0.30	0.40	171.60	14.10	9.90	6.20	Z = -1.973 p = 0.048
ALT (IU/L)	13.00	13.50	6.00	9.00	34.50	53.00	7.50	8.50	Z = - 0.714 p = 0.475
GGT (IU/L)	18.50	18.00	8.00	10.00	371.50	71.00	15.40	25.30	Z = -0.732 p = 0.464
ALP (IU/L)	73.50	73.50	28.00	34.50	1404.00	630.00	37.60	59.00	Z = - 0.174 p = 0.862
iPTH (pg/mL)	155.00	129.50	7.70	1.00	1866.00	1643.00	221.30	506.00	Z = -0.037 p = 0.970
PMDG (mg)	200.00	100.00	50.00	50.00	800.00	800.00	250.00	100.00	Z = -2.095 p = 0.036

MCH - mean hemoglobin content in red blood cell, FER - serum ferritin concentration, CRP - C-reactive protein, ALT - alanin aminotransferase, GGT - gama glutamil transferase, ALP - alkaline phosphatase, iPTH - intact parathormone, KDE-M - average monthly dose of erythropoietin, PMDG - average monthly dose of i.v. iron, Med - mediana, Min - minimum, Max - maximum, IQR - interquartile range

serum, malnutrition and inadequate hemodialysis (type and dose of dialysis modality) [26-34].

In the last decade, the number of patients treated with hemodiafiltration has increased significantly. The examination included 28 patients treated with regular hemodiafiltration over a period of more than three months (23.33%). Hemodiafiltration is better at removing uremic toxins of a moderate molecular weight, which have been shown to block erythropoiesis in the bone marrow, compared to standard hemodialysis. It also reduces microinflammation and increases the availability of iron for bone marrow erythropoiesis [35-37]. The results of this study have shown that patients undergoing hemodiafiltration have statistically significantly higher concentration of hemoglobin in blood, the value of hematocrit and parameters of hemodialysis adequacy, as well as statistically significantly lower concentration of C-reactive protein and ferritin in the serum, compared to patients treated with standard hemodialysis. These results are in accordance with the previously reported results of studies carried out so far which show that patients who are being treated with hemodiafiltration have a statistically significant higher concentration of hemoglobin in blood and a lower concentration of C-reactive protein and interleukin-6, compared to patients treated with conventional hemodialysis [35-37]. Patients treated with regular hemodialysis with microinflammation require a higher dose of erythropoietin in order to optimally control anemia (achieving and maintaining the target value of hemoglobin of 100-120 g/L) [35-37]. Patients treated with standard hemodialysis have a higher average amount of short-acting erythropoietin and an average higher index of resistance of short-acting erythropoietin compared to patients treated with hemodiafiltration, but this difference is not statistically significant. Considering that patients who are treated with regular hemodialysis have a statistically significantly lower of blood hemoglobin concentration, and that there is no statistically significant difference in the average monthly doses of short-acting and long-acting erythropoietin, it can be concluded that these patients require a higher dose of short-acting erythropoietin to achieve and maintain the target values of hemoglobin (optimal control of anemia). This indicates that hemodiafiltration reduces microinflammation, improves the availability of iron for the synthesis of hemoglobin in erythrocytes and corrects the response to erythropoietin activity. These results are in accordance with the results of other authors who have shown that hemodiafiltration is better at cleansing the blood of patients from uremic toxins of a moderate molecular weight, reduces microinflammation, increases the sensitivity to erythropoietin activity and is better at providing optimal control of anemia, compared to the patients treated with standard hemodialysis [35-37]. The results of the clinical study of REDERT show that online hemodiafiltration statistically significantly reduces inflammation, oxidative stress, concentration of β2-microglobulin in serum and hepcidin in serum, and the resistance to erythropoietin compared to patients treated with "low-flux" bicarbonate hemodialysis, [35-37]. The results of this examination showed that patients treated with hemodiafiltration had a statistically significantly lower concentration of serum ferritin (better iron availability), compared to the patients treated with standard hemodialysis. These results are in accordance with the results of other authors who have shown that there is a statistically

significant positive correlation between the concentration of 25-hepcidin and ferritin in serum, and a statistically significant positive correlation was also found between the concentration of 25-hepcidin and the index of resistance to the erythropoietin effect. Reducing the concentration of 25-hepcidin in serum in patients treated with hemodiafiltration results in the resistance to erythropoietin activity [35-37]. The examined patients treated with hemodiafiltration have statistically significantly lower level of C-reactive protein in serum. These results are in accordance with the results of the clinical study CONTRAST (CONvective TRAnsport STudy), which also show that online hemodiafiltration with ultra-pure dialysis solution reduces microinflammation, compared to conventional hemodialysis [35-37]. Patients treated with online hemodiafiltration during the period of 3-6 months have a statistically significantly lower concentration of C-reactive protein and interleukin-6 in the serum, compared to the patients treated with standard bicarbonate "low-flux" hemodialysis [35-37]. The results of clinical studies show that patients who are treated with online hemodiafiltration have a statistically significant lower mortality rate compared to patients treated with standard "low-flux" hemodialysis [38-40].

CONCLUSION

Patients treated with regular hemodiafiltration have a lower level of microinflammation, a better status of iron in the body (smaller functional defect) and an optimal control of anemia compared to the patients treated with standard hemodialysis. The modality of dialysis is a significant factor for the treatment of anemia in patients with end-stage kidney disease.

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REFERENCES

1. Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group. KDIGO Clinical Practice Guideline for Anemia in Chronic Kidney Disease. Kidney Int Suppl 2012; 2(4): 279-335.
2. Rossert JA, Wauters JP. Recommendation for the screening and management of patients with chronic kidney disease. Nephrol Dial Transplant 2002; 17(Suppl 1): 19-28.
3. Levin A. Anaemia and left ventricular hypertrophy in chronic kidney disease populations: A review of the current state ofknowledge. Kidney Int 2002; 61(Suppl 80): 35-8.
4. Stojimirović B, Petrović D, Obrenović R. Hipertrofija leve komore kod bolesnika na hemodijalizi: značaj anemije. Med Pregl 2007; LX (Supl 2): 155-9.
5. Petrović D, Miloradović V, Poskurica M, Stojimirović B. Dijagnostika i lečenje ishemijske bolesti srca kod bolesnika na hemodijalizi. Vojnosanit Pregl 2009; 66(11): 897-903.
6. Petrović D, Miloradović V, Poskurica M, Stojimirović B. Slabost srca bolesnika na hemodijalizi: procena i lečenje. Srp Arh Celok Lek 2011; 139(3-4): 248-55.
7. Petrović D, Trbojević-Stanković J, Stojanović-Marjanović V, Nikolić A, Miloradović V. Iznenadna srčana smrt bolesnika na hemodijalizi: procena rizika i prevencija. Ser J Exp Clin Res 2013; 14(1): 29-32.
8. Murray AM, Knopman DS, Tupper DE, Kane R. Cognitive impairment in hemodialysis patient is common. Nephrology 2006; 67(2): 216-23.
9. Murray AM, Pederson SL, Tupper DE, Hochhalter AK, Miller WA, Li Q, et al. Acute variation in cognitive function in hemodialysis patients: a cohort study with repeated measures. Am J Kidney Dis 2007; 50(2): 270-8.
10. National Kidney Foundation K/DOQI. Clinical Practice Guidelines for Anemia of Chronic Kidney Disease: Update 2000. Am J Kidney Dis 2001; 37(Suppl 1): 182-238.
11. Goodnough LT. The role of iron in erythropoiesis in the absence and presence of erythropoietin therapy. Nephrol Dial Transplant 2002; 17(Suppl 5): 14-18.
12. Cavill I. Iron and erithropoetin in renal disease. Nephrol Dial Transplant 2002; 17(Suppl 5): 19-23.
13. Hörl WH. Clinical Aspect of Iron Use in the Anemia of Kidney Disease. J Am Soc Nephrol 2007; 18(2): 382-93.
14. Wish J. Assessing Iron Status: Beyond Serum Ferritin and Transferrin Saturation. Clin J Am Soc Nephrol 2006; 1(Suppl 1): 4-8.
15. Drüke T. Hyporesponsiveness to recombinant human erythropoietin. Nephrol Dial Transplant 2001; 16 (Suppl 5): 50-5.
16. Good LT, Nemeth E, Ganz T. Detection, evaluation, and management of iron-restricted erythropoiesis. Blood 2010; 116(23): 4754-61.
17. Kiss Z, Ambrus C, Almasi C, Berta K, Deak G, Horonyi P, et al. Serum 25(OH)-cholecalciferol concentration is associated with hemoglobin level and erythropoetin resistance in patients on maintenance hemodialysis. Nephron Clin Pract 2011; 117(4): 373-8.
18. Icardi A, Paoletti E, De Nicola L, Russo R, Cozzolino M. Renal anemia and EPO hyporesponsiveness associated with vitamin D deficiency: the potential role of inflammation. Nephrol Dial Transplant 2013; 28(7): 1672-7.
19. Tattersal JE, Ward RA. Online haemodiafiltration: definition, dose quantification and safety revisited. Nephrol Dial Transplant 2013; 28(3): 542-50.

20. Canaud B, Barbieri C, Marcelli D, Bellocchio F, Bowry S, Mari F, et al. Optimal convection volume for improving patient outcomes in an international incident dialysis cohort treated with online hemodiafiltration. Kidney Int 2015; 88(5): 1108-16.
21. Marcelli D, Scholz C, Ponce P, Sousa T, Kopperschmidt P, Grassmann A, et al. High-Volume Postdilution Hemodiafiltration Is a Feasible Option in Rutine Clinical Practice. Artif Organs 2015; 39(2): 142-9.
22. De Roij van Zuijdewijn CLM, Chapdelaine I, Nube MJ, Blankestijn PJ, Bots ML, Konings CJAM, et al. Achieving high concentration volumes in postdilution online hemodiafiltration: a prospective multicenter study. Clin Kidney J 2017; 10(6): 804-12.
23. Rosati A, Ravaglia F, Panichi V. Improving Erythropoiesis Stimulating Agent Hyporesponsivenessin Hemodialysis Patients: The Role of Hepcidin and Hemodiafiltration Online. Blood Purif 2018; 45(1-3): 139-46.
24. Rossert JA, McClellan WM, Roger SD, et al. Contribution of anaemia to progression of renal disease : a debate. Nephrol Dial Transplant 2002; 17(Suppl 1): 60-6.
25. Tamura MK, Vittinghoff E, Yang J, Go AS, Seliger SL, Kusek JW, et al. Anemia and risk for cognitive decline in chronic kidney disease. BMC Nephrol 2016; 17(1): 13. doi:10.1186/s12882-016-0226-6.
26. Roger SD. Practical considerations for iron therapy in the management of anemia in patients with chronic kidney disease. Clin Kidney J 2017; 10(Suppl 1): 9-15.
27. Drüeke TB, Eckardt KU. Role of secondary hyperparatireoidism in erythropoetin resistance of chronic renal failure patients. Nephrol Dial Transplant 2002; 17(Suppl 5): 28-31.
28. Brancaccio D, Cozzolino M, Gallieni M. Hyperparathyroidism and Anemia in Uremic Subjects: A Combined Therapeutic Approach. J Am Soc Nephrol 2004; 15(Suppl 1): 21-4.
29. Jean G, Souberbielle JC, Chazot C. Vitamin D in Chronic Kidney Disease and Dialysis Patients. Nutrients 2017; 9(4): 328.
30. Stenvinkel P. The role of inflammation in the anaemia of end-stage renal disease. Nephrol Dial Transplant 2001; 16(Suppl 7): 36-40.
31. Akchurin OM, Kaskel F. Update on Inflammation in Chronic Kidney Disease. Blood Purif 2015; 39(1): 84-92.
32. Nassar GM, Fishbane S, Ayus JC. Occult infection of old non functioning arteriovenous grafts: A novel cause of erythropoietin resistance and chronic inflammation in hemodialysis patients. Kidney Int 2002; 61(Suppl 80): 49-54.
33. Chawla LS, Krishnan M. Causes and consequences of inflammation on anemia management in hemodialysis patients. Hemodialysis Int 2009; 13(2): 222-34.
34. De Oliveira Junior WV, de Paula Sabino, Figueiredo RC, Rios DRA. Inflammation and poor response to treatment with erythropoietin in chronic kidney disease. J Bras Nefrol 2015; 37(2): 255-63.
35. Panichi V, Rocchetti MT, Scatena A, Rosati A, Migliori M, Pizarelli F, et al. Long term variation of serum levels of uremic toxins in patient treated by post-dilution high volumen on-line hemodiafiltration in comparison to standard low-flux bicarbonate dialysis: results from the REDERET study. J Nephrol 2017; 30(4): 583-91.
36. Den Hoedt CH, Bots ML, Grooteman MPC, Der Weerd NC, Mazairac AHA, Penne EL, et al. Online hemodiafiltration reduces systemic inflammation compared to low-flux hemodialysis. Kidney Int 2014; 86(2): 423-32.
37. Panichi V, Scatena A, Rosati A, Giusti R, Ferro G, Malagnino E, et al. High-volume online hemodiafiltration improves erythropoiesis-stimulating agents (ESA) resistance in comparison with low-flux bicarbonate dialysis: results of the REDERT study. Nephrol Dial Transplant 2015; 30(4): 682-9.
38. Maduell F, Moreso F, Pons M, Ramos R, Mora-Macia J, Foraster A, et al. Desing and patient characteristics of ESHOL study, a Catalonian prospective randomized study. J Nephrol 2011; 24(2): 196-202.
39. Maduell F, Moreso F, Pons M, Ramos R, Mora-Macia J, Carreras J, et al. High-Efficiency Postdilution Online Hemodiafiltration Reduces All-Cause Mortality in Hemodialysis Patients. J Am Soc Nephrol 2013; 24(3): 487-97.
40. Peters SA, Bots ML, Canaud B, Davenport A, Grooteman MP, Locatelli F, et al. Haemodiafiltration and mortality in end-stage kidney disease patients: a pooled individual participant data analysis from four randomized controlled trials. Nephrol Dial Transplant 2016; 31(6): 978-84.

INCREASED IL-33 AND IL-17 IN COLORECTAL CARCINOMA PATIENTS WITH SEVERE DISEASE

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POVEĆANE KONCENTRACIJE IL-33 I IL-17 KOD PACIJENATA SA TEŽOM FORMOM KOLOREKTALNOG KARCINOMA

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ABSTRACT

Colorectal cancer (CRC) represents one of the most common cancers. It is frequently diagnosed at advanced stages, indicating on need for new diagnostic markers. The aim of this study was to determine systemic and fecal values of IL-17 and IL-33 in patients with CRC and the relationship with clinicopathological aspects of disease.

The blood samples and feces liquid fraction of 50 patients with CRC were analyzed. Serum and fecal levels of IL-33 and IL-17 were measured using sensitive enzyme-linked immunosorbent assay (ELISA) kits.

Fecal levels of Il-33 and IL-17 were increased in CRC patients with poor tumor tissue differentiation. Serum IL-33 and fecal IL-17 were increased in patients with presence of lung/liver metastasis or peritoneal carcinomatosis, respectively, while enhanced fecal IL-33 was detected only in patients with peritoneal carcinomatosis.

Positive correlation between IL-33 and IL-17 values in sera and feces, respectively was also observed.

We believe that increased local values of IL-33 and IL-17, reflected trough higher fecal concentration, in CRC patients with poor tumor tissue differentiation and with presence of lung/liver metastasis or peritoneal carcinomatosis may be considered as a sign of the tumor's malignant progression and, consequently, of a poor prognosis for patients.

Keywords: *Colorectal carcinoma, IL-17, IL-33.*

SAŽETAK

Kolorektalni karcinom (engl. Colorecral carcinoma- CRC) predstavlja jedan od najčešćih karcinoma. Često se dijagnostikuje u uznapredovalim stadijumima, ukazujući na potrebu za novim dijagnostičkim markerima. Cilj ove studije bio je utvrđivanje sistemskih i fekalnih vrednosti IL-17 i IL-33 kod pacijenata sa CRC i odnosa sa kliničko-patološkim aspektima bolesti.

Analizirani su uzorci krvi i tečne frakcije fecesa 50 pacijenata sa CRC-om. Serumske i fekalne koncentracije IL-33 i IL-17 su merene korišćenjem senzitivnog ELISA (enzyme-linked immunosorbent assay) testa.

Koncentracije IL-33 i IL-17 u fecesu povećane su kod pacijenata sa CRC-om i slabo diferentovanim tumorskim tkivom. Serumski IL-33 i fecesni IL-17 su povećani u pacijenata sa metastazama u plućima/jetri ili peritonealnom karcinomotozom, dok je povećan IL-33 detektovan samo u fecesu pacijenata sa peritonealnom karcinomotozom.

Takođe je detektovana pozitivna korelacija između vrednosti IL-33 i IL-17 u serumu kao i u fecesu.

Verujemo da se povećane lokalne vrednosti IL-33 i IL-17 kod pacijenata sa slabo diferentovanim tumorskim tkivom kolorektalnog karcinoma i prisustvom metastazama u plućima/jetri ili peritonealnom karcinomotozom mogu smatrati znakom progresije maligne bolesti i, posledično loše prognoze za pacijente.

Ključne reči: *kolorektalni karcinom, IL-33, IL-17.*

INTRODUCTION

Colorectal cancer (CRC) is one of the most commonly diagnosed malignancy worldwide. Despite its frequency, it's still one of leading causes of death among women and men. The main cause of death is liver metastasis (1). Colorectal carcinoma is comprised of many types of carcinoma that differ in gene expresion, pathohistological characteristics, primary localisation, and, unfortunately, treatment outcomes (2). Traditonal approaches to therapy of this malignancy - surgery, radiotherapy and chemotherapy up to this day fail to significantly improve survival rate (3). Due to

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specific condition in whom CRC arises, many factors such as gut microbiota, chronic inflammation, eating habbits should be further investigated (4). Until this day, it is well known that, no mather what histological type, localisation or genetic characteristics of CRC, inflammation is positively correalted with more invasive types of cancer (5).

Interleukin-33 (IL-33) is a member of the IL-1 family of cytokines, which includes many cytokines, such as IL-1α and β, IL-18, IL-36α, β, γ, IL-37, IL-38 (6, 7). However, in contrast to other IL-1 family cytokines, IL-33 may function as a cytokine, as alarmin, or as a nuclear factor which modulates expression of many genes, especially NF-κB (8, 9). A large body of evidence indicates that IL-33 participate in tissue repair, allergy, autoimmune disease, infectious disease, and cancer. IL-17 is a member of a cytokine family composed of six cytokines and five receptors (10-13). IL-17 is secreted primarily by Th17 cells, but can also be produced by cells other than Th cells, such as invariant NKT cells, $CD8^+$ T cells, and γδ-T cells (14-16). The cytokine has pleiotropic functions with multiple targets. It is shown that IL-17 is involved in several biological processes such as inflammation and neoangiogenesis. The inflammation serves two counteracting functions: promoting tumor growth and antitumor immunity. Interleukins 33 and 17 promotes inflammation and thus may promote both tumor growth and tumor regression.

The aim of this study was to evaluate systemic and fecal values of IL-33 and IL-17 in patients with CRC and the relationship with clinicopathological aspects of disease. In this study we demonstrate enhanced fecal concentration of IL-33 and IL-17 in CRC patients with poor tumor tissue differentiation, with metastatic disease.

METHODS

Ethical Approvals.

The study was conducted at the Clinical center, Kragujevac, Serbia, and Center for Molecular Medicine and Stem Cell Research, Faculty of Medical Sciences, University of Kragujevac, Serbia. All patients gave their informed consent. Ethical approval was obtained from Ethics Committee of the Clinical Center of Kragujevac, Kragujevac, Serbia. All research procedures were made according to the Principle of Good Clinical Practice and the Declaration of Helsinki.

Subjects.

Study included 50 patients with CRC. The diagnosis was based on endoscopic and histopathological criteria, as previously described (17). The study did not include patients with no well-defined pathology, no adequate clinical document available or with previously diagnosed CRC who were treated with radiation and chemotherapy, as previously adopted (18). Clinical data about age, gender, size of cancer and pathologic reports (well/moderate/poor differentiation) and clinical stage (metastasis) were recorded and analyzed in study. Well-differentiated and moderately differentiated tumors (well/moderate) were defined as low-grade lesions, whereas poorly differentiated tumors (poor) were defined as high grade lesions according to the WHO guidelines (19). Grading was based on the evaluation of the worst area, excluding areas of focal dedifferentiation present at the invasive margin of the tumor (20). Poorly differentiated tumors have repeatedly been shown to behave more aggressively than well/moderate- differentiated carcinomas in multivariate analysis (20). Blood and stool samples were taken before the surgery and stored at -80°C until ELISA.

Feces liquid fraction preparation

Stool samples (1-10 g) were collected in the morning in sterile containers and weighed. One gram of fecal samples was diluted, mixed, homogenised in 5 mL of protease inhibitor cocktail (SIGMA, P83401), and then centrifuged, as previously described (21, 22). The supernatant fluid was collected and stored at - 80°C until ELISA.

Determination of IL-17 and IL-33 in sera and feces.

Serum and fecal concentrations of cytokines were measured, as described (23) using sensitive enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, for IL-17 and IL-33; measurement of cytokines according to the manufacturer's instructions). Briefly, the 96-well plates were coated with capture antibody, overnight. The plates were washed with a washing buffer (0.05% Tween-20 in PBS) and incubated with blocking buffer (1% bovine serum albumin in PBS) for 1 hour at room temperature. Serum/faecal samples or standard recombinant IL-17/IL-33 were introduced to the plates for 2 hours before the application of biotinylated detection antibody for 1 hour at room temperature. After introduction of streptavidin peroxidase for 1 hour, the plates were developed with substrate reagent for 20 minutes. The reaction was stopped by adding 4mol/L sulfuric acid, and the absorbance was read at 495 nm by a microplate reader. We measured the exact concentration of mentioned biomarkers by intrapolation of a standard curve made by a series of well-known concentrations as per manufacturer's instruction. Values of measured cytokines are presented as pg/ml of sera and pg/g of feces, respectively.

Statistical analysis

The data were analyzed using commercially available SPSS 20.0 software. The results were reported as mean and standard error of mean (SEM). In determining statistically significant difference between the means of two groups it was used Student's t-test for independent samples if the data had normal distribution or Mann-Whitney U-test for

Table 1. Baseline characteristics of patients

	Number
Gender (male/female)	29/21
Age (mean [range])	65 [50-82] years
Site (P/D/R)	12/29/9
Necrosis (well/moderate/absent)	13/37/0

Note: P: proximal colon; D: distal colon; R: rectum.

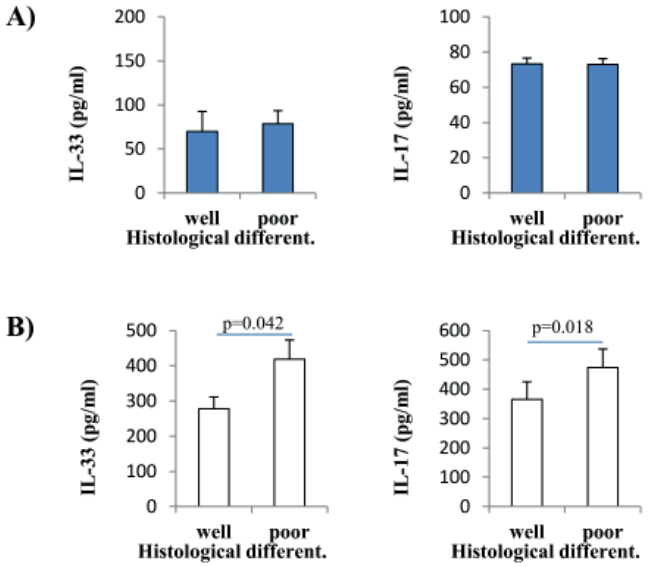

Figure 1. Serum and fecal values of IL-33 and IL-17 in patients with CRC, based on histological differentiation of tumor.
A. *No significant difference in concentration of IL-33 and IL-17 in sera, in patients according to histological differentiation of CRC.* Patients with CRC were divided in two groups, according to histological differentiation rate (well/moderate and poor). Serum and fecal levels of all mentioned biomarkers were determined by ELISA.
B. *Increased concentration of IL-33and IL-17 in feces of patients with poor histological differentiation of CRC.* Statistical significance was tested by Mann–Whitney Rank Sum test or independent samples t-test, where appropriate.

data without normal distribution. Spearman's correlation evaluated the possible relationship between the IL-33 and IL-17. Strength of correlation was defined as negative or positive weak (-0.3 to -0.1 or 0.1 to 0.3), moderate (-0.5 to -0.3 or 0.3 to 0.5) or strong (-1.0 to -0.5 or 1.0 to 0.5). P-value of 0.05 was considered as statistically significant.

RESULTS

Fifty patients with CRC were enrolled in the study. Clinical and pathologic characteristics of these patients are presented in Table 1. There was no significant difference in gender distribution (29 males and 21 females). Patients were similar in age (mean age 65 [50–82]).

Higher fecal IL-33 and IL-17 concentration in patients with poor tumor tissue differentiation

Patients with CRC were categorized into 2 groups according to histological differentiation rate: well/moderate and poor. As shown in Figure 1A, there were no differences in systemic values of IL-33 and IL-17 between defined groups. In patients with poor tumor tissue differentiation, we detected increased fecal IL-33 (poor vs. well/moderate: 418,95 ± 54,33 vs. 278,43 ± 33,50 pg/g; p=0.042) and IL-17 (poor vs. well/moderate: 473,76 ± 62,82 vs. 365,85 ± 148,67; p=0.018; Figure 1B).

Liver, lung and peritoneal metastasis associated with higher IL-33 and IL-17

Further, we divided patients in two categories based on presence of lung/liver metastasis or peritoneal cacrinomatosis, respectively, and analyzed them for values of IL-33 and IL-17. Higher IL-33 was found in sera of patients with detectable liver metastasis (81,01 ± 21,78 vs. 67,16 ± 11,71; p=0.038), lung metastasis (128,37 ± 27,69 vs. 61,51 ± 8,72; p=0.007), or peritoneal cacrinomatosis (105,56 ± 27,04 vs.

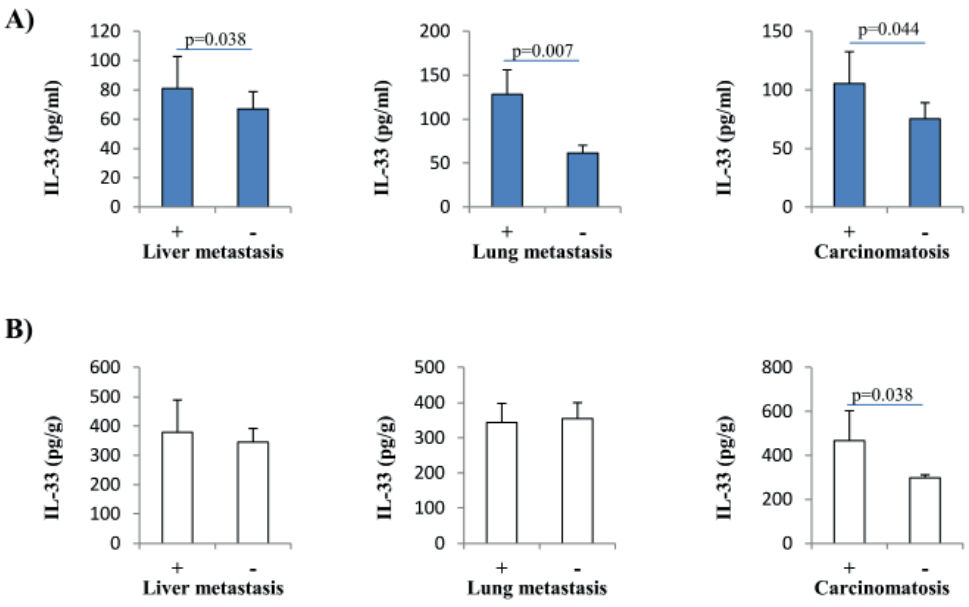

Figure 2. Sistemic and fecal values of IL-33 in patients with CRC, based on tumor progression.

A. *Increased serum IL-33 in patients with detectable liver, lung metastasis and peritoneal carcinomatosis.* Patients with CRC were divided in two groups, based on presence of liver metastasis, lung metastasis and carcinomatosis in peritoneum, respectively (+ and -). Serum and fecal levels of IL-33 were determined by ELISA.
B. *Increased fecal IL-33 in patients with detectable peritoneal carcinomatosis.* Statistical significance was tested by Mann–Whitney Rank Sum test or independent samples t-test, where appropriate.

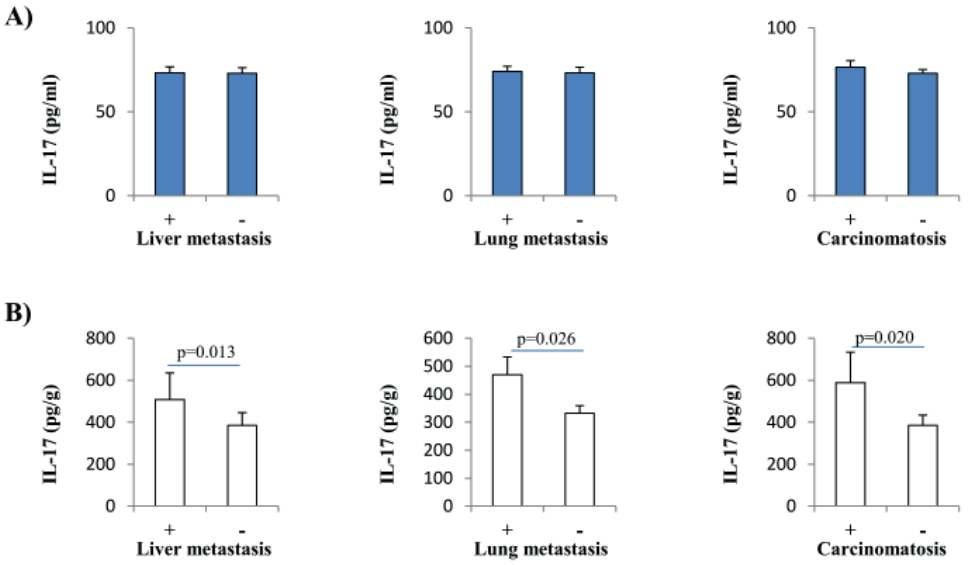

Figure 3. Sistemic and fecal values of IL-17 in patients with CRC, based on tumor progression.
A. *No significant difference in concentration of IL-17 in sera, in patients with detectable liver, lung metastasis and peritoneal carcinomatosis*. Patients with CRC were divided in two groups, based on presence of liver metastasis, lung metastasis and carcinomatosis in peritoneum, respectively (+ and -). Serum and fecal levels of IL-17 were determined by ELISA.
B. *Increased fecal IL-17 in patients with detectable liver, lung metastasis and peritoneal carcinomatosis*. Statistical significance was tested by Mann–Whitney Rank Sum test or independent samples t-test, where appropriate.

75,17 ± 13,91; p=0.044), in comparison to patients without metastasis/carcinomatosis (Figure 2A). Increased IL-33 was detected in feces of patients with detectable peritoneal cacrinomatosis (466,50 ± 136,37 vs. 297,39 ± 14,06; p=0.038; Figure 2B).

In addition, we also found higher IL-17 in feces of patients with detectable liver metastasis (508,78 ± 125,75 vs. 384,41 ± 61,54; p=0.013), lung metastasis (470,34 ± 62,89 vs. 331,76 ± 27,06; p=0.026), or peritoneal cacrinomatosis (588,40 ± 144,95 vs. 385,98 ± 47,53; p=0.020), as illustrated in figure 3.

Serum and faecal IL-33 concentrations significantly correlated with appropriate values of IL-17

Spearman correlation analysis of IL-33 concentration in sera and stool uncovered positive correlation between IL-33 value and IL-17 in sera (r=0.478; p= 0.001) and feces (r=0.675; p= 0.001), respectively.

DISCUSION

Biological role of IL-33 in tumor genesis, progression, immuno-suppression and tumor angiogenesis is well known. IL-33 is known to have protumorigenic role in many malignancies. There are sufficient data that IL-33 is overexpressed in CRC (24, 25). IL-33 is highly expressed in earlier strages of colorectal adenoma-carcinoma, implicating that it might be important for iniciating carcinogenesis. Expression of IL-33 in tumor tissue is higher than in adjacent healthy one. Authors suggest that Il-33 can work in an autocirne manner, especilly when it comes to processes of neoangiogenesis, because it can stimulate secretion of VEGF (24). In the present study, we analyzed systemic and fecal level of IL-33 in different stages of CRC. We didn`t find that sera IL-33 mean values ranged significantly different regard to histological differentiation rate of tumor, while fecal IL-33 showed significant alteration (Figure 1). Recent studies have been exploring usage of feces as a sample for testing different biomarkers (26, 27). For instance, fecal calprotectin (FC), a biomarker of intestinal inflammation that has been in clinical use for years (28), has been also shown to be elevated in CRC and suggested for screening high risk groups for CRC (29). To our knowledge, this is the first study testing fecal IL-33 and IL-17 for detection of severe and progressive forms of CRC. We found increased concentration of IL-33 in stool of CRC patients with poor tumor tissue differentiation (Figure 1). In line with our finding, it has been shown that levels of IL-33 are higher in poor-differentiated human carcinoma cells, and are connected with higher rate of metastasis. Signaling through IL-33/ST2 pathway incerases levels of metaloproteinases (MMP2 and MMP9), IL-6 and CXCR4, molecules that are important for metastasis of human CRC (25). Also, IL-33 is found to be overexpressed on tumor cells and vascular endothelial cells in tumor stroma. Signaling through its receptor ST2, IL-33 can activate JNK kinases, that further activate genes like *NANOG, NOTCH, OCT3/4* which are active in stem cells. Therefore, indirecly, IL-33 promotes poor differentiation of CRC cells and supports its invasivness. It also can recruit macrophaphages in tumor microenviroment that produce prostaglandins, molecules that are also somewhat involved in tumor cell stemness (30). IL-33 via its receptor can also induce higher expression of mRNA and protein levels of COX2 in primary CRC cells. COX2, enzyme crucial in prostaglandin synthesis, indirectly induces proliferation of cancer cells via phosphatidylinositol 3-kinase/Akt pathway (31).

Our study also revealed increased fecal concentration of IL-17 in CRC patients with poor tumor tissue differentiation (Figure 1). IL-17 can stimulate oncogenesis via activating certain genes. In a pancreatic cancer, it has been show that along with IL-17 expression, there are higher levels of stem cell genes that promote poor differentiation. Also, it has been shown that blocking of

IL-17 using a anti-IL-17 antibody significantly downregulates genes that regulate properties of a malignat cell - cellular movement, development, growth and proliferation, even cell-to-cell signaling, cellular assembly and organization (32).

Next, we tested systemic and fecal values of IL-33 and IL-17 as a reliable markers of the disease progression and showed increased IL-33 concentration in sera of patients with CRC with progressive disease: patients with presence of lung/liver metastasis or peritoneal carcinomatosis, respectively, while increased fecal IL-33 was detected only in patients with peritoneal carcinomatosis (Figure 2). Based on these findings, we believe that IL-33 could be a predictor for the advanced stages of colorectal cancer. IL-33 influences differentiation of various immune cells. These changes promote immunosuppressive environment that promotes CRC growth (33). IL-33 expressed on tumor tissue or in tumor stroma enhances infiltration of macrophages, mostly M2 type (TAMs). TAMs work together with IL-33-stimulated Th2 responses thus paving a path to a more malignant disease (34). Also, IL-33 may promote CRC progression through processes of angiogenesis and lymph angiogenesis. Vascular density of CRC is significantly higher in presence of IL-33/ST2 signaling. Also, many markers of neoangiogenesis, such as CD31, LYVE1, and α-SMA are elevated, thus suggesting a role of this cytokine in formation of new blood and lymph vessels and metastasis (35 ,35).

Interestingly, there was no difference in systemic values of IL-17 between patients with and without presence of lung/liver metastasis or peritoneal carcinomatosis, respectively (Figure 3), while increased IL-17 concentration in feces was observed in patients with CRC with progressive disease: patients with presence of lung/liver metastasis or peritoneal carcinomatosis, respectively (Figure 3). The primary role of IL-17 is to recruit and activate neutrophils during an infection. However, when put in a tumor microenviroment, attraction of neutrophiles can lead to unwanted inflammation and consequent exacerbation of the disease (37). Using immnunohistochemistry, Chen et al showed that higer expression of IL-17 in CRC tissue is associated with poorer prognosis (38). It has been shown that IL17 may facilitate progression of colorectal carcinoma by fostering angiogenesis via promoting the vascular endothelial growth factor (VEFG) production from cancer cells (39).

Finally, we found positive correlation between IL-33 and IL-17 in sera and stool, respectively of CRC patients. There is very little data on connection between these two cytokines. Recent study illustrates the ability of IL-33 to directly stimulate mast cells and enhance the Th17 response, in animal model of airway inflammation (40). According to this study, we believe that increased local IL-33 in patients with severe disease may stimulate IL-17 production, and that both cytokines facilitate disease progression.

CONCLUSION

In summary, increased local values of IL-33 and IL-17, reflected trough higher fecal concentration, in CRC patients with poor tumor tissue differentiation and with presence of lung/liver metastasis or peritoneal carcinomatosis may be considered as a sign of the tumor's malignant progression and, consequently, of a poor prognosis for patients. These observations support the idea of potential use of IL-33 and IL-17 as therapeutic targets.

DECLARATION OF INTEREST

The authors declare that they have no competing interests.

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REFERENCES

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer 3 statistics, 2012.CA Cancer J Clin 2015;65:87-108.
2. Guinney J, Dienstmann R, Wang X, et al. The consensus molecular subtypes of colorectal cancer. Nat Med 2015;21:1350-1356.
3. Jiang Q, Ma L, Li R, Sun J. Colon cancer-induced interleukin-35 inhibits beta-catenin-mediated pro-oncogenic activity. Oncotarget 2017;9:11989-11998
4. Zou S, Fang L, Lee MH. Dysbiosis of gut microbiota in promoting the development of colorectal cancer. Gastroenterol Rep (Oxf) 2018;6:1-12.
5. Zou S, Fang L, Lee MH. Epithelial Smad4 Deletion Up-Regulates Inflammation and Promotes Inflammation-Associated Cancer. Cell Mol Gastroenterol Hepatol 2018;6:257-276.
6. Garlanda C, Dinarello CA, Mantovani A. The interleukin-1 family: back to the future. Immunity 2013;39:1003-1018.
7. Günther S, Deredge D, Bowers AL, Luchini A, Bonsor DA, Beadenkopf R, Liotta L, Wintrode PL, Sundberg EJ. IL-1 Family Cytokines Use Distinct Molecular Mechanisms to Signal through Their Shared Co-receptor. Immunity 2017;47:510-523.
8. Wasmer M-H, Krebs P. The Role of IL-33-Dependent Inflammation in the Tumor Microenvironment. Frontiers in Immunology 2016;7:682.

9. Ali S, Mohs A, Thomas M, Klare J, Ross R, Schmitz ML, Martin MU. The dual function cytokine IL-33 interacts with the transcription factor NF-κB to dampen NF-κB-stimulated gene transcription. J Immunol 2011;187:1609-1616.
10. Yao Z, Fanslow WC, Seldin MF, Rousseau AM, Painter SL, Comeau MR, Cohen JI, Spriggs MK. Herpesvirus Saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor. Immunity 1995;3:811-821.
11. Hymowitz SG, Filvaroff EH, Yin JP, Lee J, Cai L, Risser P, Maruoka M, Mao W, Foster J, Kelley RF, Pan G, Gurney AL, de Vos AM, Starovasnik MA. IL-17s adopt a cystine knot fold: structure and activity of a novel cytokine, IL-17F, and implications for receptor binding. EMBO J. 2001;20:5332-5341.
12. Moseley TA, Haudenschild DR, Rose L, Reddi AH. Interleukin-17 family and IL-17 receptors. Cytokine Growth Factor Rev 2003;14:155-174.
13. Gaffen SL. Biology of recently discovered cytokines: interleukin-17--a unique inflammatory cytokine with roles in bone biology and arthritis. Arthritis Res Ther 2004;6:240-247.
14. Michel ML, Mendes-da-Cruz D, Keller AC, Lochner M, Schneider E, Dy M, Eberl G, Leite-de-Moraes MC. Critical role of ROR-γt in a new thymic pathway leading to IL-17-producing invariant NKT cell differentiation. Proc Natl Acad Sci U S A 2008;105:19845-51980.
15. Ciric B, El-behi M, Cabrera R, Zhang GX, Rostami A. IL-23 drives pathogenic IL-17-producing CD8+ T cells. J Immunol 2009;182:5296-5305.
16. O'Brien RL, Roark CL, Born WK. IL-17-producing gammadelta T cells. Eur J Immunol 2009;39:662-666.
17. Jovanovic M, Gajovic N, Zdravkovic N, Jovanovic M, Jurisevic M, Vojvodic D, Maric V, Arsenijevic A, Jovanovic I. Fecal Galectin-3: A New Promising Biomarker for Severity and Progression of Colorectal Carcinoma. Mediators Inflamm 2018;2018:8031328.
18. Jovanovic M, Gajovic N, Zdravkovic N, Jovanovic M, Jurisevic M, Vojvodic D, Mirkovic D, Milev B, Maric V, Arsenijevic N. Fecal galectin-1 as a potential marker for colorectal cancer and disease severity. Vojnosanit Pregl (2018); DOI: https://doi.org/10.2298/VSP171201007J.
19. Hamilton SR and Aaltonen LA. Pathology and genetics:tumours of the digestive system, in World Health Organization Classification of Tumours, IARC, Lyon, France, 3rd edition, 2000. 103-143.
20. Lanza G, Messerini L, Gafa R, Risio M. Colorectal tumors: the histology report. Dig Liver Dis 2011;43 Suppl 4:S344-355.
21. Heilmann RM, Cranford SM, Ambrus A, Grützner N, Schellenberg S, Ruaux CG, Suchodolski JS, Steiner JM. Validation of an enzyme-linked immunosorbent assay (ELISA) for the measurement of canine S100A12. Vet Clin Pathol 2016;45:135-47.
22. Prakash N, Stumbles P, Mansfield C. Initial Validation of Cytokine Measurement by ELISA in Canine Feces. Open Journal of Veterinary Medicine 2013;3:282-288.
23. Jovanovic M, Zdravkovic N, Jovanovic I, Radosavljevic G, Gajovic N, Zdravkovic N, Maric V, Arsenijevic N. TGF-β as a marker of ulcerative colitis and disease severity. Ser J Exp Clin Res DOI: 10.1515/sjecr-2017-0019.
24. Cui G, Qi H, Gundersen MD, Yang H, Christiansen I, Sørbye SW, Goll R, Florholmen J. Dynamics of the IL-33/ST2 network in the progression of human colorectal adenoma to sporadic colorectal cancer. Cancer Immunol Immunother 2015;64:181-190.
25. Mertz KD, Mager LF, Wasmer MH, Thiesler T, Koelzer VH, Ruzzante G, Joller S, Murdoch JR, Brümmendorf T, Genitsch V, Lugli A, Cathomas G, Moch H, Weber A, Zlobec I, Junt T, Krebs P. The IL-33/ST2 pathway contributes to intestinal tumorigenesis in humans and mice. Oncoimmunology 2015;5:e1062966.
26. Wagner M, Peterson CG, Ridefelt P, Sangfelt P, Carlson M. Fecal markers of inflammation used as surrogate markers for treatment outcome in relapsing inflammatory bowel disease. World J Gastroenterol 2008;14:5584-5589.
27. Tibble JA, Sigthorsson G, Bridger S, Fagerhol MK, Bjarnason I. Surrogate markers of intestinal inflammation are predictive of relapse in patients with inflammatory bowel disease. Gastroenterology 2000;119:15-22.
28. Tibble J, Teahon K, Thjodleifsson B, Roseth A, Sigthorsson G, Bridger S, Foster R, Sherwood R, Fagerhol M, Bjarnason I. A simple method for assessing intestinal inflammation in Crohn's disease. Gut 2000;47:506-513.
29. Johne B, Kronborg O, Tøn HI, Kristinsson J, Fuglerud P. A new fecal calprotectin test for colorectal neoplasia. Clinical results and comparison with previous method. Scand J Gastroenterol 2001;36:291-296.
30. Fang M, Li Y, Huang K, et al. IL33 Promotes Colon Cancer Cell Stemness via JNK Activation and Macrophage Recruitment. Cancer Res 2017;77:2735-2745.
31. Li Y, Shi J, Qi S, Zhang J, Peng D, Chen Z, Wang G, Wang Z, Wang L. IL-33 facilitates proliferation of colorectal cancer dependent on COX2/PGE(2). J Exp Clin Cancer Res 2018;37:196.
32. Zhang Y, Zoltan M, Riquelme E, et al. Immune Cell Production of Interleukin 17 Induces Stem Cell Features of Pancreatic Intraepithelial Neoplasia Cells. Gastroenterology 2018;155:210-223.
33. He Z, Chen L, Souto FO, Canasto-Chibuque C, Bongers G, Deshpande M, Harpaz N, Ko HM, Kelley K, Furtado GC, Lira SA. Epithelial-derived IL-33 promotes intestinal tumorigenesis in Apc (Min/+) mice. Sci Rep 2017;7:5520.
34. Italiani P, Boraschi D. From Monocytes to M1/M2 Macrophages: Phenotypical vs. Functional Differentiation. Front Immunol 2014;5:514.
35. Zhang Y, Davis C, Shah S, Hughes D, Ryan JC, Altomare D, Peña MM. IL-33 promotes growth and liver metastasis of colorectal cancer in mice by remodeling the tumor microenvironment and inducing angiogenesis. Mol Carcinog 2017;56:272-287.

36. Akimoto M, Maruyama R, Takamaru H, Ochiya T, Takenaga K. Soluble IL-33 receptor sST2 inhibits colorectal cancer malignant growth by modifying the tumour microenvironment. Nat Commun 2016;7:13589.
37. Akbay EA, Koyama S, Liu Y, et al. Interleukin-17A Promotes Lung Tumor Progression through Neutrophil Attraction to Tumor Sites and Mediating Resistance to PD-1 Blockade. J Thorac Oncol 2017;12:1268-1279.
38. Chen Y, Yuan R, Wu X, He X, Zeng Y, Fan X, Wang L, Wang J, Lan P, Wu X. A Novel Immune Marker Model Predicts Oncological Outcomes of Patients with Colorectal Cancer. Ann Surg Oncol 2016;23:826-832.
39. Liu J, Duan Y, Cheng X, Chen X, Xie W, Long H, Lin Z, Zhu B. IL-17 is associated with poor prognosis and promotes angiogenesis via stimulating VEGF production of cancer cells in colorectal carcinoma. Biochem Biophys Res Commun 2011;407:348-354.
40. Kyung-Ah Cho, Jee Won Suh, Jung Ho Sohn, Jung Won Park, Hyejin Lee, JiHee Lee Kang, So-Youn Woo, and Young Joo Cho. IL-33 induces Th17-mediated airway inflammation via mast cells in ovalbumin-challenged mice. Am J Physiol Lung Cell Mol Physiol 2012;302:429-440.

THE EFFECTS OF VALSARTAN ON CARDIAC FUNCTION AND PRO-OXIDATIVE PARAMETERS IN THE STREPTOZOTOCIN-INDUCED DIABETIC RAT HEART

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EFEKTI VALSARTANA NA FUNKCIJU SRCA I PRO-OKSIDACIONE PARAMETRE KOD PACOVA SA STREPTOZOTOCINOM-IZAZVANIM DIJABETESOM

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ABSTRACT

Diabetes mellitus is a major risk factor for cardiovascular diseases, while cardiovascular diseases are a leading cause of morbidity and mortality worldwide. The renin–angiotensin–aldosterone system controls renal, cardiovascular, adrenal function and regulates fluid and electrolyte balance as well as blood pressure. Because of his role, inhibition of renin-angiotensin-aldosteron system is another therapy approach that reduces the risk of diabetes and cardiovascular disease. In this study, our goal was to evaluate effect of valsartan,as inhibitor of angiotensin II receptor type 1, on cardiac tissue and function, with focus on cardiodynamic and oxidative stress. The present study was carried out on 20 adult male Wistar albino rats (8 week old and with body masses of 180-200 g). Rats were divided randomly into 2 groups (10 animals per group). Healthy animals treated with 1 μM of valsartan and streptozotocin-induced diabetic animals perfused with 1 μM of valsartan 4 weeks after the induction of diabetes. Our results demonstrated that acute application of valsartan has different effect on cardiodynamics in rat heart of diabetic and healthy animals but did not improve cardiac function in hyperglycemia-induced changes. A challenge for further investigations are studies with chronic or acute administration, alone or in combination with other angiotensin-converting-enzyme inhibitor in various models of diabetes.

Keywords: *cardiodynamics, redox status, renin-angiotensin-aldosteron system, diabetes, valsartan.*

SAŽETAK

Dijabetes melitus je glavni faktor rizika za nastanak kardiovaskularnih bolesti, dok su kardiovaskularne bolesti vodeći uzrok morbiditeta i mortaliteta širom sveta. Sistem renin-angiotenzin-aldosteron kontroliše bubrežnu, kardiovaskularnu, i nadbubrežnu funkciju i reguliše ravnotežu tečnosti i elektrolita, kao i krvni pritisak. Zbog svoje uloge, inhibicija renin-angiotenzin-aldosteron sistema predstavlja još jedan terapijski pristup koji smanjuje rizik od nastanka dijabetesa i kardiovaskularnih bolesti. Cilj naše studije je bio da ispita akutni efekat valsartana, kao inhibitora angiotenzin II receptora (podtipa 1) na srčano tkivo i funkciju, sa fokusom na kardiodinamiku i oksidacioni stres. Ova studija je sprovedena na 20 odraslih mužjaka Wistar albino pacova (starosti 8 nedelja, telesne mase 180-200 g). Pacovi su svrstani nasumično u 2 grupe (10 životinja po grupi): zdrave životinje tretirane sa 1 μM valsartana i dijabetične životinje tretirane sa 1 μM valsartana, 4 nedelje nakon indukcije dijabetesa streptozotocinom. Naši rezultati pokazuju da akutna primena valsartana ima različiti efekat na kardiodinamiku srca pacova dijabetičnih i zdravih životinja, ali bez pozitivnog uticaja na promene srčane funkcije koje su izazvane hiperglikemijom. Izazov za dalja istraživanja su studije sa hroničnom ili akutnom primenom, samostalno ili u kombinaciji sa drugim inhibitorima angiotenzin konvertujućeg enzima u različitim modelima dijabetesa.

Ključne reči: *kardiodinamika, redoks status, renin-angiotenzin-aldosteron sistem, dijabetes, valsartan.*

ABBREVIATIONS

ACE - Angiotensin-converting-enzyme
ARBs - Angiotensin-receptor blockers
AT1 - Angiotensin type 1
AT2 - Angiotensin type 2
ATII - Angiotensin II
CPP - coronary perfusion pressure
CVD - Cardiovascular diseases
CVS - cardiovascular system
DM - diabetes mellitus
eNOS - endothelial nitric oxide synthases
RAAS - Renin–angiotensin–aldosterone system
STZ - Streptozotocin

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INTRODUCTION

The renin–angiotensin–aldosterone system (RAAS) is a synchronized hormonal cascade that controls renal, cardiovascular and adrenal function and regulates fluid and electrolyte balance as well as arterial pressure (1). RAAS contribute in pathogenesis and development of hypertension, atherosclerosis and cardiac disease. It provokes vasoconstriction, inflammation, cardiac remodeling and sodium retention and other possible harmful effects (2). Recent study showed that beside global RAAS activation, the cellular RAAS have an important role in physiology responses in cardiovascular diseases (CVD) (3).

Angiotensin II (ATII) is the major bioactive peptide of the RAAS that has a great influence in the functioning of many cells. As a pleiotropic hormone this peptide controls many organ systems principally through redox-sensitive processes (4). It is powerful vasoconstrictor that also indicates hypertrophy, inflammation and fibrosis that leads to vascular tissue damage and remodeling in cardiovascular diseases (5). There are two different AT II receptors: Angiotensin type 1 (AT1) and angiotensin type 2 (AT2) but AT1 receptor is more important for the cardiovascular system due to the fact that it plays predominant and mediate role in harmful effects of ATII (6).

According to previous research data, it has been shown that patients with diabetes mellitus (DM) have 3.4 times higher intracellular ATII levels than those without diabetes and in diabetic patients who also have hypertension this difference can be double (7). In addition to the standard therapy in the prevention of diabetes and lifestyle modification inhibition of the RAAS is another approach that reduces the risk of diabetes and cardiovascular disease (8).

Angiotensin-converting-enzyme (ACE) inhibitors and angiotensin-receptor blockers (ARBs) may reduce the incidence of diabetes mellitus and the risk of cardiovascular events (9, 10) compared to other anti-hypertensive drugs (11, 12) in patients with hypertension, heart failure, stroke as well as myocardial infarction (13).

An important role in the pathogenesis and progression of diabetic complications plays oxidative stress. Mitochondrial superoxide overproduction in endothelial of vessels as well as in the myocardium can be involved in the pathogenesis of these complications. In addition to the above-mentioned ATII is also known as an inducer of oxidative stress in cardiovascular tissue (14). Previously, it has been reported that RAAS antagonists such as ACE inhibitors or ARBs can reduce oxidative stress and inflammation (15, 16).

Valsartan is a potent, non-peptide tetrazole derivative that selectively inhibits ATII receptor type 1. Inhibition of AT1 receptor has a plenty of beneficial effects, as antiinflamatory, antioxidative, cardioprotective and antiatherosclerosis effect (17, 18). As a ARB valsartan prevents ATII-mediated adverse effects on the cardiovascular system (CVS) and it widely used in treatment of hypertension and organ damage that hypertension can lead (19). Recent study showed that valsartan has a protective effect on myocardial injury with a reduction in myocardial enzymes and proinflammatory mediators (6) as well as beneficial effects on DM (9).

Considering the role of ATII and AT1 receptors in cardiac tissue especially in hyperglycemic conditions as well as beneficial effects of valsartan in CVD and DM, aim of our study was to estimate effect of diabetes and valsartan on cardiac tissue and function.

MATERIALS AND METHODS

Animals and experimental design

The present study was carried out on 20 adult male *Wistar* albino rats (8 weeks old and body mass of 180–200 g). They were housed under controlled environmental conditions: temperature 25°C with an established photoperiod of 12 h light/day. The rats had free access to food and tap water - *ad libitum*. Rats were divided randomly into 2 groups (10 animals per group): Non-diabetic animals perfused with 1 μM of valsartan and streptozotocin-treated diabetic animals perfused with 1 μM of valsartan four weeks after the induction of diabetes (20).

All experimental procedures were done in accordance with prescribed legislation (EU Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes 86/609/EES) and the principles of ethics. Animals were cared in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council 1996). The experimental protocol was approved by Ethics committee for experimental animal well being of the Faculty of Medical Sciences of the University of Kragujevac.

Diabetic Rat Model

Before induction of DM two weeks of adaptation were provided. To develop a rat model of experimentally-induced type 1 DM, which resembles that occurring in human population, overnight fasting rats were injected with a single intraperitoneal dose of Streptozotocin (STZ) (60 mg/kg) (Sigma, St. Louis, MO, USA) dissolved in cold fresh 0.01 M citrate buffer fresh or frozen in 1 mL aliquots at 20°C (0.1 mol/L citric acid, 0.1 mol/L sodium citrate), pH 4.5. DM was confirmed 72 hours later when blood glucose were > 11.1 mmol/l (21). Animals that developed DM were held in the same conditions and followed for the next 4 weeks.

Isolated heart perfusion

On the 29th day from beginning of experimental protocol, animals were euthanized by cervical dislocation and the chest was opened via midline thoracotomy. The hearts were immediately removed and immersed in cold saline, then mounted on a stainless steel cannula of the Langendorff perfusion apparatus to provide retrograde perfusion under gradually increasing coronary perfusion pressure (CPP) (CPP from 40 cmH_2O to 120 cmH_2O). Krebs-Henseleit buffer was used for retrograde perfusion (in mmol/l: NaCl 118, KCl 4.7, $CaCl_2$ x $2H_2O$ 2.5, $MgSO_4$ x $7H_2O$ 1.7, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11, and pyruvate 2). The buffer was bal-

anced with 95% O_2 and 5% CO_2, with a pH value of 7.4 and temperature of 37°C. Following the establishment of heart perfusion, the preparations were stabilised within 30 minutes with a basal CPP of 70 cmH_2O. Following the stabilisation period, the perfusion pressure was reduced to 50 and 40 cmH_2O and then gradually increased to 60, 80, 100 and 120 cmH_2O to establish coronary autoregulation. Testing started immediately after the control experiment to avoid unwanted time-dependent consequences. The administration of 1μM valsartan lasted until the achievement of a stable flow but not under 5 minutes for each value of perfusion pressure.

After placing the sensor (transducer BS4 73-0184, Experimetria Ltd., Budapest, Hungary) into the left ventricle (LV) following cardio-dynamic parameters were continuously registered: maximum and minimum rate of pressure development in left ventricle (dp/dt max, dp/dt min), systolic and diastolic left ventricle pressure (SLVP, DLVP), and heart rate (HR) on each of predetermined values of perfusion pressure (40, 60, 80, 100 and 120 cmH_2O). CF was measured by flowmetry. Each heart was its own control.

Biochemical analysis

Markers of oxidative stress were measured spectrophotometrically in the collected samples of coronary venous effluent. Samples were collected after stabilization of the coronary flow and after drug administration for each perfusion pressure. We performed quantification of nitrites (the amount of NO_2^- released), superoxide anion radical (O_2^-), and hydrogen peroxide (H_2O_2) and an indirect quantification of the index of lipid peroxidation via reactive thiobarbituric substances (TBARS), for all samples.

Determination of superoxide anion radicals (O_2^-)

Superoxide anion radical concentrations were measured using the NTB (Nitro Blue Tetrazolium) reagent in TRIS buffer (assay mixture) with coronary venous effluent. The measurement was performed at a wavelength of 550 nm. Distilled water was used as a blank probe (22).

Determination of nitrites (NO_2^-)

Nitric oxide quickly decomposes into stable metabolite nitrites/nitrates. Nitrites can therefore be used as an index of NO_2^- production via a spectrophotometric method using the Griess reagent. Briefly, 0.5 ml of the perfusate was precipitated with 200 μl of 30% sulfosalicylic acid, vortexed for 30 min and centrifuged at 3000 x g. Equal volumes of the supernatant and Griess reagent containing 1 % sulphanilamide in 5 % phosphoric acid/0.1 % naphthalene ethylenediamine dihydrochloride was added and stabilized for 10 min in the dark and measured spectrophotometrically at a wavelength of 550 nm. The nitrite concentrations were determined using sodium nitrite as the standard (23).

Determination of hydrogen peroxide (H_2O_2)

A measurement of hydrogen peroxide was based on the oxidation of phenol red by hydrogen peroxide in a reaction catalysed by horseradish peroxidase (HRPO). A total of 200 μl of perfusate was precipitated with 800 ml of freshly prepared phenol red solution, followed by the addition of 10 μl of (1:20) HRPO (made ex tempore). Distilled water was used as a blank probe (instead of coronary venous effluent). The level of H_2O_2 was measured at 610 nm (24).

Determination of the index of lipid peroxidation measured as TBARS

The index of lipid peroxidation was determined indirectly by measuring the products of the reaction with thiobarbituric acid (TBARS or **T**hiobarbituric **A**cid **R**eactive **S**ubstances). Briefly, 1% thiobarbituric acid (TBA) in 0.05 M NaOH was incubated with coronary venous effluent at 100˚C for 15 min and then measured at 530 nm of wavelength, spectrophotometrically. Distilled water was used as a blank probe (25).

Drugs

Streptozotocin and valsartan were purchased from Sigma–Aldrich Chemie GmbH Eschenstr. 5, 82024 Taufkirchen, Germany.

Statistics

Statistical analysis of experimental data included the following basic descriptive statistics: the mean value (X) ± standard deviation (SD). The following statistical tests were used to test the statistical significance of the results and to confirm the hypothesis: Paired – Samples *T* test and Independent *T* test. A database analysis of the results was performed using software package SPSS 18th (SPSS Inc., Chicago, IL, USA). *P* values lower than 0.05 ($p<0.05$) were considered to be significant while *P* values lower than 0.01 ($p<0.01$) were considered to be high significant.

RESULTS

Cardiodynamic parameters

Ventricular contractility assessment (dp/dt max and dp/ dt min)

The maximum rate of pressure development in LV wall (dp/dt max) was non-significantly higher in healthy relative to diabetic rats at all CPP pressures (40–120 cmH_2O) (Fig. 1a). Perfusion with valsartan in healthy animals led to decreased dp/dt max with the statistical significance at CPP 80 and 120 cmH_2O (Fig. 2a). In diabetic animals, the change in dp/dt max is absent for most CPPs. Nevertheless, after valsartan perfusion a statistically significantly lower value at CPP 100 cmH_2O was observed (Fig. 3a). The minimum rate of pressure development in LV wall (dp/dt min) was not significantly different between the healthy and diabetic animals; values of this parameter were quite similar (Fig. 1b). Perfusion with valsartan increased dp/dt min in the healthy animals with statistical significance at CPP at 80-120 cmH_2O (Fig. 2b). As well as in the healthy rats, in diabetic animals, perfusion with valsartan increased values

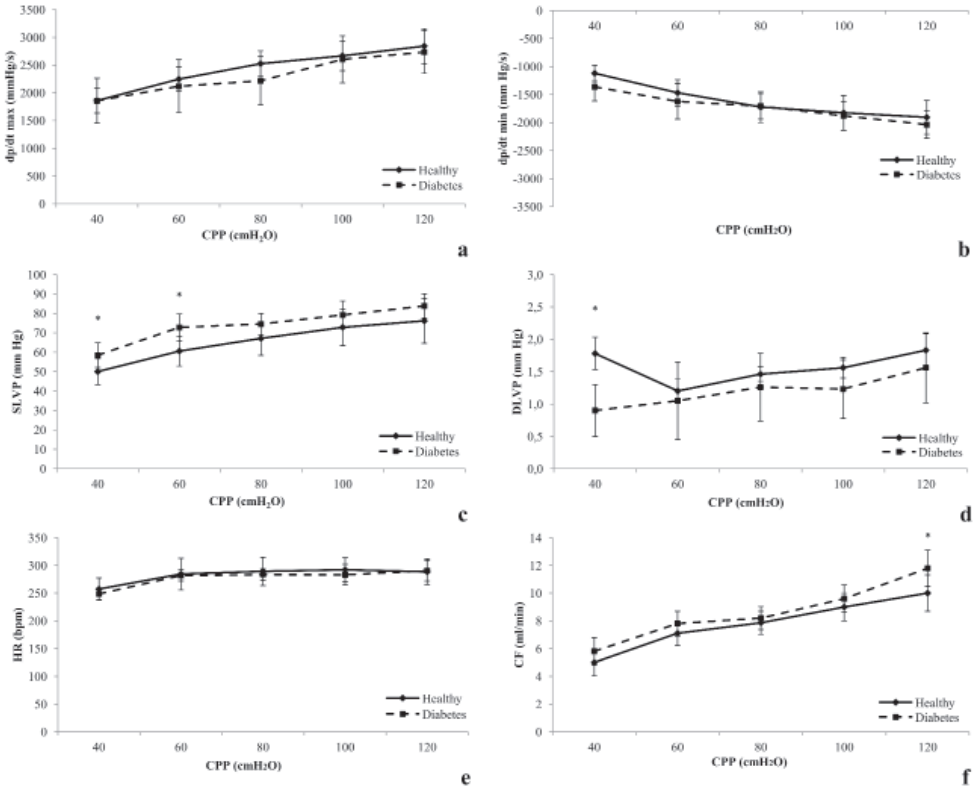

Figure 1. Cardiodynamic parameters in hearts of healthy rats compared to hearts of diabetic rats.
Data are presented as following: a – Maximum rate of development pressure in left ventricle (dp/dt max); b – Minimum rate of development pressure in left ventricle (dp/dt min); c – Systolic left ventricle pressure (SLVP); d - Diastolic left ventricle pressure (DLVP); e – Heart rate (HR); f - Coronary flow (CF). Data are presented as mean ± SD (*p<0.05; **p<0.01).

of dp/dt min with statistically significance at CPP 80-120 cmH_2O (Fig. 3b).

Left ventricular pressures (SLVP, DLVP)

Systolic left ventricle pressure was increased in diabetic group relative to healthy rats at all CPPs with statistically significance at 40 and 60 cmH_2O (Fig. 1c). SLVP was decreased after perfusion with valsartan in healthy rats at all CPPs (40–120 cmH_2O) and statistical significance was

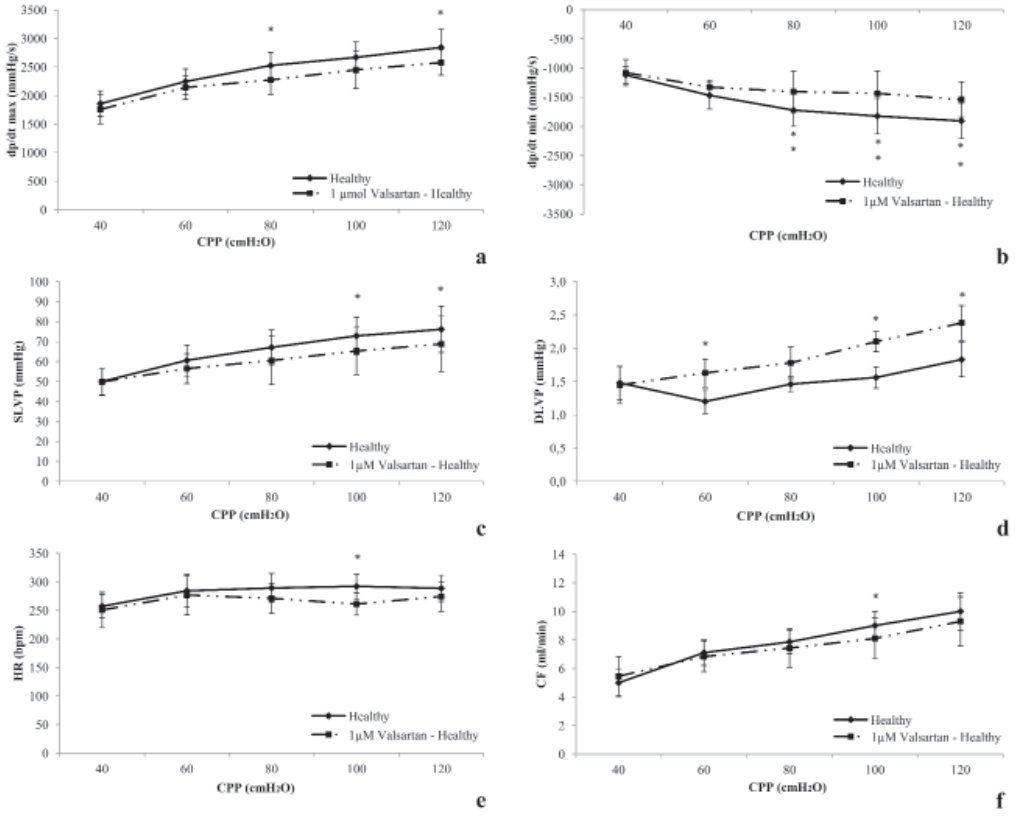

Figure 2. Cardiodynamic parameters in hearts of healthy rats compared to hearts of healthy rats perfused with 1 µM of valsartan.
Data are presented as following: a – Maximum rate of development pressure in left ventricle (dp/dt max); b – Minimum rate of development pressure in left ventricle (dp/dt min); c – Systolic left ventricle pressure (SLVP); d - Diastolic left ventricle pressure (DLVP); e – Heart rate (HR); f - Coronary flow (CF). Data are presented as mean ± SD (*p<0.05; **p<0.01).

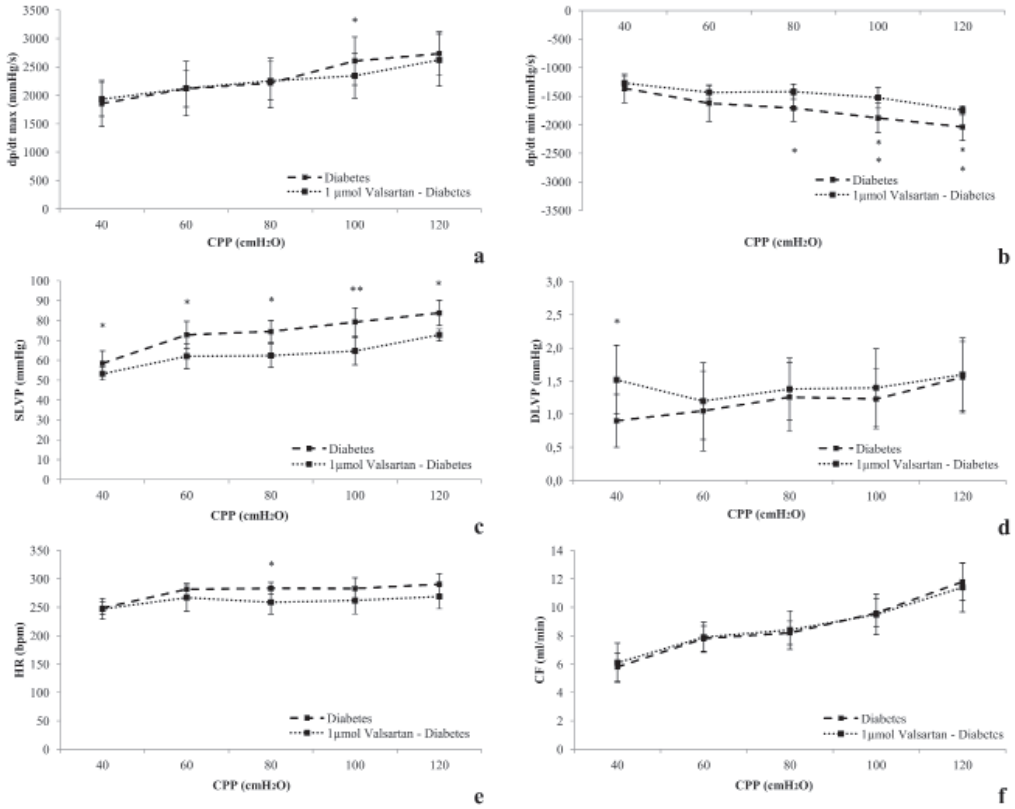

Figure 3. Cardiodynamic parameters in hearts of diabetic rats compared to hearts of diabetic rats perfused with 1 µM of valsartan.
Data are presented as following: a – Maximum rate of development pressure in left ventricle (dp/dt max); b – Minimum rate of development pressure in left ventricle (dp/dt min); c – Systolic left ventricle pressure (SLVP); d - Diastolic left ventricle pressure (DLVP); e – Heart rate (HR); f - Coronary flow (CF). Data are presented as mean ± SD (*p<0.05; **p<0.01)

noticed at CPP 100 and 120 cmH_2O (Fig. 2c). The same trend as in healthy animals was noted in diabetic group but the effect of valsartan on the SLVP reduction is even more pronounced it these animals. All values after valsartan perfusion are statistically significantly lower and at CPP 100 cmH_2O the difference is highly significant (p<0.01) (Fig. 3c). Diastolic left ventricle pressure was decreased in diabetic relative to healthy group with statistical significance at 40 cmH_2O (Fig. 1d). Additionally, in the healthy animals DLVP is statistically higher after valsartan perfusion at CPP 60, 100 and 120 cmH_2O (Fig. 2d). In the group of diabetic animals DLVP is also increased after valsartan perfusion and statistical significance is achieved only at CPP 40 cmH_2O (Fig. 3d).

Heart rate (HR) and coronary flow *(CF)*

Heart rate displayed a non-significant decrease in the diabetic group compared to healthy animals at all CPP values (Fig. 1e). Valsartan affects the heart rate by decreasing its values in both groups, healthy and diabetic rats. The values are non-significant lower at all CPP values, except at CPP 100 cmH_2O in healthy rats group (Fig. 2e) and at 80 cmH_2O in diabetic group (Fig. 3e) after valsartan perfusion. Coronary flow was increased in the diabetic animals

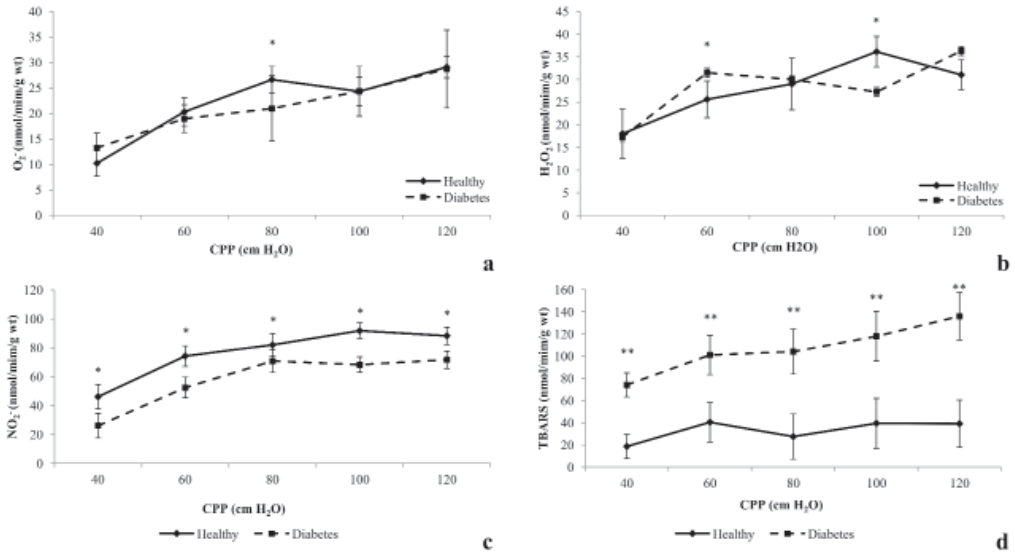

Figure 4 - Oxidative stress parameters in healthy rats compared to diabetes rats.
Data are presented as following: a - Level of superoxide anion radical (O_2-); b – Level of hydrogen peroxide (H_2O_2); c - Level of nitric oxide (NO) measured in the form of nitrite (NO_2-); d - level of index of lipid peroxidation measured as thiobarbituric acid reactive substances (TBARS). Data are presented as mean ± SD (*p<0.05; **p<0.01)

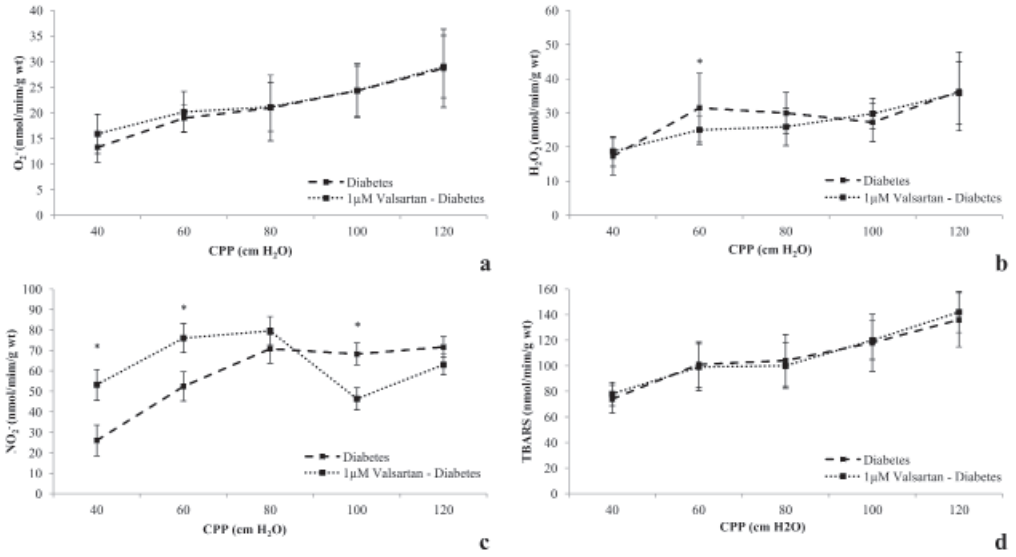

Figure 5 - Oxidative stress parameters in healthy rats compared to healthy rats with perfusion of 1 μM of valsartan.
Data are presented as following: a - Level of superoxide anion radical (O_2-); b – Level of hydrogen peroxide (H_2O_2); c - Level of nitric oxide (NO) measured in the form of nitrite (NO_2-); d - level of index of lipid peroxidation measured as thiobarbituric acid reactive substances (TBARS). Data are presented as mean ± SD (*p<0.05; **p<0.01)

at all CPP values compared to healthy rats, significantly at CPP 120 cmH_2O (Fig. 1f). After valsartan perfusion CF was discretely increased at CPP 40 cmH_2O and after that CF values began to decrease in healthy animals. Statistically significant lower CF after valsartan perfusion was detected at CPP 100 cmH_2O (Fig. 2e). In diabetic rats group there was no statistical significance after valsartan perfusion at all CPP (Fig. 3e).

Oxidative stress parameters

Values of O_2-

The level of superoxide anion radical in healthy relative to diabetic animals was significantly higher at CPP 80 cmH_2O while at the other CPPs these values were similar (Fig. 4a). In healthy rats valsartan firstly significantly increased and thereafter highly significantly decreased level of O_2^- at CPPs 60 – 120 cmH_2O (Fig. 5a). In diabetic animals valsartan didn't induces any statistical significance, values between groups were quite similar (Fig. 6a).

Values of H_2O_2

Between healthy and diabetic groups of animals there was difference in level of H_2O_2 at CPP 60 and 100 cmH_2O. In diabetic animals at CPP 60 cmH_2O level of H_2O_2 was significantly higher relative to healthy group while opposite to this result at CPP 100 cmH_2O in group of healthy animals level of H_2O_2 was statistically increased (Fig. 4b). Valsartan perfusion statistically reduced level of H_2O_2 in healthy rats at CPP 100 cmH_2O while at the other CPPs level of this parameter was quite similar (Fig. 5b). On the other hand, valsartan statistically decreased level of H_2O_2 at 60 cmH_2O while at the other CPPs values were slightly different (Fig. 6b).

Values of NO_2^-

NO_2^- activity was measured as nitrite. The nitrite level was significantly decreased in diabetic group relative to the healthy animals at all CPPs (Fig. 4c). In healthy animals after valsartan perfusion, nitrite level wasn't changed significantly and the values were slightly similar in both groups (Fig. 5c). In diabetic animals valsartan increased nitrite

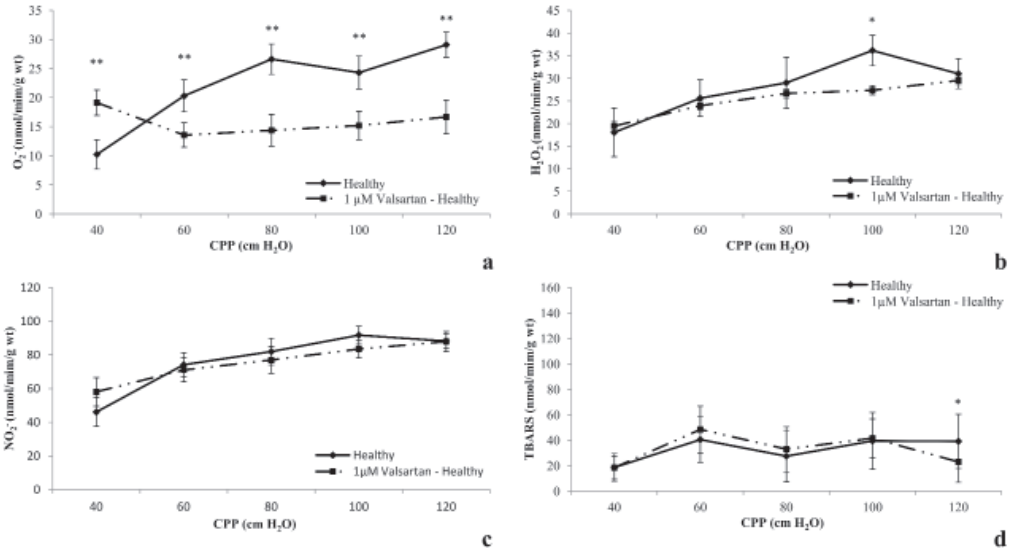

Figure 6 - Oxidative stress parameters in diabetic rats compared to diabetic rats with perfusion of 1 μM of valsartan.
Data are presented as following: a - Level of superoxide anion radical (O_2-); b – Level of hydrogen peroxide (H_2O_2); c - Level of nitric oxide (NO) measured in the form of nitrite (NO_2-); d - level of index of lipid peroxidation measured as thiobarbituric acid reactive substances (TBARS). Data are presented as mean ± SD (*p<0.05; **p<0.01)

level with statistically difference at CPP 40 and 60 cmH_2O but opposite to these results, valsartan decreased NO level at CPP 100 and 120 cmH_2O with statistical significance at CPP 100 cmH_2O (Fig. 6c).

Values of TBARS

The TBARS values were highly significant decreased in healthy animals relative to diabetic rats at all CPPs (Fig. 4d). After valsartan perfusion in healthy animals, level of TBARS was statistically decreased at CPP 120 cmH_2O (Fig. 5d) while in diabetic animals there is no notably difference, values were quite similar after valsartan perfusion (Fig. 6d).

DISCUSSION

The aim of this study was to determine acute effects of valsartan on heart function as well as on oxidative stress in STZ-induced DM isolated rat hearts. Additionally, we would like to investigate whether STZ-induced DM had influence on cardiac function or whether valsartan can reduce effects of STZ-induced DM on cardiovascular parameters and oxidative stress isolated rat heart.

There are different models developed to investigate pathophysiological mechanisms of diabetic cardiomyopathy in rats whereby STZ-induces DM is most extensively and the most similar to human DM (26). The injection of streptozotocin leads to the development of a clinical syndrome characterized by hyperglycemia, excessive diuresis and loss of weight (7).

It is interesting that although the myocardial concentrations of renin is 1–4% of those in plasma, the cardiac interstitial fluid concentrations of angiotensinogen I and angiotensinogen II are over 100-fold those of plasma. There is evidence that the cardiac interstitial fluid represents a separate compartment from the systemic circulation and that interstitial ATII derives exclusively from *de novo* biosynthesis in the heart (1).

As previously mentioned, valsartan is a potent, orally active, non-peptide antihypertensive drug that is highly selective antagonist of the ATII AT1 receptor with no significant affinity for other receptors (27, 28). It has been shown that in patients with diabetes blockade of AT1 receptors reduces cardiovascular mortality and morbidity. Previous clinical trials with ATII receptor blockers in addition to reducing cardiovascular morbidity and mortality likewise results in the consistently reduces of the incidence of new onset DM (29, 30)

Although a large number of studies investigated metabolic disorders in the heart of diabetic rats there is almost no data regarding acute influence of valsartan on cardiodynamics and functional status of the diabetic heart.

In our study, dp/dt max and dp/dt min were decreased in diabetic rats but in physiological range while systolic pressure was increased in hypoxia condition opposite to diastolic pressure which was decreased compared to healthy heart also in hypoxia condition (Figs. 1a-d). We have recently showed that myocardial contractile function is impaired in STZ- induced diabetic rats (7). Depressed cardiac function in diabetic rats is due to gross changes with increased extracellular type 1 collagen synthesis (31) and impaired Ca^{2+} release that leads to reduced contractility and slower Ca^{2+} uptake by SERCA which results in impaired relaxation of cardiac muscle (32). Increasing of systolic pressure may indicate a lower tolerance to increase tensions in the system. However, present results showed diminished cardiac function in diabetic rats mostly in condition of hypoxia which can be explained by the fact that there is no damage of the heart muscle during short-time perfusion in normoxy condition and that diabetic heart is more vulnerable in hypoxic conditions than healthy one.

On the other hand, valsartan did not significantly changed cardiac contractility and relaxation in normoglycemic conditions. Nonetheless, valsartan decreased systolic pressure and increased diastolic pressure on the higher CPPs where the oxygenation was over the normal range (Figs. 2c-d). Heart rate and coronary flow were decreased after valsartan perfusion but in physiology range. Heart rate was significantly decreased after valsartan perfusion at CPP 100 cmH_2O but at CPP 120 cmH_2O values were similar to control conditions. Withal, increase of pressure value leads to slightly flow increase (Figs. 2e-f).

In the diabetic rats systolic pressure in LV was statistically significantly lower at all CPP that can be explained by chronic hyperglycemia which affect the further reaction of the tissue after perfusion with valsartan (Fig. 3c). This reduction in value after valsartan perfusion on SLVP indicates a change in heart tissue in diabetes despite seemingly approximate values of the cardiodynamic parameters in previous comparisons of healthy and diabetic rats. Our results are in accordance with Chan and coworkers (27) who showed that valsartan applied as intravenous injection in STZ-diabetic rats also reduces systolic blood pressure. Diastolic pressure at diabetic rats was increased but less than in healthy rats after valsartan perfusion whereby that can indicate on rigidity of the chamber (Fig. 3d) as a consequence of hyperglycemia for a period of 4 weeks before valsartan perfusion. As well as in healthy rats there is no significant difference in value of HR but the changes are less in diabetic rats. Literature data suggested that ARBs have positive effects in the prevention of chronic hyperglycemia influence on tissues (33) but there is no precise mechanism that explains how acute valsartan administration affects heart ratio. With all, previous studies have found that streptozotocin-induced diabetes is characterized by downregulated endothelial nitric oxide synthases (eNOS) that localizes at the caveolae where it controls heart rate, contraction, diastolic relaxation and oxygen utilization (34). It has been snown that eNOS plays a protective role in cardiovascular system which is confirmed by Janssens et al (35). They observed that cardiomyocyte-restricted eNOS overexpression was protective against adverse LV remodeling after coronary artery ligation in transgenic mice that correlate with other studies (35-37).

It may be possible that during long-time of hyperglycemia in diabetic rats, downlegulation of eNOS induced increase diastolic pressure in LV and slightly but not significant decreased heart rate (Fig. 3d; Fig. 3f).

As the effects are more pronounced at dp/dt min than on dp/dt max (Fig. 2a-b; Fig. 3a-b) we can assume that in valsartan group the effect is more pronounced on the relaxation capability. This is indicated by changes in the diastole (Fig. 2d; Fig. 3d). Taken together, it seems that valsartan improved LV properties of the heart during hyperglycemia and thus have positive influence on diastolic function of the STZ-induced diabetic heart.

In the second part of our study we examined the oxidative status of heart in normoglycemic and hyperglycemic conditions as well as effect of valsartan on redox status. In control conditions we can notice that TBARS values were statistically higher at all CPPs in diabetic compared to healthy rats (Fig. 4d) which indicates muscle damage in diabetic heart. Opposite trend was noticed in NO_2^-, values were statistically higher in healthy group while O_2^- was statistically higher only at CPP 80 cmH_2O also in healthy group. These results may indicate that DM led to myocardial damage. One of the possible explanations for this could be excessive NO_2^- produced by iNOS which can be activated by prolonged hyperglycemia and its interaction with O_2^- to create peroxynitrite ($ONOO^-$) and to reduce O_2^- levels. O_2^- has a half-life of 10^{-6} of a second, and NO molecules last for few seconds, both of which support this possibility (7).

Based on previous data it was expected that the highest activity of reactive oxygen species would be observed in diabetic rats. Result is little bit different compared to the stated assumption. In healthy animals, at the lowest, hypoxic CPP, O_2^- statistically significantly increased than it was significantly lower in other CPPs after valsartan perfusion (Fig. 5a). Compared to these results in diabetic animals there wasn't significant increase of O_2^- (Fig. 6a) like it was expected that can be consequence of reaction with NO produced by pathological activation of iNOS due to enhancement of inducible iNOS activity because the metabolism of the L-arginine NO system is altered in diabetes (38, 39). O_2^- results indicate that valsartan has a lower impact on O_2^- reduction in diabetes than in the control group. This result is difficult to explain, and can be consequence of either higher O_2^- in normoglycemic group or modified AT1 receptors in hyperglycemic conditions, however exact mechanisms for this effect is still unknown.

Oxidative stress reducing by using the AT II AT1 receptor antagonist is expected in the pathological conditions of diabetes (40) but from some unexpected reason the effect is absent that can be explained by increased concentration of NO (Fig. 6c) that reacted with O_2^- and led to the formation of peroxynitrite, which unfortunately we did not measured. Peroxynitrite contributes to contractile dysfunction and even apoptosis. According to previously mentioned half-life of O_2^- and NO_2^-, that can explain non-significant difference in concentration of O_2^- between control and diabetic rats (Fig. 6a) (34). Continuously increasing of NO_2^- in diabetic rats may be result of inducible enzyme activation (iNOS) whereby its expression during pathological states continuously produces NO until the enzyme is degraded, and this leads to excessive reactive nitrogen species (RNS) production as well as peroxynitrite, which as previously mentioned contributes to contractile dysfunction. On the other hand, another explanation is that prolonged and significantly elevated hyperglycemia and consequent activation of the PKCβ2–iNOS pathway leading to loss of O_2^- in the reaction with NO (34) that we noticed in our results (Fig. 6a; Fig. 6c). As already mentioned, it is possible that uncontrolled iNOS activity in diabetes caused such a NO_2^- oscillations. TBARS level is expectedly higher in diabetic rats, because, normally, a diabetic heart makes greater use of free fatty acids for metabolism. Movement of values in control groups are relatively, until highest CPP, apropos in condition of hypertension and overloaded tissue. In this conditions valsartan decreases TBARS level which can be significant in hypertension without diabetes, because it represent additional antioxidative effect of valsartan.

CONCLUSION

In summary, it can be concluded that heart from STZ-induced diabetic animals were not functionally different. Acute application of valsartan failed to improve these hyperglycemia-induced changes of cardiac function. Finally, it seems that oxidative stress does not have role in these effects of valsartan. A challenge for future investigations will be to identify the effects either of acute or chronic valsartan treatment alone or in combination with other ACE inhibitors in various models of diabetes.

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CONFLICT OF INTERESTS

The authors declare that there are no competing interests associated with the manuscript.

REFERENCES

1. Carey RM, Siragy HM. Newly recognized components of the renin-angiotensin system: potential roles in cardiovascular and renal regulation. Endocr Rev 2003; 24(3): 261–271
2. Vranic A, Simovic S, Ristic P, Nikolic T, Stojic I, Srejovic I et al. The acute effects of different spironolactone doses oncardiac function in streptozotocin-induced diabetic rats. Can J Physiol Pharmacol 2017; 95: 1343–1350

3. Nguyen Dinh Cat A, Jaisser F. Extrarenal effects of aldosterone. Curr Opin Nephrol Hypertens 2012; 21: 147–156
4. Nguyen Dinh Cat A, Montezano AC, Burger D, Touyz RM. Angiotensin II, NADPH oxidase, and redox signaling in the vasculature. Antioxid Redox Signal 2013; 19: 1110-1120
5. Nguyen Dinh Cat A, Touyz RM. Cell signaling of angiotensin II on vascular tone: novel mechanisms. Curr Hypertens Rep 2011; 13: 122–128
6. Kintscher U, Marx N, Martus P, Stoppelhaar M, Schimkus J, Schneider A et al. Effect of high-dose valsartan on inflammatory and lipid parameters in patients with Type 2 diabetes and hypertension. Diabetes Res Clin Pract 2010; 89: 209–215
7. Ristic P, Srejovic I, Nikolic T, Stojic I, Ristic D, Zivkovic V et al. The effects of zofenopril on cardiac function and pro-oxidative parameters in the streptozotocin-induced diabetic rat heart. Mol Cell Biochem 2017; 426: 183–193
8. Holman RR, Haffner SM, McMurray JJ, Bethel MA, Holzhauer B, Hua TA et al. Effect of nateglinide on the incidence of diabetes and cardiovascular events. N Engl J Med 2010; 362: 1463–1476
9. Kjeldsen SE, Julius S, Mancia G, McInnes GT, Hua T, Weber MA et al. Effects of valsartan compared to amlodipine on preventing type 2 diabetes in high-risk hypertensive patients. the VALUE trial J Hypertens 2006; 24: 1405–1412
10. Yusuf S, Gerstein H, Hoogwerf B, Pogue J, Bosch J, Wolffenbuttel BH et al. Ramipril and the development of diabetes. JAMA 2001; 286: 1882–1885
11. Scheen AJ. Prevention of type 2 diabetes mellitus through inhibition of the renin-angiotensin system. Drugs 2004; 64: 2537–2565
12. Weber MA, Julius S, Kjeldsen SE, Brunner HR, Ekman S, Hansson L. Blood pressure dependent and independent effects of antihypertensive treatment onclinical events in the VALUE Trial Lancet 2004; 363: 2049–2051
13. Hollenberg NK, Parving HH, Viberti G, Remuzzi G, Ritter S, Zelenkofske S et al. Albuminuria response to very high-dose valsartan in type 2 diabetes mellitus. J Hypertens 2007; 25: 1921–1926
14. Chabrashvili T, Kitiyakara C, Blau J, Karber A, Aslam S, Welch WJ et al. Effects of ANG II type 1 and 2 receptors on oxidative stress, renal NADPH oxidase, and SOD expression. Am J Physiol Regul Integr Comp Physiol 2003; 285: 117–124
15. Hornig B, Landmesser U, Kohler C, Ahlersmann D, Spiekermann S, Christoph A et al. Comparative effect of ACE inhibition and angiotensin II type 1 receptor antagonism on bioavailability of nitric oxide in patients with coronary artery disease: role of superoxide dismutase. Circulation 2001; 103: 799–805
16. Kim HJ, Han SJ, Kim DJ, Jang HC, Lim S, Choi SH et al. Effects of valsartan andamlodipine on oxidative stress in type 2 diabetic patients with hypertension: arandomized, multicenter study. Korean J Intern Med 2017; 32: 497–504
17. Jung KH, Chu K, Lee ST, Kim SJ, Song EC, Kim EH et al. Blockade of AT1 receptorreduces apoptosis, inflammation, and oxidative stressin normotensive rats with intracerebral hemorrhage. J Pharmacol Exp Ther 2007; 322: 1051–1058
18. Navalkar S, Parthasarathy S, Santanam N, Khan BV. Irbesartan, an angiotensin type 1 receptor inhibitor, regulatesmarkers of inflammation in patients with premature atherosclerosis. J Am Coll Cardiol 2001; 37: 440–444
19. Shmiedr R, Hilgers KF, Schlaich MP, Shmidt BMW. Renin-angiotensin system and cardiovascular risk, Lancet 2007; 369(9568): 1208–1219
20. Yang ZH, Peng XD. Effects of valsartan on diabetic cardiomyopathy in rats with type 2 diabetes mellitus. Chin Med J 2010; 123: 3640–3643
21. Tesch GH, Allen TJ. Rodent models of streptozotocin-induced diabetic nephropathy. Nephrology 2007; 12: 261–266
22. Auclair C, Voisin E. Nitroblue tetrazolium reduction. In: Greenwald RA (ed) CRC handbook of methods for oxygen radical research. Boca Raton CRC Press, 1985: 123–132
23. Green LC, Wagnwr DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite and (15 N) nitrate in biological fluids. Anal Biochem 1985; 126: 131–138
24. Pick E, Keisari Y. A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture. J Immunol Methods 1980; 38: 161–170
25. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979; 95: 351–358
26. Goyal SN, Reddy NM, Patil KR, Nakhate KT, Ojha S, Patil CR et al. Challenges and issues with streptozotocin-induced diabetes—A clinically relevant animal model to understand the diabetes pathogenesis and evaluate therapeutics. Chem Biol Interact 2016; 244: 49–63
27. Chan P, Wong KL, Liu IM, Tzeng TF, Yang TL et al. Antihyperglycemic action of angiotensin II receptor antagonist, valsartan, in streptozotocin-induced diabetic rats. J Hypertens 2003; 21; 761–769
28. Criscione L, De Gasparo M, Buhlmayer P, Whitebread S, Ramjoue HP, Wood J. Pharmacological profile of valsartan: a potent, orally active, nonpeptide antagonist of the angiotensin II AT1–receptor subtype. Br J Pharmacol 1993; 110: 7617–7671
29. Julius S, Kjeldsen SE, Weber M, Brunner HR, Ekman S, Hansson L et al. Outcomes in hypertensive patients at high cardiovascular risk treated with regimens based on valsartan or amlodipine: the VALUE randomised trial. Lancet 2004; 363: 2022–2031
30. Pfeffer MA, Swedberg K, Granger CB, Held P, McMurray JJ, Michelson EL et al. Effects of candesartan on mortality and morbidity in patients with chronic heart failure: the CHARM-Overall programme. Lancet 2003; 362: 759–766

31. Ward ML, Crossman DJ. Mechanisms underlying the impaired contractility of diabetic cardiomyopathy. World J Cardiol 2014; 6: 577–584
32. Ganguly PK, Pierce GN, Dhalla KS, Dhalla NS. Defective sarcoplasmic reticular calcium transport in diabetic cardiomyopathy. Am J Physiol 1983; 244: E528–E535
33. Seeger H, Lippert C, Wallwiener D, Mueck AO. Valsartan and candesartan can inhibit deteriorating effects of angiotensin II on coronary endothelial function. J Renin Angiotensin Aldosterone Syst 2001; 2: 141–143
34. Khanna S, Singh GB, Khullar M. Nitric oxide synthases and diabetic cardiomyopathy. Nitric Oxide 2014; 1: 29–34
35. Janssen S, Pokreisz P, Schoonjans L, Pellens M, Vermeersch P, Tjwa M et al. Cardiomyocyte-specific overexpression of nitric oxide synthase 3 improves left ventricular performance and reduces compensatory hypertrophy after myocardial infarction. Circ Res 2004; 94: 1256–1262
36. Ichinose F, Bloch KD, Wu JC, Hataishi R, Aretz HT, Picard MH et al. Pressure overload-induced LV hypertrophy and dysfunction in mice are exacerbated by congenital NOS3 deficiency. Am J Physiol Heart Circ Physiol 2004; 286: H1070–H1075
37. Scherrer-Crosbie M, Ullrich R, Bloch KD, Nakajima H, Nasseri B, Aretz HT et al. Endothelial nitric oxide synthase limits left ventricular remodeling after myocardial infarction in mice. Circulation 2001; 104: 1286–1291
38. Nagareddy PR, Xia Z, McNeill JH, MacLeod KM. Increased expression of iNOS is associated with endothelial dysfunction and impaired pressor responsiveness in streptozotocin-induced diabetes. Am J Physiol Heart Circ Physiol. 2005; 289: H2144–H2152
39. West MB, Ramana KV, Kaiserova K, Sirvastava SK, Bhatngar A. L-Arginin prevents metabolic effects of high glucose in diabetic mice. FEBS let. 2008; 582: 2609–2614
40. Iglarz M, Touyz RM, Viel EC, Amiri F, Schiffrin EL. Involvement of oxidative stress in the profibrotic action of aldosterone. Interaction with the reninangiotensin system. Am J Hypertens. 2004; 17: 597–603

MESENCHYMAL STEM CELLS ATTENUATE ACUTE LIVER FAILURE BY PROMOTING EXPANSION OF REGULATORY T CELLS IN AN INDOLEAMINE 2,3-DIOXYGENASE-DEPENDENT MANNER

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MEZENHIMSKE MATIČNE ĆELIJE EKSPRIMIRAJU INDOLAMIN 2-3 DIOKSIGENAZU I PROMOVIŠU EKSPANZIJU REGULATORNIH ĆELIJA U JETRI UTIČUĆI NA SMANJENJE AKUTNOG HEPATITISA

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ABSTRACT

The influence of mesenchymal stem cells (MSCs) on the phenotype and function of CD4+CD49b+FoxP3- regulatory cells has not been elucidated. We used Concanavalin A (ConA) - and α-galactosylceramide (α-GalCer)-induced acute liver injury to estimate the effects of MSCs on liver-infiltrating CD4+CD49b+FoxP3-regulatory cells. MSCs significantly reduced ConA- and α-GalCer-mediated liver injury in C57BL/6 mice, as demonstrated by biochemical tests, reduced influx of inflammatory CD4+ T cells, and increased presence of CD4+CD49b+FoxP3- regulatory cells in the injured livers. The number of CD4+CD49b+FoxP3-regulatory cells was also significantly increased in α-GalCer-treated mice that received MSC-derived conditioned medium (MSC-CM). The presence of 1-methyltryptophan, a specific inhibitor of indoleamine 2,3-dioxygenase (IDO), in MSC-CM completely abrogated the hepatoprotective effect of MSCs and significantly decreased the total number of liver-infiltrated CD4+CD49b+FoxP3- regulatory cells, indicating the crucial importance of MSC-derived IDO for the expansion of CD4+CD49b+FoxP3- regulatory cells and the consequent MSC-dependent attenuation of acute liver injury.

Keywords: *mesenchymal stem cells, regulatory T cells, acute liver failure.*

SAŽETAK

Uticaj mezenhimskih matičnih ćelija (engl. Mesenchymal Stem Cells, MSCs) na fenotip i funkciju CD4+CD49b+FoxP3-regulatornih ćelija nije razjašnjen. Da bismo procenili uticaj MSCs na CD4+CD49b+FoxP3- regulatorne ćelije, izazvali smo oštećenje jetre konkanavalinom A (engl. ConcanavalinA, ConA) i alfa-galaktozilceramidom (engl. α-Galactosylceramide, α-GalCer).MSCs, koje su aplikovane nakon indukcije hepatičnog oštećenja, značajno su smanjile oštećenje jetre C57BL/6 miševa, što je pokazano biohemijskim analizama, uz smanjenu infiltraciju inflamacijskih (IFN-γ-, TNF-α- i IL-4 produkujućih CD4+ T ćelija) i povećale ukupan broj CD4+CD49b+FoxP3-regulatornih ćelija u jetri. Broj CD4+CD49b+FoxP3- ćelija je značajno povećan u jetri miševa koji su primili kondicionirani medijum, dobijen od MSCs (engl. MesenchymalStem-Cell-ConditionedMedium, MSC-CM). Nakon dodavanja 1-metil-DL-triptofana, specifičnog inhibitora indoleamin 2,3-dioksigenaze (eng. indoleamine 2,3-dioxygenase, IDO) u MSC-CM, koji je injektiran u miševe tretirane GalCer-om, hepatoprotektivni efekat MSCs je neutralisan dok je broj CD4+-CD49b+FoxP3- regulatornih ćelija značajno smanjen, što ukazuje da MSCs smanjuju akutno oštećenje jetre povećavajući broj CD4+CD49b+FoxP3- regulatornih ćelija posredstvom IDO-a.

Ključne reči: *mezenhimalne matične ćelije, regulatorneT ćelije, akutno oštećenje jetre.*

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INTRODUCTION

Autoimmune hepatitis, primary biliary cirrhosis, viral hepatitis, primary sclerosing cholangitis, and liver allograft rejection are induced by activated T lymphocytes, which infiltrate and destroy the liver parenchyma (1-2). Multipotent differentiation characteristics coupled to their capacity for self-renewal and capability of regulating immune responses point to mesenchymal stem cells (MSCs) as potentially new therapeutic agents for the treatment of various diseases (3). It is already known that MSCs suppress proliferation and effector functions of T lymphocytes; professional antigen presenting cells such as dendritic cells (DCs), macrophages, and B lymphocytes; and cytotoxicity of natural killer (NK) and natural killer T (NKT) cells (4-6). By suppressing immune responses in a juxtacrine or paracrine manner, MSCs attenuate liver inflammation and promote regeneration of hepatocytes. Thus, they represent promising therapeutic tools for acute liver injury (7). During recent decades, a variety of animal models have been used to study the mechanisms of MSC-based attenuation of acute hepatitis. Concanavalin A (ConA) and alpha-galactosylceramide (**α-GalCer)**-induced hepatitis are well-described experimental models of immune cell-mediated acute liver injury (8, 9). In ConA hepatitis, CD4+ T lymphocytes infiltrate the liver tissue and secrete large amounts of cytokines, such as tumour necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), interleukin (IL)-2, and granulocyte macrophage colony stimulating factor (GM-CSF) (10). CD8+ T cells, NK cells, NKT cells and macrophages can induce hepatocyte cell death by either cell-to-cell contact or in a paracrine manner, through the secretion of pro-inflammatory cytokines and reactive oxygen species (10, 11). In **α-GalCer**-induced hepatitis, liver injury is the result of interplay between DCs and NKT cells (12-13). By using these two murine models of immune-mediated acute liver failure, we have recently shown that injection of MSCs significantly reduced the hepatotoxicity of liver NKT cells and successfully increased the ratio between regulatory (NKTreg) and inflammatory IL-17 producing NKT (NKT17) cells in the injured livers (14). MSCs, through the production of indoleamine 2,3-dioxygenase (IDO), directly inhibited the secretion of inflammatory cytokines and the cytotoxic activity of NKT cells and induced increased production of IL-10 in Tregs, which in turn, in an IL-10 dependent manner, attenuated the hepatotoxicity of liver NKT cells (12,15).

Here, we describe another mechanism of MSC-based attenuation of acute liver failure relying on the interplay between MSCs and CD4+CD49b+FoxP3- regulatory cells. Our data strongly suggest that MSCs, in an IDO-dependent manner, induce expansion of hepatoprotective CD4+CD49b+FoxP3- regulatory cells that through the production of immunosuppressive IL-10 inhibit acute hepatitis.

MATERIALS AND METHODS

Cells

MSCs isolated from bone marrow of C57BL/6 mice were purchased from Gibco (catalogue no. S1502-100). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated foetal calf serum (FCS), 100 IU/mL penicillin G and 100 μg/mL streptomycin (Sigma-Aldrich, Munich, Germany), at 37°C in a 5% CO_2 incubator. MSCs in passage 4 were used throughout the experiments.

Animals

Male 6-8-week-old C57BL/6 mice were used. All animals received humane care, and all experiments were approved by and conducted in accordance with the Guidelines of the Animal Ethics Committee of the Faculty of Medical Sciences of the University of Kragujevac, Serbia. Mice were housed in a temperature-controlled environment with a 12-hour light-dark cycle and were administered standard laboratory chow and water *ad libitum.*

Con A-induced hepatitis

WT C57BL/6 mice were given a single intravenous injection of Con A (Sigma-Aldrich, St. Louis, MO) at 12 mg/kg body weight dissolved in 250 μL of saline (10). MSCs were intravenously injected (5 x 10^5 cells), via the tail vein, immediately after ConA administration (ConA+MSC-treated mice). Control animals received the appropriate amount of MSCs only or saline only. Serum levels of aspartate aminotransferase (AST) and alanine aminotransaminase (ALT) were measured 24 h after Con A administration, by a standard photometric method using the automated biochemistry analyser Olympus AU 400 (Olympus Diagnostica GMBH, Hamburg, Germany) and Olympus AU reagents, according to the manufacturer's instructions (11,16).

α-GalCer-induced hepatitis

WT C57BL/6 mice were given a single intravenous injection of α-GalCer (50 μg/kg) dissolved in 200 μL of saline (11). MSCs were intravenously injected (5 x 10^5 cells), via the tail vein, into C57BL/6 mice immediately after α-GalCer administration (α-GalCer+MSC-treated mice), while control animals received the appropriate amount of MSCs only or saline only. Serum levels of AST and ALT were measured 16 h after intravenous injection of α-GalCer (17).

Isolation of hepatic mononuclear cells and analysis with flow cytometry

The isolation of liver-infiltrating mononuclear cells was conducted as previously described (11). Hepatic mononuclear cells were screened for various cell surface and intracellular markers with flow cytometry 8 h after Con A and 2 h after α-GalCer injection. Briefly, MNC (1x10^6) were incubated with anti-mouse CD3, CD4, CD25, or CD49b mono-

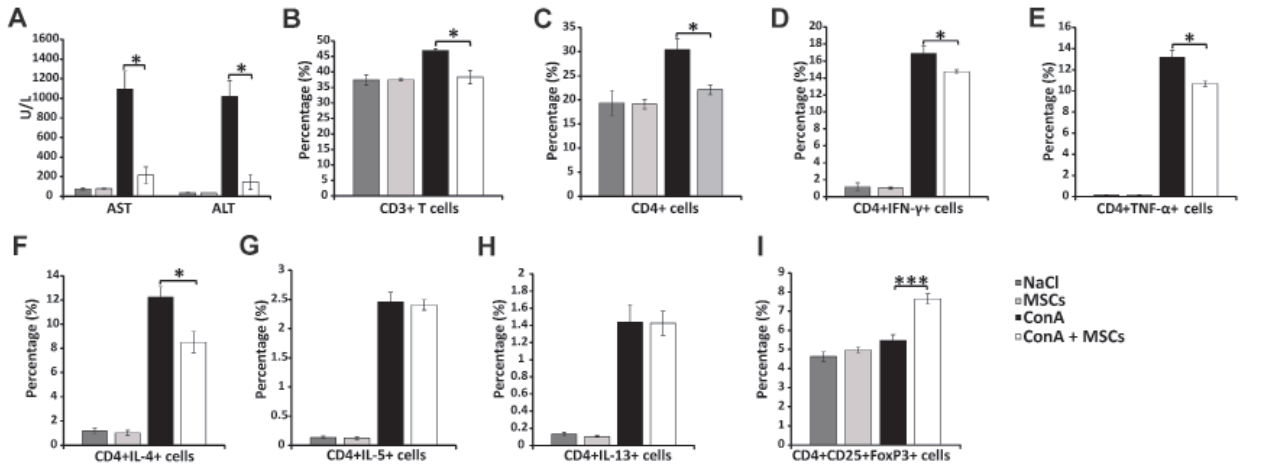

Figure 1. MSCs ameliorate Con A-induced hepatitis by reducing the total number of inflammatory CD4+ T cells and by increasing the presence of CD4+CD25+FoxP3+ T regulatory cells in the injured livers
(A) Serum levels of AST and ALT in Con A-treated mice. (B-I) Percentages of liver-infiltrating CD3+ T cells, CD4+ T cells, IFN gamma, TNFα, IL-4, IL-5, and IL-13-producing CD4+ T cells and CD4+CD25+FoxP3+ T regulatory cells. Data are presented as the mean ± SEM; n=10 mice per experimental group. **p<0.05, ***p<0.001.*

clonal antibodies conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP) or allophycocyanin (APC) (all from BD Biosciences, San Jose, CA, USA) following the manufacturer's instructions. MNCs were concomitantly stained for their intracellular content of TNF-α, IFN-γ, IL-4, IL-5, IL-10 and IL-13 by using the fixation/permeabilization kit and anti-mouse monoclonal antibodies conjugated with FITC, PE, PerCP and APC (BD Bioscience). For intracellular cytokine staining, cells were stimulated with 50 ng/mL phorbol-12-myristate-13-acetate (PMA) and 500 ng/mL ionomycin for 5 h, and GolgiStop (BD Biosciences) was added. Cells were fixed in Cytofix/Cytoperm, permeated with 0.1% saponin, and stained with fluorescent Abs. Flow cytometric analysis was conducted on a BD Biosciences FACSCalibur flow cytometer and analysed by using the Flowing software analysis program.

Generation of MSC-conditioned medium (MSC-CM)

MSCs were first cultured in serum-containing complete medium and incubated at 37°C in a humid atmosphere with 5% CO_2 in order to generate the MSC-CM. At 80% confluence, the cells were washed twice with 1X phosphate buffered saline (PBS, Invitrogen), and the medium was then changed to serum-free medium. The media were collected, 48 h later, centrifuged at 13 000×g at 4°C for 10 min and stored at -80°C until used (18).

Pharmacological inhibition of IDO

MSCs were cultured for 48 h in culture medium which contained 1 mM 1-methyltryptophan, (1-MT, Sigma-Aldrich, St-Louis, MO), a well-known inhibitor of IDO enzymatic activity (19).

Statistical analysis

Results were analysed using the Student's t test and SPSS 22.0 for Windows software (SPSS Inc., Chicago, IL). All data in this study were expressed as the mean ± standard error of the mean (SEM). Values of $p<0.05$ were considered statistically significant.

RESULTS

MSCs ameliorate acute hepatitis by reducing the capacity of liver-infiltrated CD4+T cells to produce inflammatory cytokines

MSCs efficiently attenuated Con A-induced acute liver injury as determined by liver enzyme tests (Figure 1A) and by flow cytometric analysis. Levels of serum AST and ALT were significantly lower ($p<0.05$) in Con A+MSC-treated mice compared to mice that received Con A only (Figure 1A). Flow cytometric analysis revealed a significant decrease in the percentage of CD3+ T cells ($p<0.05$), CD4+ T cells, IFN-γ-, TNF-α-, and IL-4 producing CD4+ T cells in the livers of Con A+MSC-treated mice ($p<0.05$, Fig. 1 B- F). Transplanted MSCs successfully and notably increased the presence of CD4+CD25+FoxP3+ T regulatory cells in the livers of Con A+MSC-treated mice (Fig. 1I). Since Tregs are able to attenuate acute liver failure (20,21), MSC-mediated increases of CD4+CD25+FoxP3+ T regulatory cells might be responsible for beneficial effects of MSCs. There was no significant difference in the percentage of IL-5 and IL-13 producing CD4+ cells ($p>0.05$; Fig. 1 G, H) between Con A+MSC- and Con A-only treated mice, indicating that MSCs did not induce polarization of naive T cells into effector Th2 cells.

MSCs significantly reduced acute liver injury by promoting expansion of IL-10-producing CD4+CD49b+FoxP3- regulatory cells

Similar to what we saw in Con A-induced hepatitis, the serum levels of AST and ALT were significantly reduced ($p<0.05$) in α-GalCer +MSC-treated mice compared to

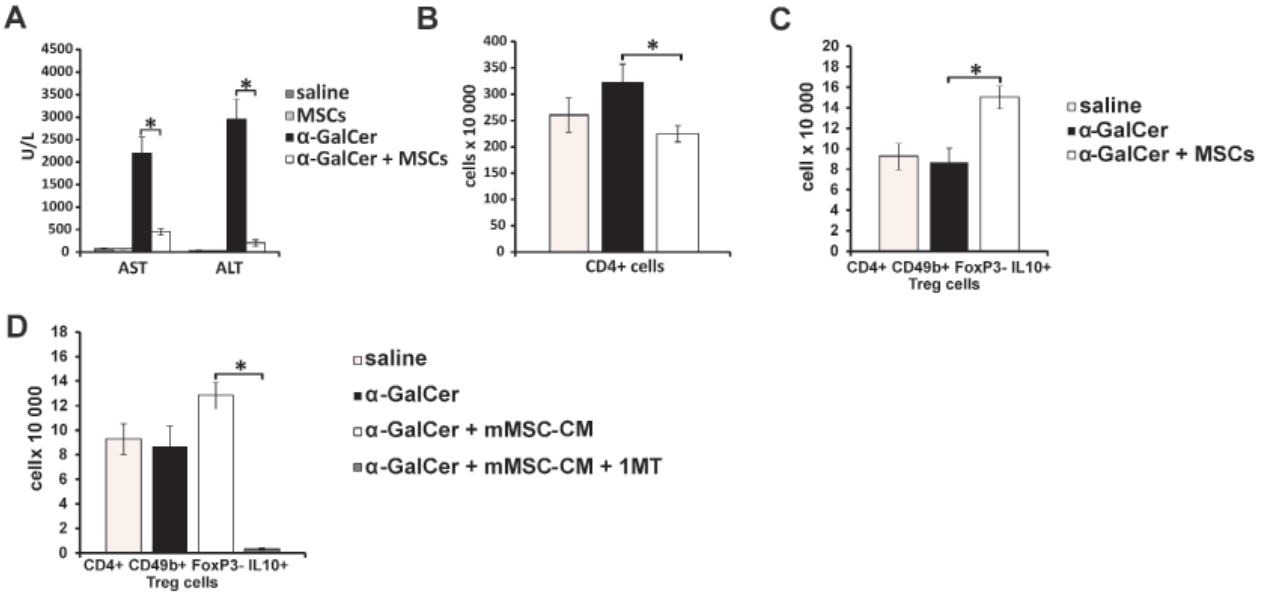

Figure 2. IDO is critically involved in MSC-based expansion of CD4+CD49b+FoxP3- T regulatory cells
(A) Serum levels of AST and ALT in α-GalCer-treated mice. (B) Total number of liver-infiltrating CD4+ T cells and (C) IL-10-producing CD4+CD49b+FoxP3- T regulatory cells in Con A and MSCs+α-GalCer-treated mice. (D) Total number of liver-infiltrating IL-10-producing CD4+CD49b+FoxP3- T regulatory cells in α-GalCer, MSC+CM+α-GalCer and 1-MT+MSC-CM+α-GalCer-treated mice. Data are presented as the mean ± SEM; n=10 mice per experimental group. *$p<0.05$.

mice that received α-GalCer only (Figure 2A). The total number of CD4+ T cells was significantly decreased in the livers of α-GalCer+MSC-treated mice compared to animals treated with α-GalCer only ($p<0.05$; Fig. 2B). Most importantly, intravenous injection of MSCs successfully significantly increased the number of CD4+CD49b+FoxP3-IL10+ cells in the livers of α-GalCer-treated mice ($p<0.05$; Fig. 2C), confirming that the beneficial effects of MSCs are at least partially reliant on MSC-dependent expansion of regulatory cells in the injured livers.

To investigate whether soluble factors were responsible for the immunomodulatory effects of MSCs and their capacity to induce expansion of regulatory cells in the livers, mice were intravenously injected with MSC-CM immediately after α-GalCer administration (α-GalCer+MSC-CM-treated mice). Similar to the effects observed after injection of MSCs, MSC-CM treatment significantly increased the total number of CD4+CD49b+FoxP3-IL10+T regulatory cells in the livers of α-GalCer treated mice ($p<0.05$; Fig. 2D), indicating that MSCs promoted the expansion of CD4+CD49b+FoxP3-IL10+T regulatory cells in a paracrine manner.

IDO is critically involved in MSC-based expansion of CD4+CD49b+FoxP3- T regulatory cells

Since we previously demonstrated the crucial importance of IDO for MSC-dependent expansion of IL-10-producing CD4+CD25+FoxP3+ Tregs in injured livers of α-GalCer-treated animals (15), we now examined the effects of IDO on MSC-dependent expansion of CD4+CD49b+FoxP3-IL10+Tregs. For this purpose, we used 1-MT, which inhibits mRNA expression of IDO in MSCs through p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) signalling (22). Intracellular staining of liver MNCs revealed significantly lower total numbers of CD4+CD49b+FoxP3-IL10+Tregs in the livers of α-GalCer+MSC-CM+1-MT-treated mice compared to α-GalCer+MSC-CM-treated animals, confirming that MSC-mediated expansion of IL-10-producing CD4+CD49b+FoxP3-regulatory cells in injured livers was IDO dependent.

DISCUSSION

In several recently published papers, we and others demonstrated the therapeutic potential of MSCs in acute liver failure (8,9,23). In this study, we introduce the first evidence that MSCs are able to attenuate hepatitis by promoting expansion of "non-classical" populations of T regulatory cells (CD4+CD49b+FoxP3-cells) in a paracrine IDO-dependent manner.

MSCs significantly reduced Con A- and α-GalCer-mediated hepatitis in C57BL/6 mice by inducing the conversion of inflammatory T cells (Fig. 1B-F) into immunosuppressive regulatory T cells (Fig. 1I), which is in line with our previous findings (3, 15, 23). We recently described the molecular mechanisms involved in the crosstalk between MSCs and regulatory cells during acute liver failure (15). MSC-dependent attenuation of α-GalCer-induced acute liver injury in mice was accompanied by an increased presence of IL-10-producing CD4+ CD25+ FoxP3+ T regulato-

ry cells and IL10- and TGFβ-producing marginal zone-like B regulatory cells in the liver (15). Among a variety of immunosuppressive factors which were produced by MSCs, the interplay between nitric oxide (NO) and IDO was responsible for MSC-based expansion of liver-infiltrated CD4+ CD25+ FoxP3+ T regulatory cells (15).

In addition to conventional CD4+CD25+Foxp3+T regulatory cells (24), another "non-classical" type of T regulatory cells, named type 1 T regulatory cells (Tr1 cells) was identified several years ago (25). Tr1 cells are found in both humans and mice and are characterized by their copious secretion of IL-10 and their lack of Foxp3 expression. Human and mouse Tr1 clones co-express CD49b, the α2 subunit of the adhesion molecule very late antigen (VLA)-2, which specifically binds to collagens I, II, and X, and lymphocyte activation gene 3 (LAG-3), which differentiates Tr1 cells from other CD4+ T cells, including Th1, Th2, Th17, and Foxp3+ T regulatory cells (26). We found that administration of MSCs attenuated α-GalCer-induced liver injury by increasing the total number of CD4+CD49b+FoxP3- Tr1 cells (Figure 2 C). Additionally, MSCs as well as MSC-CM increased the capacity of Tr1 cells to produce immunosuppressive IL-10, leading to the attenuation of acute hepatitis (Figure 2D). It is well known that Tr1 cells suppress tissue inflammation, graft-versus-host disease, and autoimmunity by producing anti-inflammatory cytokine IL-10 in an antigen-specific manner (27,28). Tr1-derived IL-10 enhances the production of human leukocyte antigen (HLA)-G5 in MSCs and promotes MSC-dependent expansion of CD4+CD25+FoxP3+ T regulatory cells (29). CD4+CD49b+FoxP3- Tr1 cells exhibit a suppressive function in a hepatitis B virus (HBV)-carrier mouse model (29) and human hepatitis C virus (HCV) infection (30). Interestingly, their suppressive effect is even higher than that of the classical CD4+CD25+FoxP3+ T regulatory cells (31). It has been reported that naïve T cells can be induced to become Foxp3-Tr1 cells by IL-10, and these Foxp3-Tr1 cells secrete high levels of IL-10 and TGF-β to modulate the inflammatory microenvironment.

It has been elucidated that IDO promotes the degradation of tryptophan into kynurenine and toxic metabolites (quinolinic acid and 3-hydroxy-anthranillic acid), which suppress proliferation or induce apoptosis of T cells through activation of the stress response kinase GCN2 (32). Additionally, IDO triggers the production of immunosuppressive IL-10 in activated lymphocytes, while 1-MT significantly impairs the capacity of stimulated lymphocytes to secrete IL-10 (33). In our recently published paper we demonstrated that MSC-derived IDO is crucially important for the expansion of IL-10-producing CD4+CD25+FoxP3+ T regulatory cells and consequent attenuation of acute liver injury (12). In line with these findings, here we demonstrated that IDO inhibition completely abrogated the capacity of MSC-CM to induce expansion of IL-10-producing CD4+CD49b+FoxP3- Tr1 cells (Figure 2 D) and to attenuate acute hepatitis.

In conclusion, the capacity of MSCs to promote expansion of immunosuppressive CD4+CD49b+FoxP3- Tr1 cells in an IDO-dependent manner should be explored in future studies as new therapeutic approach for the treatment of liver diseases which are mediated by T cells.

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CONFLICT OF INTEREST

None.

REFERENCES:

1. Zhou Y, Dai W, Lin C, Wang F, He L, Shen M, Chen P, Wang C, Lu J, Xu L, XuX,Guo C. Protective effects of necrostatin-1 against concanavalin A-induced acute hepatic injury in mice. Mediators Inflamm. 2013;2013:706156.
2. Eggink HF, Houthoff HJ, Huitema S, Gips CH, Poppema S. Cellular and humoral immune reactions in chronic active liver disease. I. Lymphocyte subsets in liver biopsies of patients with untreated idiopathic autoimmune hepatitis, chronic active hepatitis B and primary biliary cirrhosis. ClinExpImmunol. 1982;50:17-24.
3. Volarevic V, Arsenijevic N, Lukic ML, Stojkovic M. Concise review: Mesenchymal stem cell treatment of the complications of diabetes mellitus. Stem Cells. 2011 (1):5-10.
4. Li N, Hua J. Interactions between mesenchymal stem cells and the immune system. Cell Mol Life Sci. 2017;74:2345-2360.
5. Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC,Largaespada DA, Verfaillie CM. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature. 2002;418:41-9.
6. Spaggiari GM, Capobianco A, Abdelrazik H, Becchetti F, Mingari MC, Moretta L. Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2. Blood. 2008;111:1327-33.
7. Nikolic A, SimovicMarkovic B, Gazdic M, Randall Harrell C, FellabaumC,Jovicic N, Djonov V, Arsenijevic N, L Lukic M, Stojkovic M, VolarevicV.Intraperitoneal administration of mesenchymal stem cells ameliorates acute dextran sulfate sodium-induced colitis by suppressing dendritic cells. Biomed Pharmacother. 2018;100:426-432.
8. Biburger M, Tiegs G. Alpha-galactosylceramide-induced liver injury in mice is mediated by TNF-alpha but independent of Kupffer cells. J Immunol. 2005;175:1540-50.

9. Tiegs G, Hentschel J, Wendel A. A T cell-dependent experimental liver injury in mice inducible by concanavalin A. J Clin Invest. 1992;90:196-203.
10. Volarevic V, Misirkic M, Vucicevic L, Paunovic V, SimovicMarkovicB,Stojanovic M, Milovanovic M, Jakovljevic V, Micic D, Arsenijevic N, Trajkovic V, Lukic ML. Metformin aggravates immune-mediated liver injury in mice. Arch Toxicol. 2015;89:437-50.
11. Volarevic V, Mitrovic M, Milovanovic M, Zelen I, Nikolic I, MitrovicS,Pejnovic N, Arsenijevic N, Lukic ML. Protective role of IL-33/ST2 axis in Con A-induced hepatitis. J Hepatol. 2012;56:26-33.
12. Gazdic M, SimovicMarkovic B, Vucicevic L, Nikolic T, Djonov V, ArsenijevicN,Trajkovic V, Lukic ML, Volarevic V. Mesenchymal stem cells protect from acute liver injury by attenuating hepatotoxicity of liver natural killer T cells in an inducible nitric oxide synthase- and indoleamine 2,3-dioxygenase-dependent manner. J Tissue EngRegen Med. 2018;12:e1173-e1185.
13. Biburger M, Tiegs G. Alpha-galactosylceramide-induced liver injury in mice is mediated by TNF-alpha but independent of Kupffer cells. J Immunol. 2005;175:1540-50.
14. Milosavljevic N, Gazdic M, SimovicMarkovic B, Arsenijevic A, NurkovicJ,Dolicanin Z, Djonov V, Lukic ML, Volarevic V. Mesenchymal stem cells attenuate acute liver injury by altering ratio between interleukin 17 producing and regulatory natural killer T cells. Liver Transpl. 2017;23:1040-1050.
15. Gazdic M, Markovic BS, Arsenijevic A, Jovicic N, Acovic A, Harrell CR,Fellabaum C, Djonov V, Arsenijevic N, Lukic ML, Volarevic V. Crosstalk between mesenchymal stem cells and T regulatory cells is crucially important for the attenuation of acute liver injury. Liver Transpl. 2018;24:687-702.
16. Volarevic V, Milovanovic M, Ljujic B, Pejnovic N, Arsenijevic N, Nilsson U, Leffler H, Lukic ML. Galectin-3 deficiency prevents concanavalin A-induced hepatitis in mice. Hepatology. 2012;55:1954-64.
17. Volarevic V, Markovic BS, Bojic S, Stojanovic M, Nilsson U, Leffler H, Besra GS, Arsenijevic N, Paunovic V, Trajkovic V, Lukic ML. Gal-3 regulates the capacity of dendritic cells to promote NKT-cell-induced liver injury. Eur J Immunol. 2015;45:531-43.
18. Linero I, Chaparro O. Paracrine effect of mesenchymal stem cells derived from human adipose tissue in bone regeneration. PLoS One. 2014;9:e107001.
19. Yang SH, Park MJ, Yoon IH, Kim SY, Hong SH, Shin JY, Nam HY, Kim YH, Kim B, Park CG. Soluble mediators from mesenchymal stem cells suppress T cell proliferation by inducing IL-10. ExpMol Med. 2009;41:315-24.
20. Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood. 2005;105:1815-22.
21. Milosavljevic N, Gazdic M, SimovicMarkovic B, Arsenijevic A, NurkovicJ,Dolicanin Z, Jovicic N, Jeftic I, Djonov V, Arsenijevic N, Lukic ML, Volarevic V. Mesenchymal stem cells attenuate liver fibrosis by suppressing Th17 cells – an experimental study. Transpl Int. 2018;31:102-115
22. Lim JY, Im KI, Lee ES, Kim N, Nam YS, Jeon YW, Cho SG. Enhanced immunoregulation of mesenchymal stem cells by IL-10-producing type 1 regulatory T cells in collagen-induced arthritis. Sci Rep. 2016;6:26851.
23. Volarevic V, Nurkovic J, Arsenijevic N, Stojkovic M. Concise review: Therapeutic potential of mesenchymal stem cells for the treatment of acute liver failure and cirrhosis. Stem Cells. 2014;32:2818-23.
24. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science. 2003;299:1057-61.
25. Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE, Roncarolo MG. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature. 1997;389:737-42.
26. Gagliani N, Magnani CF, Huber S, Gianolini ME, Pala M, Licona-Limon P, et al. Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. Nature medicine. 2013; 19:739–46.
27. Ahangarani RR, Janssens W, VanderElst L, Carlier V, VandenDriessche T, Chuah M, Weynand B, Vanoirbeek JA, Jacquemin M, Saint-Remy JM. In vivo induction of type 1-like regulatory T cells using genetically modified B cells confers long-term IL-10-dependent antigen-specific unresponsiveness. J Immunol. 2009;183:8232-43.
28. Xu L, Yin W, Sun R, Wei H, Tian Z. Liver type I regulatory T cells suppress germinal center formation in HBV-tolerant mice. ProcNatlAcadSci U S A. 2013;110:16993-8.
29. Nasef A, Mazurier C, Bouchet S, François S, Chapel A, Thierry D, Gorin NC, Fouillard L. Leukemia inhibitory factor: Role in human mesenchymal stem cells mediated immunosuppression. Cell Immunol. 2008;253:16-22.
30. Carpentier A, Conti F, Stenard F, Aoudjehane L, Miroux C, Podevin P, Morales O, Chouzenoux S, Scatton O, Groux H, Auriault C, Calmus Y, Pancre V, DelhemN. Increased expression of regulatory Tr1 cells in recurrent hepatitis C after liver transplantation. Am J Transplant. 2009;9:2102-12.
31. Wildbaum G, Netzer N, Karin N. Tr1 cell-dependent active tolerance blunts the pathogenic effects of determinant spreading. J Clin Invest. 2002;110:701-10
32. Munn DH, Sharma MD, Baban B, Harding HP, Zhang Y, Ron D, Mellor AL. GCN2 kinase in T cells mediates proliferative arrest and anergy induction in response to indoleamine 2,3-dioxygenase. Immunity. 2005;22:633-42.
33. Eleftheriadis T, Pissas G, Karioti A, Antoniadi G, Liakopoulos V, Dafopoulou K, Pournaras S, Koukoulis G, Stefanidis I. The indoleamine 2,3-dioxygenase inhibitor 1-methyl-tryptophan suppresses mitochondrial function, induces aerobic glycolysis and decreases interleukin-10 production in human lymphocytes. Immunol Invest. 2012;41:507-20.

AN OVERVIEW OF PHARMACOLOGICAL AND NON-PHARMACOLOGICAL TREATMENT AS A USEFUL TOOL FOR THE PROTECTION FROM CARDIOTOXICITY OF ANTINEOPLASTIC DRUGS

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PREGLED FARMAKOLOŠKIH I NEFARMAKOLOŠKIH TRETMANA U PREVENCIJI KARDIOTOKSIČNOSTI USLED PRIMENE ANTINEOPLASTIČNIH LEKOVA

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ABSTRACT

Unfortunately, in patients with cancer disease, clinical application of antineoplastic drug results in severe side effects of cardiotoxicity.

We aim to review the research focused on elimination or reduction of antineoplastic drug-induced cardiotoxicity without affecting its anticancer efficacy by different agens.

This study is based on pertinent papers that were retrieved by a selective search using relevant keywords in PubMed and ScienceDirect. Based on mentioned purpose, various strategies were investigated and proposed, and thousands of compounds were screened. The literature mainly focusing on drugs, natural products and herb extracts with therapeutic efficacies as well as non-pharmacological treatment against differently induced cardiotoxicity during treatment in patients with cancers.

Larger future studies are necessary to reach a point of secure cytostatic therapy, improved patient survival and quality of life. Until that moment, baseline and serial cardiac evaluation is recommended to facilitate early identification and treatment of cardiotoxicity.

Keyword: *cardiotoxicity, anti-neoplastic therapy, pharmacological, herbal, natural cardioprotection, physical exercise, heart.*

SAŽETAK

Nažalost, kod pacijenata sa obolelih od karcinoma, klinička primena antineoplastičnih lekova rezultiraozbiljnim neželjenim efektima i kardiotoksičnošću.

Cilj ovog preglednog rada je sveobuhvatan prikaz informacija a koje su usmerene na eliminaciju ili smanjenje kardiotoksičnosti izazvane antineoplastičnim lekovima i bez uticaja na njegovu efikasnost protiv raka različitim agensima.

Ova studija zasnovana je na relevantnim i dostupnim radovima koji su preuzeti selektivnom pretragom koristeći relevantne ključne reči u PubMed i ScienceDirect-u. U vezi sa ciljem rada, u prethodnim studijama istražene su i predložene razne strategije, a na hiljade jedinjenja je prikazano. Literaturni podaci se fokusiraju na lekove, prirodne proizvode i ekstrakte biljaka sa terapijskim efektima, kao i na nefarmakološkom tretmanu indukovane kardiotoksičnosti tokom lečenja kod pacijenata sa kancerom.

Opsežnije buduće studije su neophodne da bi se postigla tačka sigurne citostatske terapije, bolje opšte stanje pacijenta i kvalitet života. Do tog trenutka, preporučuje se osnovna i obavezna procena funkcije srca kako bi se olakšala rana identifikacija i lečenje kardiotoksičnosti.

Ključne reči: *kardiotoksičnost, anti-neoplastična terapija, farmakološka, biljna, prirodna kardioprotekcija, vežbanje, srce.*

INTRODUCTION

Last decades, cancer has become one of the leading causes of death worldwide in both sexes, with significant geographic variations in frequency and distribution. In 2012, an estimated 14.1 million new cases of cancer occurred worldwide and worldwide there will be 23.6 million new cases of cancer each year by 2030 (1). According to previosly published epidemiology results, Serbia is the country with the highest cancer mortality in the world last years. In the period from 1991 to 2015, approximately 266,000 males and 200,000 females died from cancer in Serbia (2).

In that sense, recently data emphaze that the 28% of patients diagnosed with cancer (all cancers combined) in England in 2013-2014 had curative or palliative chemotherapy, as part of their primary cancer treatment (2). This includes patients who had chemotherapy alone, and those

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Table 1. Antineoplastic Agents Associated With Cardiotoxicity (4, 5, 7-9, 12, 13).

Antineoplastic agent	*Major cardiac side effect*	*Incidence*
Daunorubicin/doxorubicin	*Acute/chronic CHF*	*18%–65%*
Cyclophosphamide/ifosfamide	*Myocarditis, CHF*	*17%-25%*
Paclitaxel/docetaxel	*Hypotension, hypertension, bradycardia, atrial and ventricular arrhythmia*	*0.5%*
Fluorouracil	*MI,angina, hypotension, coronary vasospasm*	*1.6%–68%*
Rituximab	*Hypotension, hypertension, arrhythmia*	*25%*
Arsenic trioxide	*QT prolongation, tachycardia*	*8%–55%*
Trastuzumab	*CHF*	*7%–28%*
Bevacizumab	*CHF*	*4%-6%*
Etoposide	*MI, hypotension*	*1%–2%*
Vinca alkaloids	*MI,autonomic cardioneuropathy*	*25%*
Pentostatin	*MI, CHF, acute arrhythmia*	*3%-10%*
Cytarabine	*Arrhythmia, pericarditis, CHF*	*Unknown*
Interferon (at high doses)	*Arrhythmia, dilated cardio-myopathy, ischemic heart disease*	*Unknown*
Busulfan	*Endocardial fibrosis*	*Unknown*
Cisplatin	*Acute MI*	*Unknown*
Thalidomide	*Pulmonary hypertension*	*Unknown*

who also had other treatments such as tumour removal surgery or radiotherapy (3).

Because of widely used drugs, it is important to patients well enought tolerate the treatment. More than 100 chemotherapy or chemo drugs are used to treat cancer – either alone or in combination with other drugs or treatments. These drugs are very different in their chemical composition, how they are taken, their usefulness in treating specific forms of cancer, and their side effects (4). Cancers treatment by chemo-drug induce different side effects on various organ system, from central nervous to cardiovascular, gastrointestinal, skin to fertility and endocrinological problems (5, 6).

This review is aimed to introduces and briefly summarizes the information about present and possible pharmacological and non-pharmacological treatment as a useful tool to for the protection from chemotherapeutic drug-indiced cardiotoxicity.

Cardiac toxicity as an chemo-drug`s side effect: Incidence, Pathophysiology and Mechanisms

An ever-increasing array of chemotherapeutic agents is being used in the treatment of solid organ or hematologic malignancies. The success of many of these agents has led to an increasing survival of patients with cancer. However, many of these agents, particularly anthracyclines and trastuzumab, are associated with the development of cardiotoxicity (6, 7).

Cardiotoxicity is one of the most important adverse reactions of chemotherapy, leading to an important increase of morbidity and mortality (7, 8). The most studied chemotherapeutic agents associated with adverse cardiac events are anthracyclines (Doxorubicin), used in the treatment of many adult malignancies like breast cancer, sarcoma, lymphoma, or gynecological cancer. They also play an important role in the treatment of childhood cancers, anthracyclines are currently used in more than 50% of regimens contributing to the overall survival rates in excess of 75% (6-8). Other cytostatics more frequently correlated with cardiotoxic side effects are taxanes (paclitaxel, docetaxel), alkylating agents (Carboplatin, Cisplatin, Cyclophosphamide), small molecule tyrosine kinase inhibitors (lapatinib, imatinib, sorafenib, sunitinib) and trastuzumab, a monoclonal antibody directed against the human epidermal growth factor receptor-2 (HER2), used in the treatment of metastatic breast neoplasm (Table 1).

Cardiotoxicity can appear early or late in the course of the disease, and may vary from subclinical myocardial dysfunction to irreversible heart failure or even death (9). This definition refers to a direct effect of the chemotherapy on the entire cardiovascular system, but also to an indirect effect due to a thrombogenic status or to a hemodynamic flow alteration (10-13).

A committee of the cardiac review and evaluation supervising trastuzumab clinical trials clinically defined chemotherapy-induced cardiotoxicity as one or more of the following: 1) reduction of LVEF, either global or specific in the interventricular septum; 2) symptoms or signs associated with heart failure (HF); 3) reduction in LVEF from baseline ≤ to 5% to <55% in the presence of signs or symptoms of HF, or a reduction in LVEF ≥10% to <55% without signs or symptoms of HF (9-13).

Accumulated data revealed that oxidative stress, iron metabolism, inflammation, and other mechanisms participate in this multifactorial process. A hallmark of anthracycline-induced chronic cardiotoxicity is the reduction of left ventricular wall thickness due to the loss of cardiomyocytes, resulting in restricted LVEF. Anthracycline-induced cardiomyocyte cell death is likely mediated through caspase-3-related apoptotic pathways activated by p53 and/or TNF-signalling (11). The trigger stimuli ultimately causing cardiomyocyte cell death are uncertain and controversially discussed. Suggested mechanisms for the development of cardiomyopathy include accumulation of toxic metabolites (e.g., doxorubinicol), autophagy, production of peroxynitrite and ROS, TOP2B inhibition, and disruption of mitochondrial homeostasis/integrity (12, 13).

Pharmacological treatment of chemotherapeutic-induced cardiotoxicity

Dexrazoxane

Dexrazoxane is a cardioprotective agent which was discovered by Kurt Hellmann in 1972 (14). In July 2011 the US Food and Drug Administration released a statement restricting use only in adult patients with cancer who have received >300 mg/m^2 doxorubicin or >540 mg/m^2 epirubicin and general approval for use for cardioprotection (14). As the use of the only clinically approved cardioprotectant dexrazoxane has been limited, there is an urgent need for alternative cardioprotective measures. Approved indication for its use is extravasation of anthracyclines, and because the number of patients who have extravasation of anthracyclines is low, the condition is considered 'rare', dexrazoxane was designated an 'orphan medicine' (15).

As a derivative of ethylene diamine tetra acetic acid (EDTA), dexrazoxane chelates iron and thus reduces the number of metal ions complexed with anthracycline and, consequently, decrease the formation of superoxide radicals (16). The exact chelation mechanism is unknown, but it has been postulated that dexrazoxane can be converted into ring-opened form intracellularly and interfere with iron-mediated free radical generation that is in part thought to be responsible for anthracycline induced cardiomyopathy (16, 17).

Renin-angiotensin-aldosterone system antagonists

RAS involvement in the pathophysiology of chemotheraputic drug-mediated cardiac dysfunction has raised the question as to whether the prophylactic use of RAS antagonists could potentially mitigate these cardiotoxic effects. Previous basic science studies have demonstrated that the prophylactic administration of angiotensin converting enzyme inhibition (ACEI), including Captopril, Enalapril, and Lisinopril, was partially cardioprotective in both acute and chronic animal models of DOX induced cardiomyopathy (18, 19). In a rabbit model of DOX mediated cardiomyopathy, 1 mg/kg/day oral Lisinopril for a total of 10 weeks attenuated cardiomyocyte loss and ANP mRNA expression, in comparison to rabbits receiving DOX alone (20). Furthermore, intragastric administration of Captopril (10 mg/kg) or Enalapril (2 mg/kg) for 7 days resulted in a decline in lipid peroxidation, and enzymatic indicators of acute cardiac toxicity in a rat model of DOX induced cardiomyopathy (20, 21).

The therapeutic benefit closely depends on the improvement of left ventricular function. The ACE inhibitor enalapril and the beta-blocker carvedilol are the most effective drugs in achieving normalization of anthracycline-caused decrease in LVEF. Due to these promising therapeutic results, a preventive study was initiated. In the OVERCOME trail, 42% of the patients showed a preservation of LVEF by prophylactic enalapril and carvedilol treatment, and 10% of patients responded partially (22). However, these cardioprotective effects are less marked than in the case of dexrazoxane-based prevention.

Statins

Besides the most common hypothesis that anthracycline-induced congestive heart failure (CHF) is mainly caused by generation of reactive oxygen species (7-11), recent data point to a critical role of topoisomerase II beta (TOP2B), which is a primary target of anthracycline poisoning, in the pathophysiology of CHF (12, 14). Statins are anti-inflammatory and anti-oxidative drugs that are clinically well established for the prevention of cardiovascular diseases. They exhibit pleiotropic beneficial properties beyond cholesterol-lowering effects that most likely rest on the indirect inhibition of small Ras homologous (Rho) GTPases. The Rho GTPase Rac1 has been shown to be a major factor in the regulation of the pro-oxidative NADPH oxidase as well as in the regulation of type II topoisomerase.

Riad et al. suggested both anti-oxidative and anti-inflammatory effects of statins to contribute to cardioprotection. The statin enhanced SOD_2 levels, reduced caspase-3-mediated apoptosis and mitigated cardiac inflammation following doxorubicin treatment (23). Regarding their anti-inflammatory properties, statins, predominately atorvastatin, simvastatina and rosuvastatin, are described to inhibit nuclear translocation of Nf-kappaB by RhoA/ROCK inhibition, in vitro (24). Huelsenbeck et al demonstrated that a statin co-treatment attenuates acute anthracycline-induced cardiotoxicity in BALB/c mice as mirrored by reduced mRNA levels of pro-fibrotic and pro-inflammatory cytokines. It also protected from doxorubicin-induced sub-acute cardiac damage (25). In a similar study, atorvastatin protected mice from doxorubicin-induced DNA damage, lipid peroxidation and glutathione depletion (26).

Taken together, attenuation of Rho GTPase signalling seems to mainly contribute to the anti-atherosclerotic properties of statins and might also be of relevance beyond the maintenance of cardiovascular health (27).

β-blockers

Non-selective beta blockers (metoprolol, carvedilol and nebivolol) are cardioprotective drugs which could be effective into prevent chemotherapy-induced left ventricular systolic dysfunction (LVSD) in patients with hematological malignancies. In a randomized controlled trial of 50 patients in whom anthracycline therapy was planned, a 10% drop in LVEF occurred in most of the 25 placebo recipients in the study, although LVEF remained >50% in many of them. LVEF was preserved in the vast majority of the 25 patients randomized to receive carvedilol, demonstrating its protective effect. LVEF declined to <50% in only one carvedilol patient but in five of the controls (28).

This finding was confirmed in a later study randomizing patients to carvedilol and enalapril or placebo prior to starting anthracycline-based therapies. A significantly lower rate of death, heart failure, or final LVEF <45% occurred among the group receiving dual therapy versus placebo (6.7% versus 24.4%, p=0.02) (29). In the largest clinical trial of β-blockers for prevention of cardiotoxicity conduceted by Mônica Samuel et al (30), under contemporary anthracycline chemotherapy dosage, the authors noted a 13.5% to 14.5% incidence of cardiotoxicity. In this scenario, carvedilol had no impact on the incidence of early onset of LVEF reduction. However, the use of carvedilol resulted in a significant reduction in troponin levels and diastolic dysfunction (30, 31). The benefit of the use of pre-chemotherapy beta-blockers for prevention of chemo-induced cardiotoxicity remains unclear still. It is possible that the anti-oxidant effects of specific beta-blockers is what is preventing the toxic effects of anthracyclines/trastuzumab and not the beta-blockade itself.

Calcium channel blockers

Calcium channel blockers have a various potential beneficial effect which could be a treatment tool in preventing of cardiotoxicity induced by antineoplastic drugs. Well, we know that these drugs induce vasodilation of blood vessels and have anti-ischemic potential. Because of that, in an attempt to reduce the adverse cardiac effects, prophylaxis with a calcium channel blocker was therefore tested in a similar group of patients receiving similar induction chemotherapy.

Calcium channel blockers have previously been used as prophylaxis during 5-fluorouracil (5-FU) treatment only in a limited number of patients. These attempts have so far yielded conflicting results. A combination of nifedipine and isosorbide-dinitrate was found ineffective in the prevention of 5-FU cardiotoxicity in two patients reported by Escudier et al (32). Furthermore, verapamil did seem to modify the adverse cardiac effects of 5-FU by preventing arrhythmia (32). Also, one key and simple approach to monitor the effects of chemotherapy is arterial pressure measurement to identify hypertension. Hypertension is frequently seen in patients who are treated with several antiangiogenic agents (such as bevacizumab, sorafenib, and sunitinib) and can be severe (33). Hypertension in the cancer patient under therapy needs to be promptly and adequately treated, and calcium channel blockers can be a potential preventive therapy (33).

Natural and herbal products as treatment of chemotherapeutic-induced cardiotoxicity

As is known, antioxidants may neutralize free radicals generated by anthracyclines and potentially reduce cardiotoxicity. In that sense, natural antioxidants such as vitamin E, vitamin C, carotenoids,vitamin A, coenzyme Q, flavonoids, antioxidant components of virgin olive oil and selenium from plants are in focus laste decades in preventing of cardiovascular disorders.

Vitamin E and A as an antioxidants can protect from both acute and chronic cardiotoxicity caused by DOX, and it increases antioxidant capacity in the heart. With the aim of testing the cardioprotective effect of vitamin E in doxorubicin- induced acute cardiotoxicity in rats, Puri et al pretreated them with a high dose of vitamin E intraperitoneally followed by DOX (34).

The results show that vitamin E pre-treatment prevents the electrocardiographic changes caused by doxorubicin; moreover, it helps to lower the levels of creatine phosphokinase and lactate dehydrogenase raised by DOX. At high doses (>90 mg/kg), vitamin E also reduces lipid peroxidation and chromosomal aberrations (34).

Vitamin C (ascorbic acid) is an effective water soluble antioxidant against lipid peroxidation, scavenging ROS in the aqueous fraction before these molecules can give rise to lipid oxidation. Vitamin A and C also have a protective dose-dependent effect against the chromosomal aberrations induced by doxorubicin (35, 36).

Coenzyme Q (CoQ), or ubiquinone, plays a critical role in the mitochondrial respiratory chain, acting as a redox link between flavo-proteins and cytochromes, being an essential component in extramitochondrial redox chains. Its concentration in blood and tissues depends on biologic requirements, endogenous biosynthesis, and of course the dietary intake. Preclinical studies have shown that both supplementation and treatment with CoQ10 prior to DOX administration decreases lipid oxidation and heart toxicity without interfering with the anti-tumour activity of DOX. Clinical studies have also shown oral CoQ10 to have a protective effect against the chronic cardiotoxicity induced by anthracyclines (37, 38).

Flavonoids, polyphenols, and other natural antioxidants also is investigated as a potential beneficial factors in preventing various drug induced-cardiotoxicity. Flavonoids are characterized by high antioxidant power, and have been considered potential protectors against the chronic cardiotoxicity associated with DOX This protective effect of flavonoids is closely related to their antioxidant, iron chelating and carbonyl reductase 1 (CBR1)-

inhibitory properties (39). The flavonoid inhibits negative cardiac effects in a dose-dependent manner, in accordance with the essential properties of all flavonoids, i.e. their iron chelating and antioxidant characteristics (39, 40).

Other flavonoids, such as catechins, have cardioprotective properties at low doses, exhibiting an iron chelating activity. Quercetin, in addition to its high antioxidant capacity, can inhibit TOP2 and intercalate into DNA strands, thereby boosting the anti-tumour effect. Oral garlic supplementation decreases the oxidative stress provoked by chronic administration of DOX, and protects against free radicals, improving the clinical efficacy of adriamycin (41). Moreover, chronic garlic administration (250 and 500 mg/kg daily, orally, for 30 days) has been shown to prevent acute adriamycin- induced cardiotoxicity and decreases myocardial TNFa expression (42).

Furthermore, genistein, a soy isoflavone with high antioxidant capacity, can increase cellular antioxidant status by scavenging ROS and augmenting the activity of antioxidant enzymes like glutathione peroxidase, glutathione reductase (43).

The protective role of resveratrol, polyphenolic compound against DOX cardiotoxicity is being studied. It is known that pre-treatment with resveratrol and subsequent treatment with doxorubicin in H9c2 cardiomyocytes protects against the toxicity generated by DOX and can decrease the intracellular accumulation of ROS induced by xanthine oxidase/ xanthine (44).

Weak protective activity of selenium has also been reported against the nephrotoxicity (46) and hepatotoxicity (45) induced by DOX in rats. Finally, a recent study shows that a commercial mixture of vitamins (C, E and b-carotene) and minerals (copper, selenium and zinc) administered to Drosophila melanogaster larvae treated with DOX, was not genotoxic and it also protected against the genotoxic effects of chemotherapeutic agents (45, 46).

This cardioprotective effect of oleuropein (47) and curcumin (48) also was investigated, and results indicate that these agents could be beneficial in CVD-preventing, but further investigations are necessary to confirm this.

Non-Pharmacological treatment of chemotherapeutic-induced cardiotoxicity

Hyperbaric oxygen therapy (HBOT)

The relatively high levels of oxygen deliverable with HBO make this approach attractive, and the results of many studies support the hypothesis that HBO reduces the radioresistance of certain types of tumors. So, it is very difficult to find clear borderline between toxic and preventive dose od oxygen in patients with antioneplastic drugs treatment. Hyperbaric oxygen (HBO) therapy was studied in two animal models. In one animal study HBO was found to potentiate the cytotoxicity of doxorubicin due to its free-radical formation properties (49). The second animal study showed a beneficial effect in ulcer healing compared to mice that received no HBO therapy (50).

Karagoz et al investigated the effects of HBOT on DOX-induced cardiotoxicity in rats. Well, Wistar rats were treated with either HBO2 or doxorubicin or a combination of both treatments for 4 consecutive weeks and followed up for an additional 4 weeks. Cardiomyopathy was evaluated using two-dimensional and M-mode echocardiography at baseline, at the fourth, sixth and eighth weeks, and by histopathological investigation of the rat hearts at the eighth week. The concluded that HBOT markedly reduced ejection fraction and fractional shortening, this reduction was significantly less than that of doxorubicin treatment and attenuated doxorubicin-induced histopathological changes in rat hearts (51).

Other studies, concluded that HBO_2 therapy does not potentiate doxorubicin-induced cardiotoxicity in rats and that potential cardioprotection conferred by HBO_2 against doxorubicin warrants further investigation (51, 52).

Physical exercise

Regular and vigorous physical exercise has been scientifically established as providing strong preventative medicine against cancer with the potential to reduce incidence by 40% (53). It is well established that exercise capacity is an important prognostic factor for survival in cancer and non-cancer populations (53, 54).

There has been growing interest in evaluating the benefits of exercise training to improve oncologic outcomes. The use of exercise therapy to reduce cardiotoxicity in patients undergoing chemotherapy has yielded mixed results. Potential mechanisms by which exercise influence on cardiac function are mitigates the multiple molecules and signaling pathways, such as oxidative stress, iron metabolism, and inflammation, which are associated with chemotherapeutic drug -induced cardiotoxicity (53).

In a prospective study, 2973 women undergoing treatment for nonmetastatic breast cancer were asked to complete a questionnaire about their leisure time physical activity (55). Women who exercised for ≥9 metabolic equivalent task (MET)-hours/week had a 23% reduction in the risk of cardiovascular events, including heart failure, compared to those who exercised <9 MET-hours/week. Conversely, an uncontrolled study showed that aerobic exercise as an adjunctive therapy with trastuzumab did not prevent left ventricular dilation or a reduction in LVejection fraction (56).

Physical exercise at different intensities performed before, during, or after chemotherapy treatment increases cardiovascular reserve, reduces cardiotoxicity in mouse models,and increases peak Vo2 in patients treated with doxorubicin and cyclophosphamide (55, 56). However, a small study reported no beneficial effects on LVEF during adjuvant trastuzumab treatment (57). Ongoing small trials are currently studying the effect of different levels of training and the preventive efficacy of exercise hours before every chemotherapy cycle.

While the role of exercise therapy to improve chemotherapy-induced cardiovascular outcomes has been promising in animal models, large-scale randomized control trials are needed to evaluate its effectiveness in preventing anthracycline-induced cardiomyopathy in cancer patients (56-59).

Summary and perspectives

Chemotherapy-induced cardiotoxicity include a combination of mechanisms which influence several intracellular signaling cascades, critical to both cancer progression and the normal functioning of the heart. Larger future studies are necessary to reach a point of secure cytostatic therapy, improved patient survival and quality of life. Until that moment, baseline and serial cardiac evaluation is recommended to facilitate early identification and treatment of cardiotoxicity.

Conflict of interests

None.

REFERENCES

1. Cancer Research UK, http://www.cancerresearchuk.org/health-professional/cancer-statistics, Accessed: May, 2018.
2. Ilic M, Ilic I. Cancer mortality in Serbia, 1991-2015: an age-period-cohort and joinpoint regression analysis. Cancer Commun (Lond). 2018 Apr 10;38(1):10.
3. Albini A, Pennesi G, Donatelli F, Cammarota R, De Flora S, Noonan DM. Cardiotoxicity of anticancer drugs: the need for cardio-oncology and cardio-oncological prevention. J Natl Cancer Inst. 2010;102(1):14-25.
4. Vignot S, André T, Caux C, Bouleuc C, Evrard S, Gonçalves A, Lacroix M, Magné N, Massard C, Mazeron JJ, Orbach D, Rodrigues M, Thariat J, Wislez M, L'Allemain G, Bay JO. [Hot topics in 2017 in oncology and hematology. A selection by the editorial board of Bulletin du Cancer]. Bull Cancer. 2018;105(1):6-14.
5. Pai VB, Nahata MC. Cardiotoxicity of chemotherapeutic agents: incidence, treatment and prevention. Drug Saf. 2000;22(4):263-302.
6. Albini A, Pannesi G, Donatelli F, et al. Cardiotoxicity of anticancer drugs: the need for cardio-oncology and cardio-oncological prevention. J Natl Cancer Inst. 2010;102:14–25.
7. Brana I, Tabernero J. Cardiotoxicity. Ann Oncol. 2010;21:173–179.
8. Cardinale D, Colombo A, Lamantia G, et al. Anthracycline-induced cardiomyopathy: Clinical relevance and response to pharmacologic therapy. JACC. 2010;55:213–220.
9. Steinherz LJ, Steinherz PG, Tan CT, et al. Cardiac toxicity 4 to 20 years after completing anthracicline therapy. JAMA. 1991;266:1672–1677.
10. Stevens PL, Lenihan DJ. Cardiotoxicity due to Chemotherapy: the Role of Biomarkers. Curr Cardiol Rep. 2015 Jul;17(7):603.
11. ltena R, Perik PJ, van Veldhuisen DJ, de Vries EG, Gietema JA. Cardiovascular toxicity caused by cancer treatment: strategies for early detection. Lancet Oncol. 2009;10:391–399.
12. Alexandre J, Moslehi JJ, Bersell KR, Funck-Brentano C, Roden DM, Salem JE. Anticancer drug-induced cardiac rhythm disorders: Current knowledge and basicunderlying mechanisms. Pharmacol Ther. 2018; S0163-7258(18)30072-X.
13. Smith LA, Cornelius VR, Plummer CJ, Levitt G, Verrill M, Canney P, et al. Cardiotoxicity of anthracycline agents for the treatment of cancer: Systematic review and meta-analysis of randomised controlled trials. BMC Cancer. 2010;10:337.
14. SNPC. European Medicine Agency. Available in: http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/000682/human_med_001047.jsp&mid=WC0b01ac058001d124
15. Cvetkovic RS, Scott LJ. Dexrazoxane: A review of its use for cardioprotection during anthracycline chemotherapy. Drugs. 2005;68:1005–1024.
16. Csapo M, Lazar L. Chemotherapy-Induced Cardiotoxicity: Pathophysiology and Prevention. Clujul Medical. 2014;87(3):135-142.
17. SNPC. Food and Drug Agency. Available in: https://www.fda.gov/Drugs; https://www.accessdata.fda.gov/scripts/cder/daf/index.cfm?event=overview.process&ApplNo=020212
18. Boucek RJ, Jr, Steele A, Miracle A, Atkinson J. Effects of angiotensin-converting enzyme inhibitor on delayed-onset doxorubicin-induced cardiotoxicity. Cardiovasc Toxicol. 2003;3:319–29.
19. Abd El-Aziz MA, Othman AI, Amer M, El-Missiry MA. Potential protective role of angiotensin-converting enzyme inhibitors captopril and enalapril against adriamycin-induced acute cardiac and hepatic toxicity in rats. J Appl Toxicol. 2001;21:469–73.
20. Hiona A, Lee AS, Nagendran J, Xie X, Connolly AJ, Robbins RC, et al. Pretreatment with angiotensin-converting enzyme inhibitor improves doxorubicin-induced cardiomyopathy via preservation of mitochondrial function. J Thorac Cardiovasc Surg. 2011;142:396–403.
21. Ibrahim MA, Ashour OM, Ibrahim YF, El-Bitar HI, Gomaa W, Abdel-Rahim SR. Angiotensin-converting enzyme inhibition and angiotensin AT(1)-receptor antagonism equally improve doxorubicin-induced cardiotoxicity and nephrotoxicity. Pharmacol Res. 2009;60:373–81.
22. Bosch X, Rovira M, Sitges M, Domenech A, Ortiz-Perez JT, de Caralt TM et al. Enalapril and carvedilol for preventing chemotherapy-induced left ventricular systolic

dysfunction in patients with malignant hemopathies: the OVERCOME trial (prevention of left ventricular dysfunction with enalapril and carvedilol in patients submitted to intensive chemotherapy for the treatment of malignant hemopathies). J Am Coll Cardiol 2013; 61: 2355–2362.
23. Riad A, Bien S, Westermann D, Becher PM, Loya K, Landmesser U et al. Pretreatment with statin attenuates the cardiotoxicity of Doxorubicin in mice. Cancer Res 2009; 69: 695–699.
24. Gnad R, Kaina B, Fritz G. Rho GTPases are involved in the regulation of NF-kappaB by genotoxic stress. Exp Cell Res 2001; 264: 244–249.
25. Huelsenbeck J, Henninger C, Schad A, Lackner KJ, Kaina B, Fritz G. Inhibition of Rac1 signaling by lovastatin protects against anthracycline-induced cardiac toxicity. Cell Death Dis 2011; 2: e190.
26. Ramanjaneyulu SV, Trivedi PP, Kushwaha S, Vikram A, Jena GB. Protective role of atorvastatin against doxorubicin-induced cardiotoxicity and testicular toxicity in mice. J Physiol Biochem 2013; 69: 513–525.
27. Payne DL, Nohria A. Prevention of Chemotherapy Induced Cardiomyopathy. Curr Heart Fail Rep. 2017;14(5):398-403.
28. Kalay N, Basar E, Ozdogru I, Er O, Cetinkaya Y, Dogan A, Inanc T, Oguzhan A, Eryol NK, Topsakal R, Ergin A. Protective effects of carvedilol against anthracycline-induced cardiomyopathy. J Am Coll Cardiol. 2006;48(11):2258-62.
29. Pimprapa V, Edward Y. Prevention of Anthracycline-Induced Cardiotoxicity: Challenges and Opportunities. Journal of the American College of Cardiology. 2014; 64(9): 938-945.
30. Mônica Samuel Avila, Silvia Moreira Ayub-Ferreira, Mauro Rogerio de Barros Wanderley, Fatima das Dores Cruz, Sara Michelly Gonçalves Brandão, Vagner Oliveira Carvalho Rigaud, et al. Carvedilol for Prevention of Chemotherapy-Related Cardiotoxicity Journal of the American College of Cardiology. 2018; 71 (20) 2281-2290.
31. Purva Sharma, Stephanie Hakimian, Juan Camacho and Robert Chait. Prevention of chemo-induced cardiotoxicity with beta-blockers. Journal of the American College of Cardiology, 2018;71(11); DOI: 10.1016/S0735-1097(18)32344-1.
32. Escudier B, Alexandre JB, Leclercq B, Morin P, Guyot JM, Nitenberg G. Cardiotoxicitt du 5-fluorouracil. Caract Cristiques, mkanisme, conduite pratique. Presse Med 1986; 15:6-11.
33. Yeh ET, Bickford CL. Cardiovascular complications of cancer therapy: incidence, pathogenesis, diagnosis, and management. J Am Coll Cardiol. 2009;53(24):2231–2247.
34. Puel C, Mathey J, Agalias A, Kati-Coulibaly S, Mardon J, et al. Dose–response study of effect of oleuropein, an olive oil polyphenol, in an ovariectomy/ inflammation experimental model of bone loss in the rat. Clin. Nutr 2006; 25, 859–868.
35. Gülkaç MD, Akpinar G, Ustün H, Ozön Kanli A. Effects of vitamin A on doxorubicin-induced chromosomal aberrations in bone marrow cells of rats. Mutagenesis 2004; 19, 231–236.
36. Santos RV, Batista Jr, ML, Caperuto EC, Costa Rosa LF. Chronic supplementation of creatine and vitamins C and E increases survival and improves biochemical parameters after doxorubicin treatment in rats. Clin. Exp. Pharmacol. Physiol. 2007; 34:1294–1299.
37. Conklin KA. Coenzyme q10 for prevention of anthracycline-induced cardiotoxicity. Integr. Cancer Ther 2005; 4:110–130.
38. Huertas JR, Battino M, Lenaz G, Mataix FJ. Changes in mitochondrial and microsomal rat liver coenzyme Q9 and Q10 content induced by dietary fat and endogenous lipid peroxidation. FEBS Lett. 1991; 287:89–92.
39. Athar M, Back JH, Kopelovich L, Bickers DR, Kim AL. Multiple molecular targets of resveratrol: anticarcinogenic mechanisms. Arch. Biochem. Biophys. 2009;486:95–102.
40. Goulas V, Exarchou V, Troganis AN, Psomiadou E, Fotsis T, Briasoulis E, Gerothanassis IP. Phytochemicals in olive-leaf extracts and their antiproliferative activity against cancer and endothelial cells. Mol. Nutr. Food Res. 2009; 53, 600–608.
41. Quiles JL, Huertas JR, Battino M, Mataix J, Ramírez-Tortosa MC. Antioxidant nutrients and adriamycin toxicity. Toxicology. 2002; 180:79–95.
42. Mukherjee S, Banerjee SK, Maulik M, Dinda AK, Talwar KK, Maulik SK. Protection against acute adriamycin-induced cardiotoxicity by garlic: role of endogenous antioxidants and inhibition of TNF-alpha expression. BMC Pharmacol. 2003; 3:16–24.
43. Lim HA, Kim JH, Kim JH, Sung MK, Kim MK, Park, JH, Kim JS. Genistein induces glucose-regulated protein 78 in mammary tumor cells. J. Med. Food 2006; 9:28–32.
44. Udenigwe CC, Ramprasath VR, Aluko RE, Jones PJ. Potential of resveratrol in anticancer and anti-inflammatory therapy. Nutr. Rev. 2008; 66:445–454.
45. Bulucu F, Ocal R, Karadurmus N, Sahin M, Kenar L, Aydin A, et al. Effects of N-acetylcysteine, deferoxamine and selenium on doxorubicin-induced hepatotoxicity. Biol. Trace Elem. Res. 2009; 14:25-31.
46. Bulucu F, Oktenli C, Kenar L, Ocal R, Koc B, Inal V, Yamanel L, Yaman H, Sanisoglu YS, Aydin A. Efficacy of deferoxamine, N-acetylcysteine and selenium treatments in rats with adriamycin-induced nephrotic syndrome. J. Nephrol. 2008; 21: 576–583.
47. Jemai H, El Feki A, Sayadi S. Antidiabetic and antioxidant effects of hydroxytyrosol and oleuropein from olive leaves in alloxan-diabetic rats. J. Agric. Food Chem. 2009; 57:8798–8804.
48. Choi BH, Kim CG, Lim Y, Shin SY, Lee YH. Curcumin down-regulates the multidrug-resistance mdr1b gene by inhibiting the PI3K/Akt/NF kappa B pathway. Cancer Lett. 2008; 259:111–118.

49. Monstrey SJ, Mullick P, Narayanan K, et al. Hyperbaric oxygen therapy and free radical production: An experimental study in doxorubicin (Adriamycin) extravasation injuries. Ann Plast Surg 1997; 38:163-168.
50. Akta S, Toklu AS, Olgac V. Hyperbaric oxygen therapy in Adriamycin extravasation: An experimental animal study. Ann Plast Surg 2000; 45:167- 171.
51. Karagoz B, Suleymanoglu S, Uzun G, Bilgi O, Aydinoz S, Haholu A, Turken O,Onem Y, Kandemir EG. Hyperbaric oxygen therapy does not potentiatedoxorubicin-induced cardiotoxicity in rats. Basic Clin Pharmacol Toxicol. 2008;102(3):287-92.
52. Goolsby, Tiffany V. et al. Extravasation of Chemotherapeutic Agents: Prevention and TreatmentSeminars in Oncology . 2006; 33(1); 139-143.
53. Newton RU, Galvão DA. Exercise in prevention and management of cancer. Curr Treat Options Oncol. 2008;9(2-3):135-46.
54. Rajarajeswaran P, Vishnupriya R. Exercise in cancer. Indian Journal of Medical and Paediatric Oncology : Official Journal of Indian Society of Medical & Paediatric Oncology. 2009;30(2):61-70.
55. Payne DL, Nohria A. Prevention of Chemotherapy Induced Cardiomyopathy. Curr Heart Fail Rep. 2017;14(5):398-403.
56. Jones LW, Habel LA, Weltzien E, Castillo A, Gupta D, Kroenke CH, et al. Exercise and risk of cardiovascular events in women with nonmetastatic breast cancer. Jpn J Clin Oncol. 2016;34:2743–9.
57. Haykowsky MJ, Mackey JR, Thompson RB, Jones LW, Paterson DI. Adjuvant trastuzumab induces ventricular remodeling despite aerobic exercise training. Clin Cancer Res. 2009; 15:4963–7.
58. Varga ZV, Ferdinandy P, Liaudet L, Pacher P. Drug-induced mitochondrial dysfunction and cardiotoxicity. American Journal of Physiology - Heart and Circulatory Physiology. 2015; 309(9):H1453-H1467.
59. Fandeev OA, Vasechkin SS, Alekhin MN, Odintsov SV, Kallistov VE, Sidorenko BA. Clinical value of antracycline toxicity: modern approaches to diagnosis,prevention, and treatment. Kardiologiia. 2011; 51(7):40-6.

MYOID ANGIOENDOTHELIOMA OF THE SPLEEN - CASE REPORT AND LITERATURE REVIEW

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MIOIDNI ANGIOENDOTELIOM SLEZINE- PRIKAZ SLUČAJA I PREGLED LIERATURE

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ABSTRACT

Myoid angioendothelioma (MA) represents an extremely rare nonhaematopoietic proliferation of the spleen. MA is a rare, benign, vascular tumour that consists of vascular elements and arranged stromal cells. Due to an absence of specific clinical signs and symptoms, MA is considered challenging to diagnose. Although the radiological presentation can indicate the vascular nature of the tumour, the diagnosis of MA is almost exclusively obtained from the use of histopathology after surgical excision and immunohistochemistry of the tissue. Due to its completely unclear biological behaviour and relationship with other primary and secondary tumours, the only effective therapy for MA is splenectomy and a regular postoperative follow-up. Herein, we report a case of a 26-year-old male patient with nonspecific abdominal pain and a radiologically detected tumour of the spleen who underwent a laparoscopic splenectomy. Histopathologic and immunohistochemical examinations confirmed a myoid angioendothelioma of the spleen.

Keywords: *myoid angioendothelioma, splenic tumour, immunohistochemistry, laparoscopic splenectomy.*

SAŽETAK

Mioidni angioendoteliom (MA) slezine predstavlja veoma retku nehematološku proliferativnu promenu slezine. MA je redak, benigni, vaskularni tumor slezine koji se sastoji od vaskularnih elemenata i stromalnih ćelija. Zbog odsustva specifičnih kliničkih znakova i simptoma, dijagnotifikovati MA predstavlja izazov. Iako radiološka prezentacija može ukazati na vaskularnu prirodu tumora , dijagnoza MA se gotovo uvek dobija histopatološki i imunohistohemijom nakon operacije. Zbog potpuno nejasnog biološkog ponašanja i odnosa sa drugim primarnim i sekundarnim tumorima, jedina efikasna terapija za MA je splenektomija uz redovno postoperativno praćenje. Mi smo predstavili slučaj 26-to godišnjeg muškog pacijenta sa nespecifičnim bolom u abdomenu i radiološkim dijagnostifikovanim tumorom slezine kome je učinjena laparoskopska splenektomija. Histopatološkim i imunohistohemijskim pregledom je potvrđen mioidni angioendoteliom slezine.

Ključne reči: *Mioidni angioendoteliom, tumor slezine, imunohistohemija, laparoskopska splenektomija.*

INTRODUCTION

Myoid angioendothelioma (MA) of the spleen is uncommon and is a recently described entity that is classified as a type of nonhaematopoietic tumour of the spleen (1, 2, 3). MA is a benign, vascular tumour of the spleen that is composed of vascular endothelial cells and stromal cells with myoid and myofibroblastic features. A contemporary immunohistochemical analysis enables the successful distinction of MA from other splenic vascular tumours, such as haemangiomas, angiosarcomas, lymphangiomas, littoral cell angiomas (LCA), haemangioendotheliomas and hamartomas (1, 2, 3). MA was first described by Kraus and Dehner in 1999, who documented 3 patients with this disorder (1). The disease occurs with an *equal frequency* in all age groups. Due to non-specific clinical presentations, MA is often accidentally discovered, which is similar to other vascular lesions of the spleen. According to the literature, after the radiological detection of a mass of an unclear origin in the spleen, patients underwent splenectomies, and the diagnosis of MA was obtained after histopathologic and immunohistochemical analyses of the extracted spleen (1, 2, 3, 4).

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CASE REPORT

A 26-year-old male patient was admitted to our hospital with a two-year history of intermittent abdominal pain in the epigastric and right subcostal regions. Previous medical and family histories revealed no prior malignancies or other significant comorbidities. The patient was a non-smoker and had not recently consumed beverages or illicit drugs.

Two years prior to admission, the patient underwent an oesophagogastroduodenoscopy, after which a hiatal hernia and signs of chronic *Helicobacter pylori*-negative gastroduodenitis were diagnosed. He was periodically treated with the use of proton pump inhibitors.

The laboratory findings and tumour markers were within reference ranges. An abdominal magnetic resonance imaging (MRI) scan revealed no splenomegaly (12x6 cm) and the presence of a solitary, well-circumscribed lesion (28 mm) in the lower pole of the spleen, with a peripheral rim enhancement on the arterial phase and a centripetal pattern of enhancement on the portal phase (Figure 1). The differential diagnosis included splenic tumours of different aetiologies, as well as a possible haemangioma of the spleen.

The patient received an operation that utilized a laparoscopic approach, and a splenectomy was performed. Intraoperatively, the splenic tumour, with a diameter of approximately 3 cm, was observed in the lower pole. After the division of the ligamentar attachments and the complete mobilization of the spleen, a division of the hilar splenic vessels was performed with the use of an endoscopic stapler. The splenic specimen was placed in a large, plastic, impermeable bag and was retrieved through the umbilical port. Due to the fact that the tumour had to remain undamaged during the extraction process (in order to allow for a precise histopathological analysis), the intact spleen was carefully removed through the use of a mini-laparotomy that was created by the slight extension of the umbilical incision. The splenic specimen was fixed with 10% buffered formalin, and the representative sections were embedded in paraffin. The postoperative period was uneventful, and the patient was discharged on the fifth postoperative day.

After the histopathologic assessment, the microscopic findings revealed the presence of a well-circumscribed tumour that was composed of numerous capillary calibre vessels that were implanted in an eosinophilic matrix with pulp stromal cells (Figure 2-a; 2-b). Further immunohistochemical examinations revealed a strongly positive staining of the vascular lining cells for CD34 and CD31, while the stromal cells stained positive for SMA, desmin, actin and myosin SM (Figure 2-c; 2-d). Staining for S100, CD8 and CD21 were not present in either of the linings. Therefore, a diagnosis of myoid angioendothelioma of the spleen was confirmed. Regular postoperative check-ups after one, three, six and twelve months demonstrated that the patient was completely symptom free and lacked any other abdominal imaging disturbances.

DISCUSSION

The spleen, which is anatomically composed of red and white pulp, plays an important role in the complex modulation of the immune system by filtering the blood and recycling iron. The red pulp consists of a network of venous sinuses and Billroth cords, while the white pulp includes T and B lymphocytes. The imaging appearances of certain entities in the spleen, due to its complex structural morphology and function, can be deceiving and often cannot be distinguished with the utmost certainty (6,7,8). The most common nonlymphoid tumours of the spleen originate from the vascular endothelium (7,9). MA is an extremely rare, splenic, vascular tumour, with only 7 cases reported so far in the literature (1,2,3,4). A proposed diagnostic algorithm for MA includes the use of preoperative multidetector computed tomography (MDCT), MRI and positron emission tomography–computed tomography (PET-CT) of the abdomen, as well as subsequent histopathological and immunohistochemical examinations, to determine the nature of the tumour cells (2,3,4). A PET CT can reveal splenic myoid angioendothelioma as a hypermetabolic mass that has an increased uptake of fluorodeoxyglucose (FDG), which then indicates a neoplastic process (2).

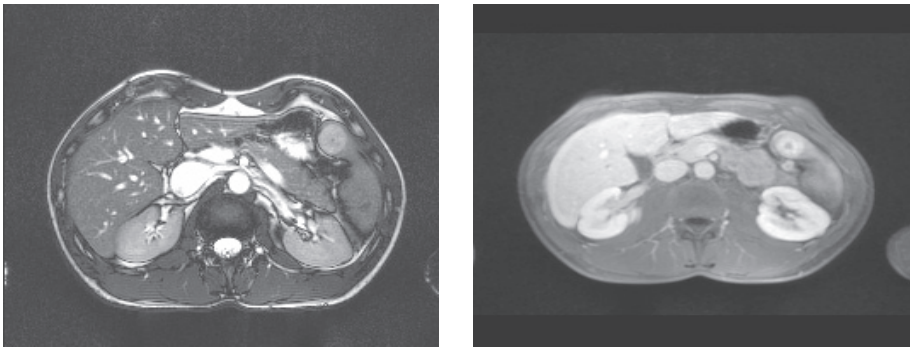

Figure 1: T1W and T2W axial abdominal MRI images
A solitary, well-circumscribed lesion (28 mm) in the lower pole of the spleen, with a peripheral rim enhancement on the arterial phase and a centripetal pattern of enhancement on the portal phase.

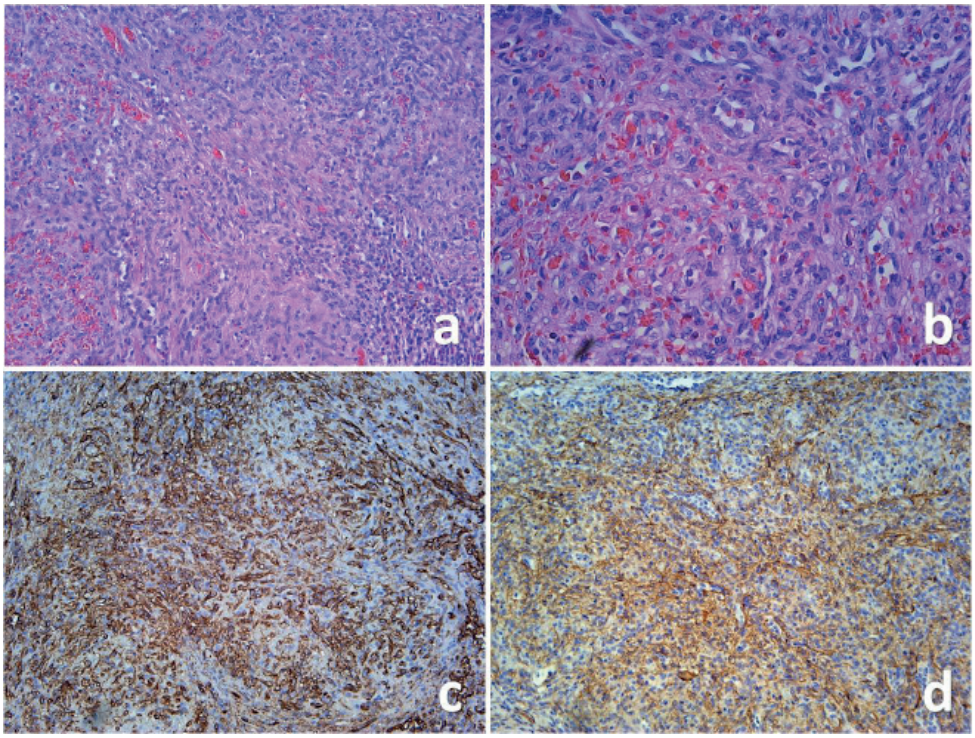

Figure 2: The histological presentation of a vascular tumour with characteristic compact proliferation of vascular and stromal elements. (a) Sieve-like, focally dilated vessels (b), which showed strong CD34 immunoexpression (c) accompanied by smooth muscle actin immunopositive stromal cells (d).

MDCT and MRI examinations usually describe MA as a round, well-circumscribed lesion, with a peripheral rim enhancement on the arterial phase and a centripetal pattern of enhancement on the portal phase, which can be attributed to the vascular nature of this disorder (2). The pathohistological findings of MA show a solid, well-circumscribed tumour that contains numerous capillary calibre vessels that are embedded in the stromal cells of the pulp [(2,3). The immunohistochemical findings reveal the strongly positive staining of stromal cells for SMA, CD31, CD34 and F VII, as well as negative staining for S100; additionally, the vascular lining cells stained positive for CD 31 and CD34, and stained negative for CD8 (2,3).

The first individuals to study myoid angioendothelioma were Kraus and Dehner in 1999, wherein they provided a description of a new, vascular tumour of the spleen by documenting 3 case reports (1). Splenomegaly was detected in only one patient who had a previous clinical presentation of abdominal pain. The other two cases were discovered by using ultrasound screening for Beckwith Wiedemann syndrome in one of the patients, and after a pancreatectomy, due to the incidence of a mucinous cystic pancreatic tumour, in the other patient. The tumour size did not exceed 4 cm in diameter in any of these patients (1). The immunohistochemical findings confirmed vascular cell lining positive staining for CD34 and F VIII, with variable reactivity for CD31, while the stromal cells were positive for SMA, MSA and desmin. The described phenotype indicated a vascular nature of the lining cells, while the SMA+ and MSA+ polygonal cells represented the proliferation of the myoid lineage. Staining for S100, CD8 and CD21 was not seen in either of the linings (1). However, in 2004, Karim et al. reported a case of splenic MA in a 51-year-old male patient, with unexpected immunohistochemical findings of S100 positive cells in the stroma (3).

Despite the reported morphological and histological similarities to splenic epithelioid and spindled haemangioendothelioma, this immunophenotype enabled the differentiation of MA tumours of the spleen (10,11, 12). In 2013, Jang et al. described a case of rectal adenocarcinoma in a female patient with a radiologically registered metastatic disease in the liver and a splenic mass mimicking a metastasis. After a splenectomy, the pathohistological findings confirmed the diagnosis of MA, with immunochemical positive staining of the stromal cells for SMA and positive staining of the vascular channels for CD 34, CD31 and factor VIII (2). Chan et al. reported another case of splenic MA that was diagnosed in a child after a successful treatment of a Wilms' tumour of the kidney (4). Out of all of the reported cases of MA in the literature, even though 3 cases had previous malignant diseases, such as rectal adenocarcinoma, a pancreatic neoplasm and a Wilms' tumour, the biological behaviour of myoid angioendothelioma is still considered to be benign. All of these data suggest that further clinical and histopathological investigations should be performed and that patients should be closely monitored, due to a lack of precise biological behaviour of MA of the spleen.

CONCLUSION

New cases of MA are expected to be reported in the future, as it is clearly defined as an independent morphological entity. Nevertheless, further research is needed for the evaluation of biological behaviour, the determination of aetiology, the clinical presentation, the pathohistology and the therapeutic options of splenic myoid angioendothelioma, due to its low incidence. Until recently, splenectomies were performed in all of the reported cases of MA patients, which present the only therapeutic options that are currently available. Furthermore, the association of MA with other neoplasms is uncertain, and further studies are necessary. All of the authors agree that the postoperative, regular follow-up of patients with MA is required.

REFERENCES

1. Kraus MD, Dehner LP. 1999. Benign vascular neoplasms of the spleen with myoid and angioendotheliomatous features. Histopathology;35(4):328–36.
2. Jang KY, Chung MJ, Moon WS, Sohn MH, Hwang SB, Lee MR, Lee H, Park HS. 2013. Myoid angioendothelioma of the spleen mimicking metastatic disease in a patient with rectal cancer: a radiologic-pathologic correlation. Ann Diagn Pathol;17(1):108-12.
3. Karim RZ, Ma-Wyatt J, Cox M, Scolyer RA. 2004. Myoid angioendothelioma of the spleen. Int J Surg Pathol;12(1):51–6.
4. Chan YF, Kumar B, Auldist A, Waters K. 2005. Myoid angioendothelioma of the spleen in a child after successful treatment of a Wilms' tumour. Pathology;37(2):181–4.
5. Pinkus GS, Warhol MJ, O'Connor EM, Etheridge CL, Fujiwara K. 1986. Immunohistochemical localization of smooth muscle myosin in human spleen, lymph node, and other lymphoid tissues. Unique staining patterns in splenic white pulp and sinuses, lymphoid follicles, and certain vasculature, with ultrastructural correlations. Am J Pathol;123(3):440–53
6. Ferrozzi F, Bova D, Draghi F, Garlaschi G. 1996. CT findings in primary vascular tumors of the spleen. AJR American Journal of Roentgenology;166(5): 1097–101.
7. Thipphavong S, Duigenan S, Schindera ST, Gee MS, Philips S. 2014. Nonneoplastic, benign, and malignant splenic diseases: cross-sectional imaging findings and rare disease entities. AJR Am J Roentgenol;203(2):315-22.
8. Karlo CA, Stolzmann P, Do RK, Alkadhi H. 2013. Computed tomography of the spleen: how to interpret the hypodense lesion. Insights Imaging;4(1):65-76.
9. Fotiadis C, Georgopoulos I, Stoidis C, Patapis P. 2009. Primary tumors of the spleen. Int J Biomed Sci;5(2):85-91. Page 7 of 39 https://mc.manuscriptcentral.com/eajm The Eurasian Journal of Medicine 123456789 10 -60 For Review Only
10. Suster S. 1992. Epithelioid and spindle-cell hemangioendothelioma of the spleen. Report of a distinctive splenic vascular neoplasm of childhood. Am J Surg Pathol;16(8):785-92.
11. Requena L, Kutzner H. 2013. Hemangioendothelioma. Semin Diagn Pathol;30:29–44.
12. Zhendan wang, Liang Zhang,Bo Zhang,Dianbin Mu, Kai Cui, Sheng Li. 2015.Hemangioendothelioma arising from the spleen: A case report and literature review Oncol Lett. 9(1): 209–212.

LETHAL OUTCOME IN A HEALTHY MAN INFECTED WITH COVID-19

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SMRTNI ISHOD KOD ZDRAVOG MUŠKARCA SA COVID-19 INFEKCIJOM

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ABSTRACT

COVID-19 is defined as a respiratory infection which is spread by droplets and immediate contact with an infected person. The first case of COVID-19 infection in Serbia was reported on March 6th, 2020.

Herein, we present the case of confirmed COVID-19 infection in a previously healthy man, whose three other family members showed mild symptoms of coronavirus disease, without the need for hospitalization. The patient was treated at the tertiary medical center, four days after the onset of symptoms. During the hospitalization, he developed serious complications and fatal outcome. In this case, hypoxia-induced cardiac arrest was secondary to severe COVID-19 pneumonia with the development of acute respiratory distress syndrome and sepsis. Laboratory and flow cytometry results indicate the presence of the cytokine storm, while the mechanical ventilation might potentially increase the risk of lethal outcome.

This case report is important because it should give clinicians the insight into the treatment of the previously healthy individuals with COVID-19, especially in terms of possible laboratory markers that could indicate the presence of the cytokine storm phenomenon.

Keywords: *COVID-19; coronavirus; epidemic; pandemic; ARDS; mechanical ventilation; cytokine storm.*

SAŽETAK

COVID-19 je definisan kao respiratorna infekcija koja se širi kapljicama i neposrednim kontaktom sa zaraženom osobom. Prvi slučaj zaraze COVID-19 infekcije u Srbiji je prijavljen 6. marta 2020. godine.

U ovom radu, predstavljamo slučaj potvrđene infekcije COVID-19 kod prethodno zdravog čoveka, kod koga su preostala tri člana uže porodice pokazala blage simptome koronavirusne bolesti, bez potrebe za hospitalizacijom. Pacijent je lečen u tercijarnom medicinskom centru, četiri dana nakon pojave simptoma. Tokom hospitalizacije razvio je ozbiljne komplikacije i smrtni ishod. U ovom slučaju, srčani zastoj izazvan hipoksijom razvio se sekundarno, usled teškog oblika COVID-19 pneumonije i razvoja sindroma akutnog respiratornog distresa i sepse. Rezultati laboratorijske i flow citometrije ukazuju na prisustvo citokinske oluje, dok je mehanička ventilacija na kojoj je pacijent bio, takođe mogla da potencijalno poveća rizik od smrtnog ishoda.

Ovaj prikaz slučaja je važan jer treba da omogući kliničkim lekarima uvid u tretman prethodno zdravih pacijenata sa COVID-19, posebno u smislu laboratorijskih markera koji mogu indikovati prisustvo fenomena citokinske oluje.

Ključne reči: *COVID-19; korona virus; epidemija; pandemija; ARDS; mehanička ventilacija; citokinska oluja.*

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INTRODUCTION

After the onset of epidemic in China since December 2019, the World Health Organization labeled the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) as the pandemic coronavirus disease 2019 (COVID-19) on February 11th, 2020 (1). COVID-19 is defined as a respiratory infection which is spread by droplets and immediate contact with an infected person (2,3). SARS-CoV-2 is a new, previously unknown, RNA virus whose primary receptor is most likely angiotensin-converting enzyme 2 (3). The incubation period for this virus varies from 0 to 24 days, with the mean value of 5.2 days (3,4,5). The reference standard for the diagnosis of this infection is done by the real-time reverse transcriptase polymerase chain reaction (rRT-PCR) assay (4). In addition to that, radiological imaging modalities are used, such as radiography and computed tomography (3,5).

The first case of COVID-19 infection in Serbia was reported on March 6th, 2020. To this day, May 19th,10699 Serbian citizens have a confirmed coronavirus disease with the rRT-PCR test, whereas there are 231 lethal cases and 4799 cured patients (6). COVID-19 mortality rate at this point in Serbia is 2.17% (6). The National Department of Health in Serbia approved the Handbook of COVID-19 prevention and treatment, written by the The First Affiliated Hospital, Zhejiang University School of Medicine in China, as the official handbook for coronavirus disease treatment (7).

Herein, we present the case of COVID-19 infection in a previously healthy man, with serious complications during the hospitalization and fatal outcome. Also, the route of coronavirus transmission is thoroughly explained, due to the fact that the patient was the only one of four family members who had a severe clinical manifestation of the infection and due to that, was hospitalized. The other three members, his wife and children had only mild symptoms and were isolated at home located in the central Serbia municipality. All four of them were non-smokers with regular physical activity and no comorbidities.

CASE PRESENTATION

A 25-year old man, a student and professional dancer from Serbia, experienced fatigue, fever (38.2℃), anosmia and impaired sense of taste, on March 7th,2020. The fever and fatigue lasted only for two days, while the other two symptoms lasted for the next 15 days. He is a professional athlete with no medical history and great health. He reported that he didn't have flu or flu-like symptoms in the last 15 years. On March 3rd and 4th, he had a direct contact with his dancing coach, who was confirmed with COVID-19 infection with the rRT-PCR test, on March 12th, 2020. On March 9th, his 28-year old sister experienced the same symptoms as her brother, with a slightly higher fever (38.8℃). She was home medicated with Paracetamol and Vitamin C. She is also a professional dancer and architect with no previous history of illness. The similar case was with their mother, 51-year old woman, an entrepreneur, who had a fever (37.4℃) only on March 10th, while anosmia and impaired sense of taste lasted for the next 15 days, just like in her children.

A 51-year old man, father of the family and an entrepreneur, developed a fever (38.5℃), fatigue, myalgia, headache and abdominal pain, on March 10th, 2020. The patient had no previous medical history or any known chronic diseases. On March 12th, after his son got information that his coach was confirmed with COVID-19, he informed an epidemiologist by telephone call and got the instruction to isolate himself and in the case of progression of symptoms to come to the nearest tertiary medical facility. In the following day, his blood analysis at the local laboratory showed Lymphocytopenia ($1.2x10^9$/L), while the other parameters were in normal range. In the next two days, he started dry coughing, developed bone tenderness and a recurrent fever from 38.5℃ to 39℃ which didn't respond well to the use of Paracetamol and Vitamin C combination. Due to this, the epidemiologist indicated an examination at the Clinical Center Kragujevac, by the on call infectologist on March 14th. The physical examination showed a fever (38.4℃) and blood oxygen saturation (SPO2) 98%. On the same day, late in the evening, the patient was admitted to the isolated area of the Clinic for Infectious Diseases, Clinical Center Kragujevac. Right after the admission, the patient was tested using the rRT-PCR, and the results came positive. After that point, he was under a constant supervision by trained medical staff and his treatment was according to the Handbook of COVID-19 prevention and treatment (6).

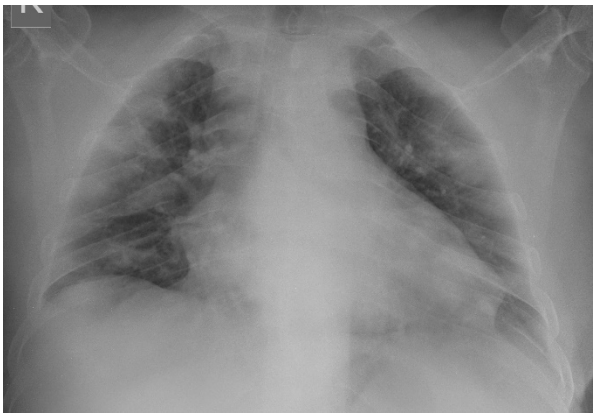
Body temperature fluctuations during the hospital stay are shown in Figure 1, whereas only values ≥ 37.5°C were presented, while on other days, the patient had a normal body temperature. Blood oxygen saturation is displayed in Figure 2. Laboratory blood results are shown in Table 1, divided by days of the hospitalization. Flow cytometry was done on the 25th hospitalization day (Table 2), and prior to that, on the 4th day, CD4 lymphocyte count was 999cells/μl and CD8 lymphocyte count was 440 cells/μl, which was in normal range. Lymphocyte gating strategy in the whole human blood is presented in Figure 3.

The initial chest radiograph was done on the 2nd day of hospitalization (Picture 1), which revealed ground-glass opacities perihilar and bilateral. On the 4th day of hospitalization, the patient was sedated, intubated and mechanically ventilated, due to the worsening of the respiratory condition and decrease of SPO2 (Figure 2). A day later, there was a radiological progression in the lungs, with bilateral alveolar consolidation and presence of endotracheal tube (Picture 2). Three days after that, on the 8th day of the hospital treatment, the patient was admitted to the Intensive care unit (ICU), specially organized for patients infected with coronavirus, on the account of illness intensification which can be well observed in Table 1. On the 16th day, there was a radiological improvement comparing to the previous chest x-ray (CXR), endotracheal tube and central venous line were required (Picture 3). On the following day, which was the 17th day of hospitalization, the patient was extubated, but his condition worsened

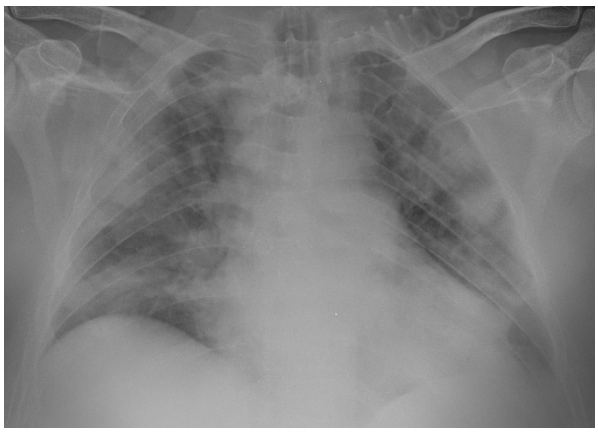
and he was intubated on the 18th day and mechanically ventilated again. On the same day, the bacteriological examination of tracheal aspirate was done and Acinetobacter spp was isolated, which confirmed the blood culture result, two days before. On the nineteenth day, lung radiography in Picture 4 showed radiological worsening with bilateral alveolar consolidation. On this day, laboratory results showed a noticeable rise in CRP (108.5), which was twice as higher comparing to the result on the 16th day (53.5 mg/L). On the 21st day of hospitalization, tracheotomy was performed. Two days later, CRP had its maximum value (234.5 mg/L) and CXR reported bilateral alveolar consolidation with panlobar affectation, the typical radiological findings of the acute respiratory distress syndrome (ARDS) (Picture 5). On the April 9th 2020, CXR showed presence of the subcutaneous emphysema. A thoracic surgeon was consulted and thoracic drainage was performed. Nevertheless, on the April 10th, which was the 27th day of hospitalization, the patient's condition worsened, he was in bradycardia, hypotensive and SPO2 was 60%. Even though intensive measures of resuscitation were performed, the patient underwent cardiac arrest and the outcome was lethal. Picture 6 shows final CXR with ARDS, subcutaneous emphysema, thoracic drains, MV and tracheotomy and central venous line. In this case, hypoxia-induced cardiac arrest was secondary to severe COVID-19 pneumonia with the development of ARDS and sepsis.

During the hospitalization, the patient was treated with a combined pharmacological treatment. The initial treatment included: antimalarial drug (Chloroquine phosphate, specific antiviral drug (Lopinavir-Ritonavir), antipyretic (Paracetamol), antibiotics (Ceftriaxone, Ertapenem, Azithromycin), vitamin C and intravenous fluids. Still, the progression of the disease required ICU care and the treatment was corrected, so the new antibiotic treatment included Piperacillin / Tazobactam and Tigecycline, while Colistin was added after the bacteriological examination of tracheal aspirate. In addition to that, due to the progression of illness, ICU treatment included: corticosteroid (Methylprednisolone), bronchodilators (Ipratropium bromide, Aminophylline), beta-blocker (Labetalol), calcium-channel blocker (Nifedipine), anticoagulant (Enoxaparin sodium), diuretic (Furosemide), vitamins (B1, D3), intravenous fluids, parenteral and enteral nutrition.

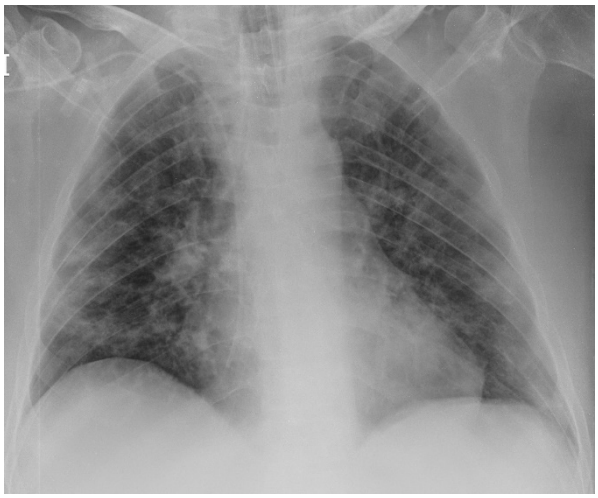
Picture 1. Initial chest radiography



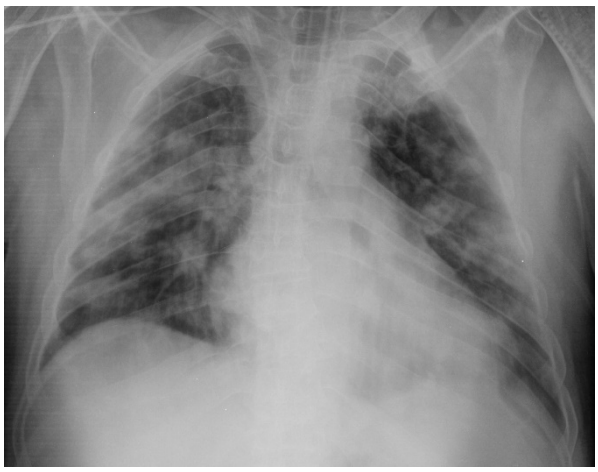
Picture 2. Chest radiography after the mechanical ventilation



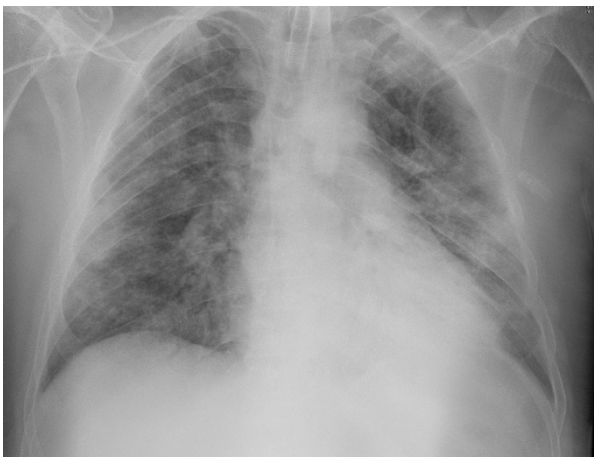
Picture 3. Chest radiography showing the improvement before extubation



Picture 4. Chest radiography, patient is mechanically ventilated and CRP is critically rising



Picture 5. Chest radiography after tracheotomy, CRP is at max level on this day



Picture 6. Final chest radiography before death

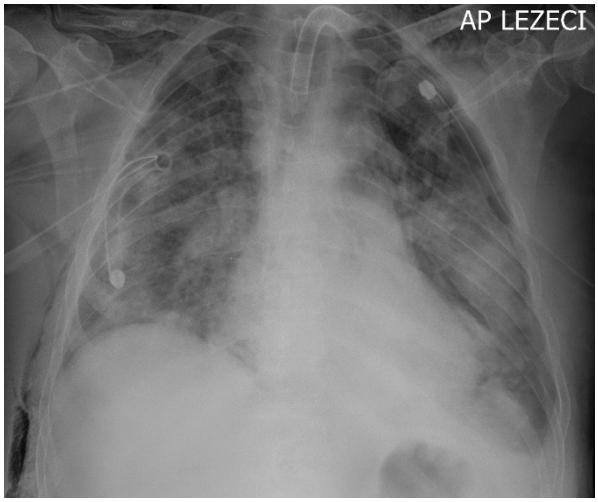


Figure 1. Body temperature fluctuations, recorded during each day of hospitalization

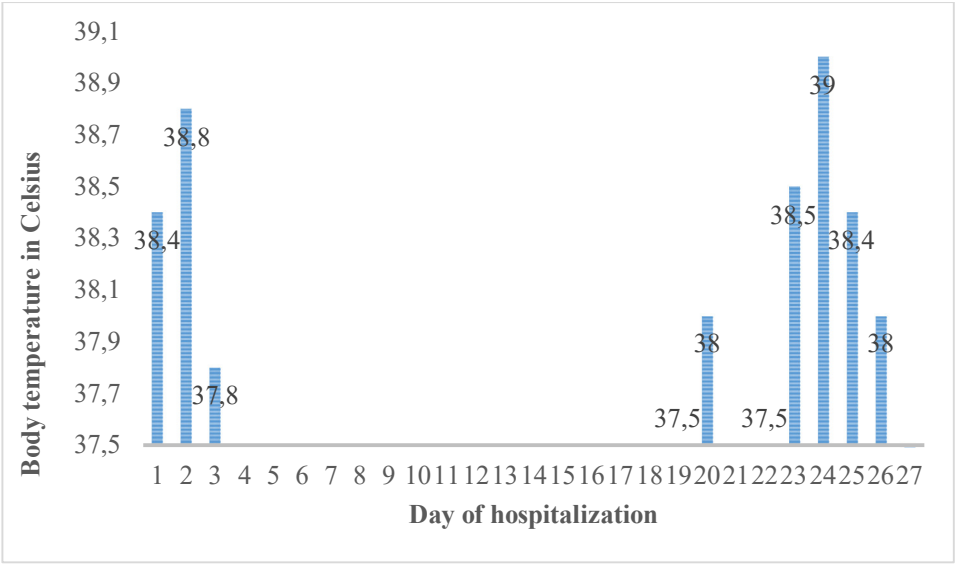
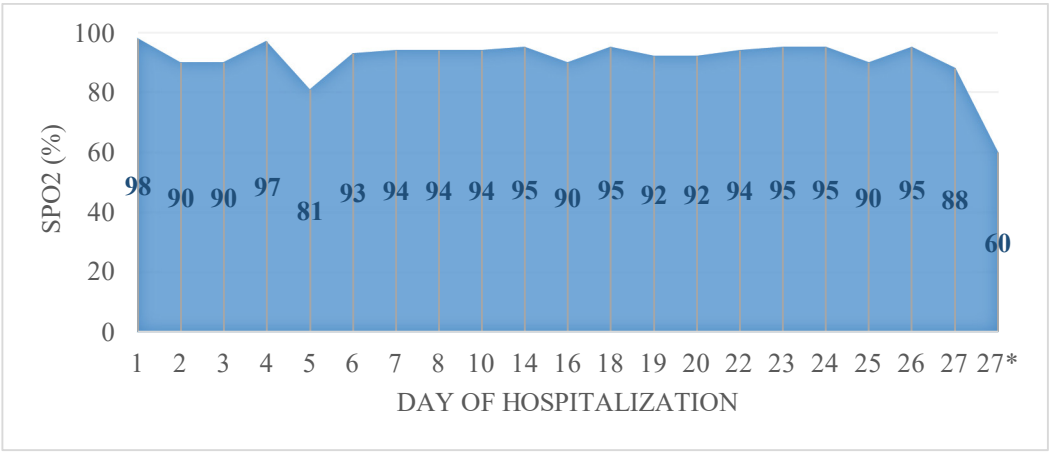
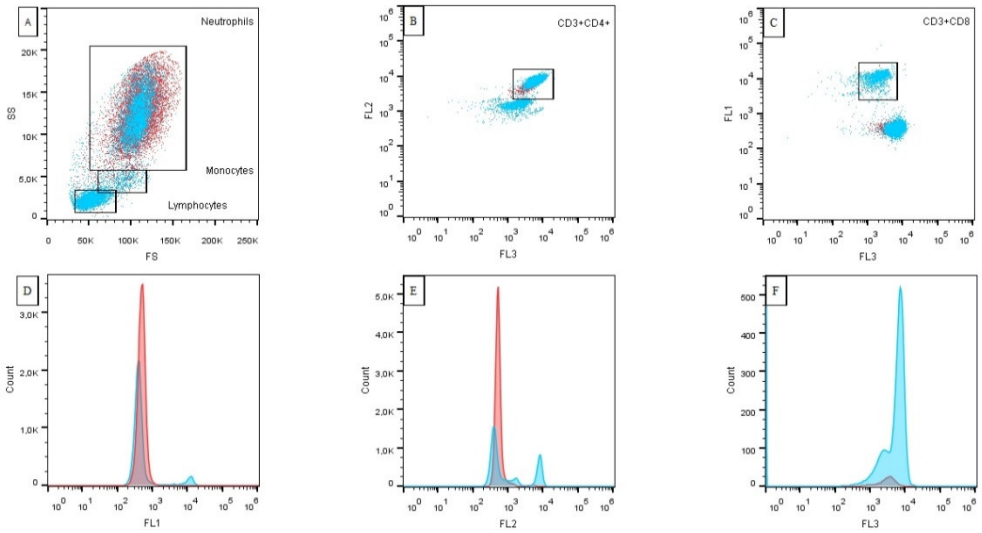


Figure 2. Blood oxygen saturation during each day of hospitalization



* On the 27th hospitalization day, SPO2 was recorded twice

Figure 3. Lymphocyte gating strategy in whole human blood



* (**A**) FSC *vs.* SSC plot: Broad selection of neutrophils, monocytes and lymphocytes based on their SSC/FSC properties. (**B-C**) Dot plots: Identification of CD3+CD4+ and CD3+CD8+ population after gating with color representing patient (red) and control (blue). (**D-F**) Smoothed histograms:
Lymphocyte FL1/CD8, FL2/CD4 and FL3/CD3 expression showing patient (red) and control (blue).

Table 1. Main laboratory findings

	Reference range	Day 1	Day 7	Day 17	Day 22	Day 27
WBC	3.7-10.0 (10^9/L)	6.6	14.1 ↑	22.59 ↑	20.7 ↑	12.25 ↑
LYM	1.2-3.4 (10^9/L)	1.3	0.6 ↓	0.58 ↓		
GRAN	2.1-6.5 (10^9/L)	4.4	12.4 ↑	15.2 ↑		
RBC	4.34-5.72 (10^{12}/L)	5.1	4.17 ↓	4.05 ↓	3.61 ↓	2.98 ↓
HGB	138-175 (g/L)	154	125 ↓	123 ↓	111 ↓	91 ↓
PLT	135-450 (10^9/L)	181	224	205	119 ↓	134 ↓
PT	11.8-15.3 (s)	13.2	13.9	17.7 ↑	22.8 ↑	38 ↑
INR	0.9-1.1	0.97	1.22 ↑	1.28 ↑	1.63 ↑	2.66 ↑
PT%	70-120	106	67 ↓	64 ↓	44 ↓	23 ↓
D-DIMER	<0.50 (ug/ml FEU)	0.36	2.48 ↑	2.22 ↑	4.34 ↑	72.2 ↑
AST	0-40 (IU/L)	23	72 ↑	44 ↑	45 ↑	59 ↑
ALT	0-40 (IU/L)	22	82 ↑	57 ↑	56 ↑	64 ↑
GLUC	3.8-6.1 (mmol/L)	6.1	6.8 ↑	7.7 ↑	6.2 ↑	6.9 ↑
UREA	3.0-8.0 (mmol/L)	4.7	5.7	7.6	8.3 ↑	16 ↑
CREA	49-106 (mmol/L)	92	61	50	40 ↓	71
CK	0-171 (U/L)	250 ↑	686 ↑	73	657 ↑	
CK-MB	<25.0 (U/L)	26 ↑	15	15	12	
CRP	0.0-5.0 (mg/L)	21.5 ↑	86.4 ↑	72.8 ↑	189.8	203.7 ↑
TROPONIN	<0.0342 (ng/mL)	0.0089	0.0237	0.0067	0.0409 ↑	
Procalcitonin	0.5-2.0 >2.0 risk for sepsis(ng/mL)	0.143	0.05	0.113	2.16 ↑	1.98
proBNP	negative <125,	130	1496	443	591	

	Reference range	Day 1	Day 7	Day 17	Day 22	Day 27
	grey area 125-450, insufficiency >450 (pg/mL)					
LDH	220-450 (U/L)	448	662 ↑	706 ↑		
GGT	7-50 (IU/L)		35	256 ↑	61 ↑	56 ↑
Potassium	3.5-5.3 (mmol/L)	4	4.8	4.1	4.4	5.4 ↑
Sodium	137-147 (mmol/L)	136 ↓	137	135 ↓	137	135 ↓
Albumin	35-52 (g/L)		29 ↓	27 ↓	27 ↓	27 ↓

* WBC-white blood cells, LYM-lymphocytes, GRAN- granulocytes, RBC-red blood cells, HGB-hemoglobin, PLT- platelets, PT-prothrombin time, INR-international normalized ration, AST- aspartate aminotransferase, ALT- alanine transaminase, GLUC-glucose, CREA-creatinine, CK-creatin kinase, CRP-C reactive protein, proBNP-brain natriuretic peptide, LDH- lactate dehydrogenase, GGT-gamma-glutamyl transferase, ↑-increased level, ↓-decreased level

Table 2. Flow cytometry

	Observed values	Reference range
Neutrophils CD15+	**94.41**	**30-80%**
T lymphocytes CD3+	**0.44**	**7-24%**
Helper T lymphocytes CD3+CD4+	**0.40**	**4-20%**
Cytotoxic T lymphocytes CD3+CD8+	**0.05**	**2-11%**
Monocytes CD14+	**1.75**	**2-12%**
B lymphocytes CD19+	**2.09**	**1-7%**
NK cells CD3-CD56+CD57-	**0.89**	**1-6%**
Activated NK cells CD3-CD56+CD57+	**0.42**	
Dendritic cells Lyn-HLADR+	**0.51**	**0,3-0,9%**
Myeloid DC Lyn-HLADR+CD11c+	**0.01**	
Plasmacytoid DC Lyn-HLADR+CD123+	**0.23**	
pDC/mDC	**20.92**	**3-9**
Ne/Li	**25.24**	**1-3**

*DC-dendritic cells, pDC- Plasmacytoid dendritic cells, mDC- Myeloid dendritic cells, Ne/Li- *neutrophil to lymphocyte ratio*

DISCUSSION

There are different explanations for the lethal outcome in this patient. The study on a large number of COVID-19 patients has shown that 76.4% patients 18-65 years old, who were on the mechanical ventilation, have died, while the mortality rate of patients in the same age group, without MV, was 19.8% (8). Our patient was 408 hours mechanically ventilated during the hospitalization, which might potentially increase the risk of the fatal outcome. Also, it is hypothesized that the presence of multiple pathogens can influence the progression of the present viral respiratory infection, which was the case in this patient with Acinetobacter spp superinfection (9). Furthermore, it is important to pay attention to the fact that the other three family members of the presented patient had only mild symptoms even though all of them were infected by the same source, almost at the same time and none of them had history of previous diseases. The reason for that may lie in the genetic susceptibility, since there are papers that described similar outcome in blood-related family members (10,11). It could be argued that the father was the only one with a specific genetic predisposition to this illness, while children and their mother had similar genetic predisposition and thus similar clinical presentation and good outcome. Finally, the outcome may be the result of the cytokine storm which is observed in COVID-19 patients and not easy to treat (12). It is described that the cytokine storm contributes to the severity of COVID-19 infection, but more importantly, depletion of CD8+ lymphocytes and high levels of CRP and D-dimer were marked as important markers (13). In our patients, there were noticeable high levels of CRP and D-dimer, as well as the lower count of CD8+ lymphocytes, which confirms the suspicion of the cytokine storm presence and its influence on the outcome.

CONCLUSION

COVID-19 is an unpredictable pandemic infection with possible serious complications such as severe pneumonia, ARDS and death. Further studies are needed to explain the mechanisms that lead to the progression of the disease and its complications, in order to prevent them. More importantly, this case report should give clinicians the insight into the treatment of healthy individuals with COVID-19, especially in terms of possible laboratory markers that could indicate the presence of the cytokine storm phenomenon.

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REFERENCES

1. World Health Organization. World experts and funders set priorities for COVID-19 research. Available online: https://www.who.int/news-room/detail/12-02-2020-world-experts-and-funders-set-priorities-for-covid-19-research
2. Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, et al. Clinical Characteristics of Coronavirus Disease 2019 in China. N Engl J Med 2020; 382(18):1708-1720. doi:10.1056/NEJMoa2002032.
3. Milovanović DR, Janković SM, Ružić Zečević D, Folić M, Rosić N, Jovanović D et al. Lečenje koronavirusne bolesti (COVID-19). Medicinski časopis 2020; 54(1). DOI: https://doi.org/10.5937/mckg54-25981. [Online First]
4. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J et al. A Novel Coronavirus from Patients with Pneumonia in China, 2019. N Engl J Med 2020; 382(8):727-733.
5. Deng Z, Hu Y, Yang P, et al. Diagnosis and treatment of an acute severe pneumonia patient with COVID-19: Case report [published online ahead of print, 2020 Mar 30]. *J Med Virol* 2020;10.1002/jmv.25802. doi:10.1002/jmv.25802
6. Korona virus COVID-19. Available online: https://covid19.rs
7. The First Affiliated Hospital, Zhejiang University School of Medicine. (2020). Handbook of COVID-19 Prevention and Treatment. Jack Ma Foundation and Alibaba Foundation.
8. Richardson S, Hirsch JS, Narasimhan M, Crawford JM, McGinn T, Davidson KWet al. Presenting Characteristics, Comorbidities, and Outcomes Among 5700 Patients Hospitalized With COVID-19 in the New York City Area. JAMA 2020. doi: 10.1001/jama.2020.6775. [Epub ahead of print]
9. Edrada EM, Lopez EB, Villarama JB, Salva Villarama EP, Dagoc BF, Smith C et al. First COVID-19 infections in the Philippines: a case report. Trop Med Health 2020;48:21.
10. Yousefzadegan S, Rezaei N. Case Report: Death Due to Novel Coronavirus Disease (COVID-19) in Three Brothers. Am J Trop Med Hyg 2020. doi: 10.4269/ajtmh.20-0240. [Epub ahead of print]
11. Chen S, Yin Q, Shi H, et al. A familial cluster, including a kidney transplant recipient, of Coronavirus Disease 2019 (COVID-19) in Wuhan, China. Am J Transplant 2020;20(7):1869-1874. doi:10.1111/ajt.15903
12. Douedi S, Miskoff J. Novel coronavirus 2019 (COVID-19): A case report and review of treatments. Medicine (Baltimore) 2020;99(19):e20207.
13. Kuppalli K, Rasmussen AL. A glimpse into the eye of the COVID-19 cytokine storm. EBioMedicine 2020;55:102789. doi: 10.1016/j.ebiom.2020.102789. [Epub ahead of print]

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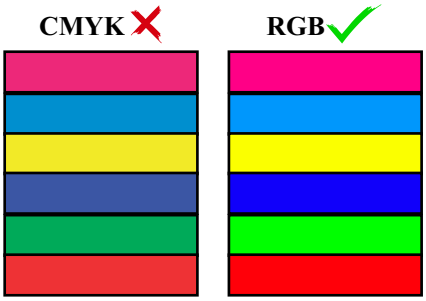

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