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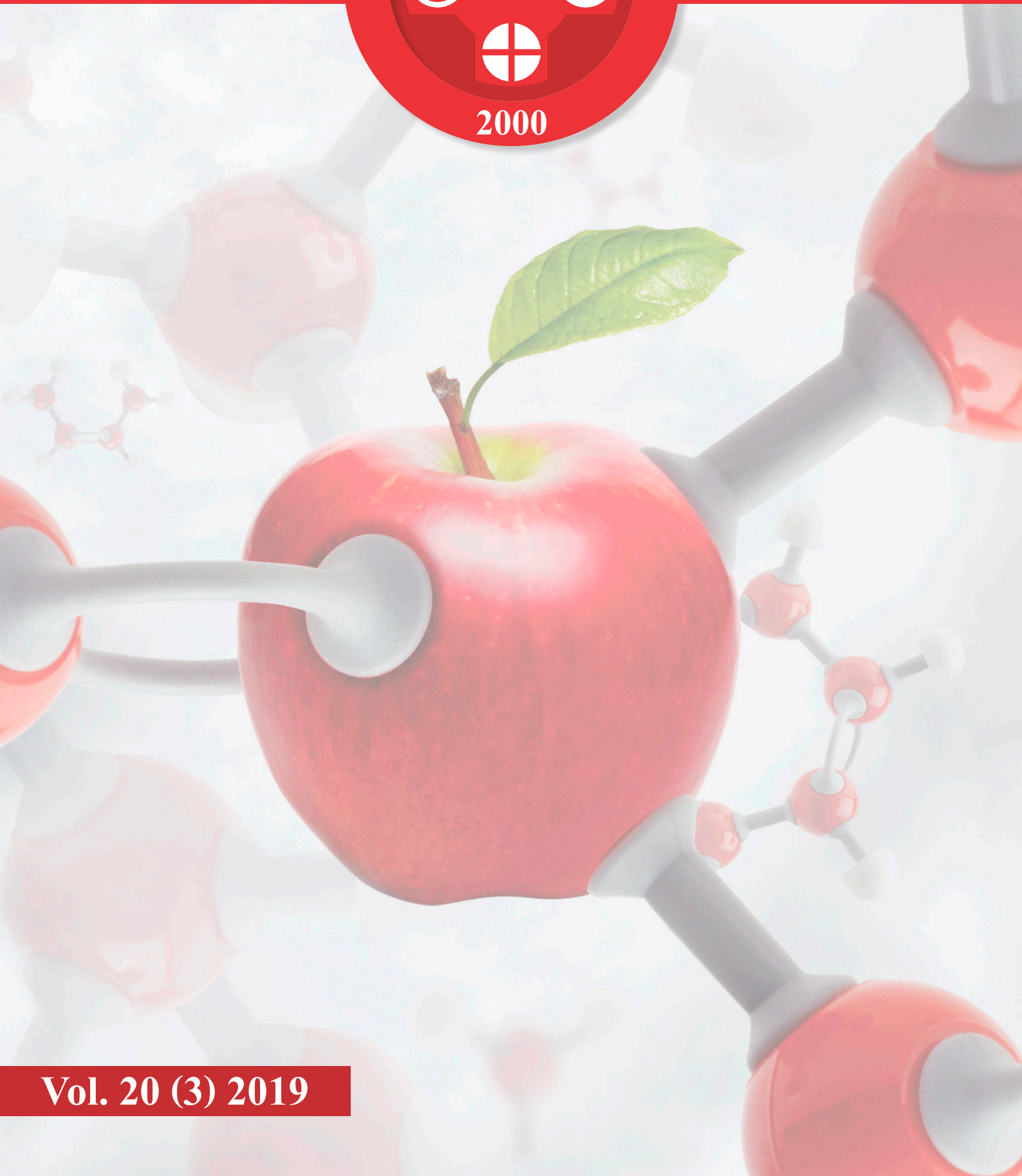

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TERAPIJSKI POTENCIJAL "EGZOZOMALNIH MULTIPLIH ALOGENIH PROTEINA ZA PARAKRINU SIGNALIZACIJU, EGZOZOM D-MAPPS" JE ZASNOVAN NA EFEKTIMA EGZOZOMA, IMUNOSUPRESIVNIH I TROFIČKIH FAKTORA

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ABSTRACT

Due to their differentiation capacity and potent immunosuppressive and pro-angiogenic properties, mesenchymal stem cells (MSCs) have been considered as new therapeutic agents in regenerative medicine. Since most of MSC-mediated beneficent effects are a consequence of their paracrine action, we designed MSC-based product "Exosomes Derived Multiple Allogeneic Proteins Paracrine Signaling (Exosomes d-MAPPS), which activity is based on MSCs-derived growth factors and immunomodulatory cytokines capable to attenuate inflammation and to promote regeneration of injured tissues. Interleukin 1 receptor antagonist (IL-1Ra) and IL-27 were found in high concentrations in Exosomes d-MAPPS samples indicating strong anti-inflammatory and immunosuppressive potential of Exosomes d-MAPPS. Additionally, high concentrations of vascular endothelial growth factor receptor (VEGFR1) and chemokines (CXCL16, CCL21, CXCL14) were noticed at Exosomes d-MAPPS samples suggesting their potential to promote generation of new blood vessels and migration of CXCR6, CCR7 and CXCR4 expressing cells. Since all proteins which were found in high concentration in Exosomes d-MAPPS samples (IL-1Ra, CXCL16, CXCL14, CCL21, IL-27 and VEGFR1) are involved in modulation of lung, eye, and synovial inflammation, Exosomes d-MAPPS samples were prepared as inhalation and ophthalmic solutions in addition to injection formulations; their application in several patients suffering from chronic obstructive pulmonary disease, osteoarthritis, and dry eye syndrome resulted with significant improvement of biochemical and functional parameters. In conclusion, Exosomes d-MAPPS, due to the presence of important anti-inflammatory, immunomodulatory, and pro-angiogenic factors, represents potentially new therapeutic agent in regenerative medicine that should be further tested in large clinical studies.

Keywords: *mesenchymal stem cells, therapy, regeneration, immunosuppression, differentiation*

SAŽETAK

Mezenhimalne matične ćelije (MSCs), se zbog svojih imunomodulatornih i proangiogenskih karakteristika, primenjuju u regenerativnoj medicini. Kako MSCs parakrinim mehanizmom ostvaruju svoje imunomodulatorne i proangiogenske efekte, dizajnirali smo produkt „Egzozomalni multipli alogeni proteini za parakrinu signalizaciju (Egzozom d-MAPPS)", koji sadrži egzozome, faktore rasta i citokine koje produkuju MSCs i njima smanjuju inflamaciju i pospešuju regeneraciju oštećenog tkiva.

Antagonist receptora IL-1 (IL-1 Ra) i interleukin (IL)-27, su pronađeni u visokim koncentracijama u ovom produktu, što je ukazivalo na snažan antiinflamacijski i imunosupresivni potencijal Egzozom d-MAPPS. Uz to, u Egzozom d-MAPPS je zabeležena i visoka koncentracija receptora za vaskularni endotelijalni faktor rasta (engl. vascular endothelial growth factor receptor, VEGFR1), kao i hemokina (CXCL16, CCL21, CXCL14), što ukazuje na potencijal Egzozom d-MAPPS da indukuje neo-angiogenezu i pospeši migraciju ćelija koje ekprimiraju CXCR6, CCR7 i CXCR4.

Pošto su sve komponente Egzozom d-MAPPS (IL-1Ra, CXCL16, CXCL14, CCL21, IL-27 i VEGFR1) uključene u modulaciju zapaljenja pluca, oka i zglobova, Egzozom d-MAPPS smo davali, u vidu inhalacionih rastvora, kapi za oči, ili intraartikularnih injekcija, pacijentima obolelih od hronične opstruktivne bolesti pluća, osteoartritisa i sindroma suvog oka. Preliminarni rezultati su pokazali značajno poboljšanje kako funkcionalnih tako i biohemijskih parametara nakon primene Egzozom d-MAPPS.

Egzozom D-MAPPS, zbog egzozoma, antiinflamacijskih, imunomodulatornih i proangiogenskih faktora može da predstavlja nov terapijski agens u regenerativnoj medicini i njegov terapijski potencijal treba da se detaljnije ispita u kliničkim studijama sa velikim brojem pacijenata.

Ključne reči: *mezenhimalne matične ćelije, terapija, regeneracija, imunosupresija, diferencijacija*

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INTRODUCTION

Mesenchymal stem cells (MSCs) are adult, self-renewable stem cells which are, due to their differentiation capacity and immunomodulatory characteristics, used as new therapeutic agents in regenerative medicine (1-3). MSCs are fibroblast-like cells that express: CD105 (endoglin, also identified as SH2, a component of the receptor complex of transforming growth factor- beta (TGF-β) involved in proliferation, differentiation, and migration), CD73 (SH3/4, ectoenzyme that regulates the purinergic signaling through the hydrolysis of adenosine triphosphate (ATP)), CD44 (hyaluronan receptor involved in migration), CD90 (Thy-1, regulates differentiation of MSCs) [4]. Importantly, MSCs do not express CD14 (marker of monocytes), CD34 (marker of hematopoietic cells), CD45 (pan-leukocyte marker), CD79a and CD19 (marker of B lymphocytes) and lack expression of major histocompatibility complex (MHC) class II and co-stimulatory molecules, CD80 (B7-1), CD86 (B7-2), and CD40, suggesting a low immunogenicity *in vitro* and *in vivo* and their potential for safe allogeneic transplantation (5, 6).

MSCs have substantial differentiation potential. In addition to the cells of mesodermal origin (osteoblasts, chondroblasts, and adipocytes), MSCs are capable of generating neural cells, hepatocytes, alveolar epithelial cells, insulin-producing cells, cardiomyocytes, indicating their clinical application (7-14). Several lines of evidence suggested that MSCs have capacity to differentiate into functional cardiomyocytes (9), hepatocytes (10, 11), alveolar epithelial cells and lung precursor cells (13) contributing to the regeneration of injured myocardium, liver and lungs (12, 15-17). Additionally, in paracrine manner, through the production of immunomodulatory factors (indoleamine 2,3-dioxygenase (IDO), prostaglandin E2 (PGE2), nitric oxide (NO), transforming growth factor beta (TGF-β), interleukin (IL)-10, interleukin 1 receptor antagonist (IL-1Ra) and growth related oncogene (GRO), MSCs are able to suppress detrimental autoimmune response and to attenuate autoimmune and chronic, inflammatory diseases (3, 18, 19).

In addition to their immunomodulatory characteristics [3, 18, 19], MSCs may promote angiogenesis, as well. Through the production of several pro-angiogenic factors (vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), TGF-β, platelet-derived growth factor (PDGF), angiopoietin-1, placental growth factor (PGF), IL-6, monocyte chemotactic protein-1 (MCP-1), epidermal growth factor (EGF)), MSCs induce generation of new blood vessels having beneficial effects in the therapy of degenerative and ischemic cardiovascular and neurodegenerative diseases (20).

It was recently revealed that immunomodulatory and pro-angiogenic paracrine effects of MSCs are, at least partially, mediated by MSC-derived exosomes: nano-sized extracellular vesicles that deliver proteins, lipids, DNA fragments, mRNA to the target cells: immune cells, endothelial cells (ECs), pericytes and other tissue-resident cells (21). MSC-derived exosomes, released into the extracellular milieu, can be either taken up by neighboring cells, (residing in the microenvironment of engrafted MSCs) or may be carried to distant sites via biological fluids where, in endocrine manner, modulate function of target cells (21, 22).

MSCs reside in perivascular niches of many diverse tissues and organs (bone marrow (BM), adipose tissue (AT), peripheral blood (PB), lungs, bone, heart, dental pulp (DP), amniotic fluid (AF), placenta (PL), chorion membrane (CM), chorion vili (CV), umbilical cord (UC), Wharton's jelly (WJ)) (23). Differences in extracellular milieu (influence of neighboring cells and their products, hypoxia) as well as intracellular conditions (expression of certain micro RNAs) significantly affect function and therapeutic potential of MSCs (23).

Several lines of evidence suggest that MSCs derived from placental tissues have superior cell biological properties such as improved proliferative capacity, life span and differentiation potential than MSCs derived from adult tissues. PL-MSCs have a higher expansion and engraftment capacity than BM-MSC [23]. Moreover, clonal subpopulations of PL-MSCs have been attributed with the potential to differentiate into tissues from all three germ layers. Accordingly, due to their capacity for neuronal differentiation, PL-MSCs have been proposed as one of the main candidates for stem cell therapy of multiple sclerosis, nerve injuries, and sensorineural hearing loss (23-26).

Ethical concerns related to the derivation of PL-MSCs should be disregarded by the fact that placental tissues are normally considered medical waste and can be recovered without harm to the donor or fetus (27). Bearing in mind the simplicity of the harvesting procedure for isolation of PL-MSCs and their huge therapeutic potential (28, 29), we recently developed: "Exosomes Derived Multiple Allogeneic Proteins Paracrine Signaling, Exosomes d-MAPPS", biological product which activity is based on placental derived biomaterials, growth factors, and immunomodulatory cytokines capable to attenuate inflammation and to promote regeneration of injured tissues. Herewith, we analyzed and discussed in detail concentrations of bio-active molecules in Exosomes d-MAPPS emphasizing its therapeutic potential in regenerative medicine.

MATERIAL AND METHODS

Exosomes d-MAPPS sample acquisition

Sterile Exosomes d-MAPPS is an engineered biologic product obtained from placental tissue, previously collected from healthy human donors. Blood samples were given by the donor prior to or at the time of collection and were tested by laboratories certified under the Clinical Laboratory Improvement Amendments (CLIA) and were found negative using United States (U.S) Food and Drug Administration (FDA) licensed tests for detection of at minimum: Hepatitis B Virus, Hepatitis C Virus, Human Immunodeficiency Virus Types 1/2, Treponema Pallidum.

Placental tissue samples were obtained with patient consent as well as institutional ethical approval and kept at 4°C until processed.

Exosomes d-MAPPS sample was engineered as a sterile product, manufactured under current Good Manufacturing Practices (cGMP) regulated and reviewed by the FDA. Sterile Exosomes d-MAPPS sample incorporate Regenerative Processing Plant's (RPP) proprietary patented sterilization process to provide safe sterile product.

Exosomes d-MAPPS samples, used in this study, were manufactured under specific conditions in order to be applicable for bioavailability testing and for different therapeutic use.

Determination of cytokines, chemokines, growth factors and their receptors in Exosomes d-MAPPS samples

Concentrations of cytokines, chemokines, growth factors and their receptors in Exosomes d-MAPPS samples were determined as previously described (30). Briefly, about fifty milliliters of sample was concentrated to 1.0-ml protein with trichloroacetic acid. The acetone-washed protein pellet was resolubilized in urea, and proteins were processed with dithiothreitol and iodoacetamide and digested with trypsin. Tryptic peptides were quantified and 10 µg was loaded through pressure cell onto a biphasic column for online two-dimensional high-performance liquid chromatography (HPLC) separation (strong-cation exchange and reversed-phase) and concurrent analysis by nanospray using a hybrid mass spectrometer. Three salt cuts of 50, 100, and 500 mM ammonium acetate were performed per sample run, with each followed by a 120-min organic gradient to separate the peptides.

Resultant peptide fragmentation spectra were compared with proteome database concatenated with common contaminants and reversed sequences to control false discovery rates. Peptide spectrum matches (PSMs) were filtered and assigned matched-ion intensities (MITs) based on observed peptide fragment peaks. PSM MITs were summed on a per-peptide basis, and only those uniquely and specifically matching a particular protein were moved onto subsequent analysis. Briefly, peptide intensity distributions were log-transformed, normalized across biological replicates by LOESS, and standardized by median absolute deviation and mean centering across samples as suggested. Peptides were then filtered to maintain at least two hits in one replicate set, and missing values were imputed using a random distribution of low-level values. Peptide abundance trends for each protein were scaled to a specific, well-sampled reference peptide. Sample-to-sample variation was visualized by PCA, Pearson's correlation and hierarchically clustered using the Ward agglomeration method to generate a heat map of protein abundance trends normalized by z-score (30).

RESULTS

Exosomes d-MAPPS has strong anti-inflammatory and immunomodulatory potential

Since MSCs produce immunosuppressive and anti-inflammatory factors (3, 18, 19), we analyzed concentration of major MSC-derived immunomodulatory molecules in Exosomes d-MAPPS sample (Figure 1). For this purpose, levels of IDO, IL-1ra, IL-10, IL-4, IL-13, IL-18 binding protein (IL-18 Bpa), TGFβ1 and Latency associated peptide of TGFβ1 (LAP (TGFβ1), were measured (Figure 1A). Among

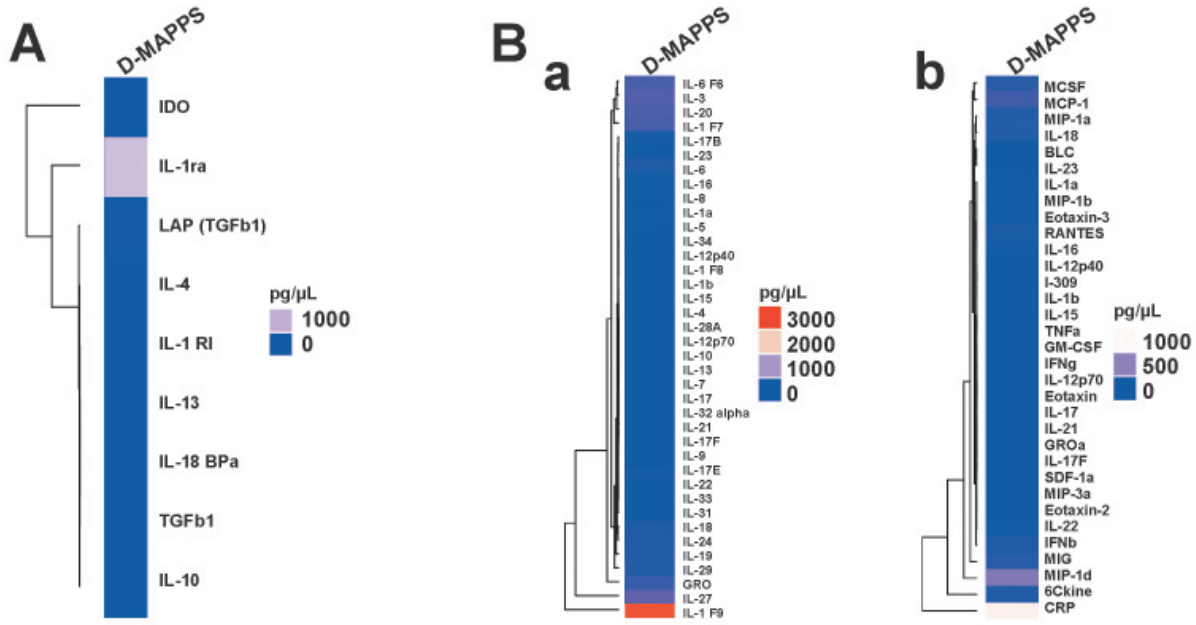

Figure 1: Inflammatory and immunomodulatory biomarkers in Exosomes d-MAPPS samples. (A) Different concentrations of 9 anti-inflammatory and immunomodulatory molecules are presented at heatmap. **(B)** Heatmap shows concentrations of 39 interleukins (a) and 33 inflammatory biomarkers (b) determined at Exosomes d-MAPPS sample.

measured immunoregulatory factors, IL-1Ra was found in high concentrations (1000 pg/μl). MSC-derived IL-1Ra is a naturally occurring cytokine which acts as an inhibitor of inflammatory cytokine IL-1. When IL-1Ra binds to the IL-1 receptor (IL-1R), binding of IL-1 is blocked and pro-inflammatory signal from IL-1 receptor is stopped. Accordingly, various pro-inflammatory events, initiated by IL-1:IL-1R binding, including the synthesis and releases of chemokines and enhanced influx of neutrophils, macrophages, and lymphocytes in inflamed tissues, are inhibited by IL-1Ra [4]. In line with these findings, high concentration of IL-1Ra, noticed in Exosomes d-MAPPS (Figure 1), indicates strong anti-inflammatory and immunomodulatory potential of this product.

In order to confirm strong anti-inflammatory properties of Exosomes d-MAPPS and to demonstrate that inflammatory mediators are not present in significant concentration in Exosomes d-MAPPS, levels of major inflammatory interleukins of innate and acquired immunity were evaluated (Figure 1B). As it is shown in Figure 1B, the main inflammatory cytokines of innate immunity (TNF-α, IL-1β, IL-12, IL-18) were not detected in Exosomes d-MAPPS sample. Similarly, Th1 (IFN-γ), Th2 (IL-4, IL-5, IL-10, IL-13) and Th17 (IL-17 and IL-22) cytokines were present in non-detectable concentrations indicating that neither one of T cell-dependent inflammatory pathways could not be elicited by Exosomes d-MAPPS. Among interleukins which might have dual (pro and anti-inflammatory) role (IL-6, IL-8, IL-27), only IL-27 was measured in Exosomes d-MAPPS sample (1000 pg/μl). IL-27 promotes regulatory and immunosuppressive effects of MSCs by enhancing MSC-dependent generation of IL-10 producing CD4+T cells within the population of activated helper T cells. Additionally, capacity of MSCs to induce apoptosis of inflammatory Th1 and Th17 cells in programmed death ligand 1 (PDL1) dependent manner is significantly enhanced by IL-27 (31). Accordingly, presence of IL-27 could be considered as an additional indicator of immunosuppressive properties and therapeutic potential of Exosomes d-MAPPS sample.

In addition to IL-27, IL-1F9 was noticed in high concentration in Exosomes d-MAPPS sample (3000 pg/μl; Figure 1Ba). Since currently there is no available information regarding the role of IL-1F9 in MSC-based immunomodulation, the role of this cytokine in Exosomes d-MAPPS-based effects should be explored and analyzed in further studies.

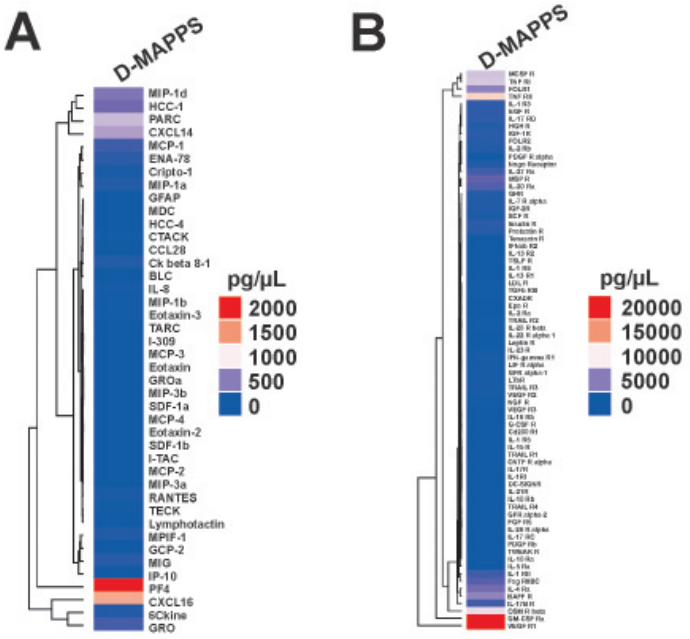

Figure 2: Chemokines and soluble receptors in Exosomes d-MAPPS sample. (A) Concentrations of 42 chemokines in Exosomes d-MAPPS samples are presented at heatmap. **(B)** Heatmap represents concentrations of 75 growth factor-related receptors and biomarkers in Exosomes d-MAPPS sample.

Exosomes d-MAPPS can promote migration of CXCR6, CCR7 and CXCR4 expressing cells

One of the main properties of MSCs is their homing capacity towards the site of the injury or inflammation where they, in juxtacrine and/or paracrine manner, suppress detrimental immune response and ongoing inflammation [3]. MSCs expressed chemokine-specific receptors (CXCR4, CX3CR1, CXCR6, CCR1, and CCR7) and are attracted by chemokines (CXCL12, CXCL14, CX3CL1, CXCL16, CCL3, CCL19, and CCL21) released from damaged tissues and inflammatory immune cells (32). Interestingly, MSCs are also able to produce chemokines which, in autocrine manner, enable migration of MSCs towards the site of injury or inflammation (32). In line with these observations, we measured high concentration of MSCs-derived chemokine CXCL16 in Exosomes d-MAPPS sample (1500 pg/μl) (Figure 2A). Since CXCR6, ligand for CXCL16, is highly expressed on MSCs and immune cells (memory/effector T cells, NK, NKT cells and plasma cells) (32), high concentration of this chemokine in Exosomes d-MAPPS sample strongly indicates that Exosomes d-MAPPS can be used as chemoattractant enabling migration of CXCR6 expressing cells into the inflamed or injured tissues.

Similarly, 6Ckine (CCL21) (ligand for CCR7 receptor) is measured at Exosomes d-MAPPS sample (500 pg/μl) (Figure 2A). Having in mind that CCL21:CCR7 axis is important for migration of MSCs in wounds, homing of naïve T cells in peripheral lymph nodes and for migration of antigen processing, activated DCs into peripheral lymph nodes and T cell-rich fields within injured lungs, synovia and eyes (33-38), high levels of CCL21 in Exosomes d-MAPPS, could be used for recruitment of CCR7 expressing MSCs and immune cells during Exosomes d-MAPPS-based modulation of skin/joint/eye/lung inflammatory diseases. In line with these findings, high concentration (2000pg/ml; Figure 2A) of platelet factor 4 (PF4), which is involved in tissue regeneration and wound repair (39), was noticed in Exosomes d-MAPPS sample confirming its potential therapeutic use in regenerative medicine.

CXCL14 was also detected in Exosomes d-MAPPS sample (500 pg/μl; Figure 2A). CXCL14 specifically binds to CXCR4 and, in a similar manner as CXCL12, is involved in CXCR4-dependant migration of MSCs into injured or inflamed tissues [40].

In addition to elevated levels of CXCL16, CCL21, PF4 and CXCL14, GRO-well known MSC-derived chemokine with strong immunosuppressive properties [41], has been detected in Exosomes d-MAPPS sample (500 pg/μl; Figure 2A). Human MSCs secrete GRO-γ which, accompanied with GRO-α, promote conversion of monocyte derived DCs (MDDCs) towards myeloid suppressive phenotype enabling generation of tolerogenic myeloid derived suppressor cells (MDSCs) (42). In line with these findings, presence of GRO in Exosomes d-MAPPS sample, strongly indicates its potential for *in vitro* generation of MDSCs and MDSCs-based cell therapy of autoimmune and chronic inflammatory diseases.

Exosomes d-MAPPS has the capacity to induce neovascularization in VEGF-dependent manner

Having in mind that generation of new blood vessels and re-vascularization are mainly responsible for MSC-dependent regeneration of ischemic tissues (20), we evaluated presence of angiogenesis-related growth factor receptors in Exosomes d-MAPPS sample in order to explore capacity of Exosomes d-MAPPS to induce neo-angiogenesis-based tissue regeneration. As it is shown in Figure 2B, high concentrations of VEGFR1 (20000 pg/μl) was determined in Exosomes d-MAPPS sample. VEGFR1 plays critical role in migration of MSCs and MSCs-based neo-angiogenesis [43]. VEGFR1 binds VEGF and is expressed by multiple bone marrow-derived cell types, including endothelial progenitor cells and MSCs. BM-derived endothelial progenitor cells and MSCs are mobilized into peripheral blood and recruited to the sites of ischemia in VEGFR1-dependent manner, where they participate in tissue repair and revascularization (42). Based on these results, it is highly expected that Exosomes d-MAPPS can modulate generation and maturation of BM-derived cells. In line with these observations are high concentrations of granulocyte-macrophage colony-stimulating factor receptor (GM-CSFR) which was also noticed in Exosomes d-MAPPS sample (20000 pg/μl; Figure 2B). Since signaling from GM-CSFR can promote an astonishing variety of cellular functions, including protection from apoptosis, progression through the cell cycle, early commitment to myelopoiesis, differentiation/maturation of committed progenitors, and multiple activation and motility functions in mature immune cells (44), Exosomes d-MAPPS can be used for controlled differentiation of BM-derived, GM-CSFR expressing cells.

DISCUSSION

Due to their differentiation capacity and potent immunosuppressive and pro-angiogenic properties, MSCs have been considered as new therapeutic agents in regenerative medicine (45, 46). Nevertheless, safety issues of MSCs-based therapy are still a matter of debate, especially in the long-term follow up (47). Several studies reported that transplanted MSCs, in response to the growth factors produced in the local microenvironment, differentiated into undesired tissues, mainly bone and cartilage (48, 49). Multiple areas of ossifications or calcifications were observed in infarcted myocardium after transplantation of MSCs (48, 49). Since most of MSC-mediated beneficent effects are consequence of their paracrine action, we designed Exosomes d-MAPPS, soluble product, which contains a broad number of MSC-derived immunomodulatory and pro-angiogenic factors (Figures 1-2). Among anti-inflammatory mediators IL-1Ra was presented in the highest concentrations in Exosomes d-MAPPS (Figure 1A). Recently, a well-characterized subpopulation of IL-1Ra expressing MSCs have been described (50). MSCs, in IL-1Ra dependent manner, were able to suppress inflammation and fibrosis in the lungs. Interestingly, therapeutic and anti-inflammatory effects of MSCs-overexpressing IL-1Ra were more effective than effects of recombinant IL-1Ra (50), indicating that MSC-derived IL-1Ra acts synergistically with other immunomodulatory cytokines and chemokines in suppression of immune response.

Among chemokines, CXCL16, CCL21 and CXCL14 were present in high concentrations in Exosomes d-MAPPS sample (Figure 2A). Each of these molecules is crucially involved in the pathogenesis of lung, synovial, and eye inflammation (51-57). Bronchial epithelium is an important source of CXCL16 (51) while its receptor CXCR6 is highly expressed on lung-infiltrated T cells (51). Moreover, an increased expression of CXCL16 was noticed in the lungs of bleomycin-treated mice while CXCR6 expression was markedly increased in the lung in patients with interstitial lung diseases (53), indicating the importance of CXCL16:CXCR6 axis in the pathogenesis of lung injury and inflammation. Similarly, CXCL16 plays an important role in recruitment of T cells in inflamed synovium of patients suffering from Rheumatoid arthritis, suggesting CXCL16 as a target molecule in biological therapy of Rheumatoid arthritis [54].

In similar manner as CXCL16, CCL21 significantly contributes to the recruitment of CCR7 expressing immune cells in inflamed synovia (55). Additionally, both CCL21 and CCR7 are significantly up-regulated in inflamed corneas [56]. CCL21 facilitate migration of inflammatory, antigen presenting dendritic cells (DCs) in CCR7 dependent manner from the cornea to draining lymph nodes, while local administration of anti-CCL21 may reduce recruitment of DCs resulting with the attenuation of corneal inflammation (56).

Smoking-induced expression of CXCL14 in the airway epithelium represents a novel potential molecular link between smoking-associated airway epithelial injury, chronic obstructive pulmonary disease (COPD) and lung cancer. Airway epithelium responds to cigarette smoking with altered CXCL14 gene expression, contributing to the dis-

ease-relevant phenotype that result with the development of COPD and lung cancer (57).

Since bone repair and regeneration depend on vasculogenesis and osteogenesis, both of these processes are essential for successful bone remodeling (58). Several lines of evidence suggest that pro-angiogenic VEGF-A and VEGFR1 play crucially important role in bone regeneration (59, 60). Despite the fact that VEGF-A: VEGFR1 axis could contribute to the regeneration of the bone, VEGFR1 mediated signaling contributes to the development of complications of ischemic retinopathies, including retinopathy of prematurity (ROP), age-related macular degeneration (AMD), and diabetic retinopathy (DR) (61). VEGFR1 expression was up-regulated during pathogenesis of choroidal neovascularization (CNV), a model of AMD. Accordingly, blockade of VEGFR1 suppresses pathological angiogenesis and vascular leakage in the eye (61).

IL-27, measured in high concentrations in Exosomes d-MAPPS sample (Figure 1B) has dual: immunomodulatory and angiostatic effect in the injured eye. It inhibits pathophysiological intraocular neovascularization by reducing VEGF production in macrophages (62) and at the same time attenuate ongoing inflammation by suppressing proliferation of IL-17 producing Th17 cells (63). In similar manner, PDF4, which was also present in high concentration in Exosomes d-MAPPS sample (Figure 2A), limits generation of Th17 cells and is crucially involved in suppression of Th17 immune response (64). In this way, we assume that Exosomes d-MAPPS, in IL-27 and PDF4-dependant manner, may attenuate potentially detrimental effects of VEGFR1 signaling in Th17 cell driven eye injury and inflammation.

Since all proteins which were found in high concentration in Exosomes d-MAPPS samples (IL-1Ra, CXCL16, CXCL14, CCL21, IL-27, PDF4 and VEGFR1) are involved in modulation of lung, eye and synovial inflammation, we analyzed effects of Exosomes d-MAPPS-based therapy in patients suffering from chronic inflammatory diseases (COPD, osteoarthritis and dry eye syndrome). Our preliminary results, obtained in several pilot trials, indicated that Exosomes d-MAPPS was well tolerated since none of undesired, side effects were observed in Exosomes d-MAPPS-treated patients. Importantly, in patients that received Exosomes d-MAPPS, we noticed significant attenuation of inflammation, accompanied with an improvement of biochemical and functional parameters of injured lungs, knees and eyes.

Having in mind that therapeutic effects of Exosomes d-MAPPS are attributed to the MSC-derived soluble factors which could be found within MSC-derived exosomes (65), we believe that, at least some of Exosomes d-MAPPS-mediated beneficent effects were related to the function of exosomes. Accumulating evidence has suggested that MSCs, via exosomes, suppress detrimental immune response, attenuate inflammation and promote tissue repair and regeneration (21). Compared with cells, exosomes have no risk of aneuploidy and are well tolerated by the immune system without the risk of rejection by immune cells after allogeneic transplantation (65). Additionally, due to their membrane-based structure, exosomes are able to cross the plasma membrane and to deliver their cargo into target cells throughout the body, indicating their potential to act in paracrine as well as endocrine manner (65). Accordingly, encouraging therapeutic effects of MSC-derived exosomes were observed in several animal models of organ specific and systemic inflammatory diseases, including acute and chronic injury of the eye and lungs (21, 66). It is well known that in early stage of corneal damage, injured epithelial cells produce IL-1 in order to elicit strong innate immune response (67). MSC-derived IL-1Ra, which was found in high concentration in Exosomes d-MAPPS sample (Figure 1A), binds to the IL-1R and prevents ongoing inflammation in the injured corneas. Similarly, it is well known that, in IL-1Ra dependent manner, MSCs are able to efficiently attenuate lung injury and inflammation (5). In line with these observations, we believe that IL-1Ra containing exosomes were responsible for the attenuated inflammation in Exosomes d-MAPPS-treated patients suffering from corneal injury and COPD.

In conclusion, Exosomes d-MAPPS, due to the presence of several important anti-inflammatory, immunomodulatory and pro-angiogenic factors, represents potentially a new therapeutic agent in regenerative medicine that should be further tested in large clinical studies.

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CONFLICT OF INTEREST

Dr. C. Randall Harrell and Dr. Crissy Fellabaum are employed at RPP.

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THE EFFECT OF THE CHRONIC ADMINISTRATION OF DPP4-INHIBITORS ON SYSTEMIC OXIDATIVE STRESS IN RATS WITH DIABETES TYPE 2

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UTICAJ HRONIČNE PRIMENE DPP4-INHIBITORA NA SISTEMSKI OKSIDACIONI STRES KOD PACOVA SA DIJABETESOM TIP 2

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ABSTRACT

Type 2 diabetes (T2DM) is characterized by well-preserved insulin secretion; however, the surrounding tissue is insensitive to insulin, resulting in increased blood glucose level due to the inability of tissues to convert glucose into energy. As a result of chronic non-regulation of glucose levels and high daily fluctuations in the blood, the micro- and macrovascular complications occur in these patients. Complications develop through two main mechanisms: induction of oxidative stress and innate immunity. In this regard, the aim of this study was to examine the effect of four week administration of DPP4 inhibitors (saxagliptin, sitagliptin and vildagliptin) to the parameters of oxidative stress and antioxidant defense in the group of rats with diabetes type 2 (T2DM). Sixty Wistar albino rats were divided randomly into 5 groups: group I: control healthy group; group II: rats with diabetes type 2; group III: rats with diabetes type 2 treated with 0.6 mg/kg of sitagliptin; group IV: rats with diabetes type 2 treated with 0.45 mg/kg of saxagliptin, group V: rats with diabetes type 2 treated with 9 mg/kg vildagliptin. The rats from experimental groups were fed with a high-fat diet for 4 weeks and after 6–8 h of starvation received one dose of streptozotocin (STZ) intraperitoneally (25 mg/kg body weight) to induce T2DM. Animals with fasting glucose above 7 mmol / L and insulin over 6 mmol / L were included in the study as rats with T2DM. Upon completion of the experiments, the blood was collected from the anesthetized animals and used for sphectrophotometrical determination of parameters of oxidative stress, and antioxidative defense. T2DM induced significant increase in production of reacitve oxygen species (ROS) (superoxide anion radical and hydrogen peroxide), but additional four-week administration of gliptins induced decrease in ROS values. On the other hand, T2DM induced decrease of nitric oxide, superoxide dismutase, catalaze, and reduced gluthation and concomitant therapy with gliptins induced increase of these parametars, suggesting significant antioxidant potential of this group of drugs.

Keywords: *type 2 diabetes, saxagliptin, sitagliptin, vildagliptin, oxidative stress, antioxidant protection*

SAŽETAK

Dijabetes tip 2 (T2DM) karakteriše očuvana sekrecija insulina; međutim okolno tkivo je neosetljivo na dejstvo insulina, što dovodi do povećanja nivoa glukoze usled nemogućnosti tkiva da pretvori glukozu u energiju. Kao posledica hronične neregulacije nivoa glukoze i visokih dnevnih fluktuacija u krvi, mikro- i makrovaskularne komplikacije se javljaju kod ovih pacijenata. Komplikacije se razvijaju kroz dva osnovna mehanizma: indukciju oksidacionog stresa i urođenog imuniteta. S tim u vezi, cilj ove studije bio je da se ispita efekat primene dipeptidil-peptidaza 4 (DPP4)-inhibitora saksagliptina, sitagliptina i vildagliptina na parametre oksidacionog stresa i antioksidacione odbrane u grupi pacova sa dijabetesom tip 2. Šezdeset Wistar albino pacova je nasumično podeljeno u 5 grupa: grupa I: kontrolna zdrava grupa; grupa II: pacovi sa dijabetesom tip 2; grupa III: pacovi sa dijabetesom tip 2 tretirani sa 0,6 mg/kg sitagliptina; grupa IV: pacovi sa dijabetesom tip 2 tretirani sa 0,45 mg/kg saksagliptina, grupa V: pacovi sa dijabetesom tip 2 tretirani sa 9 mg/kg vildagliptina. Pacovi iz eksperimentalnih grupa su 4 nedelje hranjeni hranom sa visokim sadržajem masti, a potom su nakon 6-8 sati gladovanja primili jednu dozu streptozotocina (STZ) intraperitonealno (25 mg/kg telesne težine) kako bi se indukovao T2DM. U dalju studiju su uključene životinje sa glukozom natašte iznad 7 mmol/L i insulinom preko 6 mmol/L, kao pacovi koji su razvili T2DM. Po završetku eksperimenata, krv je sakupljena od anesteziranih životinja i korišćena za spektrofotometrijsko određivanje parametara oksidacionog stresa i antioksidacione zaštite. T2DM je indukovao značajno povećanje proizvodnje reaktivnih kiseoničnih vrsta (ROS) (superoksid anjon radikal i vodonik peroksid), ali sa druge strane četvoronedeljna primena gliptina je dovela do smanjenja vrednosti ROS. Dodatno, T2DM je indukovao smanjenje azotmonoksida, superoksid dismutaze, katalaze i redukovanog glutationa, dok su gliptini doveli do povećanja ovih parametara, što govori u prilog značajnom antioksidacionom potencijalu ove grupe lekova.

Ključne reči: *dijabetes tip 2, saksagliptin, sitagliptin, vildagliptin, oksidacioni stres, antioksidaciona zaštita*

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INTRODUCTION

Diabetes belongs to a group of metabolic diseases whose main characteristic is hyperglycaemia due to the lack of secretion and/or action of insulin (1). The prevalence of this disease globally is around 8.3%, or as many as 415 million people are already diagnosed with diabetes and this number is increasing year by year especially in the developed countries (2). Diabetes Mellitus type 2 is much more common (95% of people have this type of diabetes). There is insulin secretion, but the surrounding tissue is insulin-insensitive, which results in an increase in glucose levels due to the tissue's inability to convert the available glucose into energy (3).

As a consequence of chronic non-regulation of glucose levels and high daily glucose fluctuations, micro- and macrovascular complications occur in these patients in a high percentage (4). Complications arise through two basic mechanisms: induction of oxidative stress and induction of innate immunity (5, 6). More recent studies have shown that daily fluctuations in glucose levels contribute much more to the onset of oxidative stress than chronic hyperglycemia (7). Due to the foregoing, all factors must be taken into account in order to achieve adequate control when administering antidiabetics. It is unknown whether drugs that regulate daily glucose fluctuations would have any specific benefits (reduction of oxidative stress and inflammation) compared to conventional therapy monitoring glycated hemoglobin levels as the gold standard of glycemic control. Oxidative stress in diabetes occurs as a consequence of free radical formation due to glucose oxidation, non-enzymatic glycosylation of proteins, and oxidative degradation of glycolized proteins (8, 9).

Traditionally, metformin has been used as the first drug of choice in these conditions; however, its effectiveness decreases with age, so it is necessary to administer a large number of drugs for good glycemic control (10). Gliptins (dipeptidyl peptidase 4 - DPP4 inhibitors) represent one of the additional drug groups used in diabetes therapy. Since the introduction of sitagliptin in clinical practice, DPP4 inhibitors have been increasingly used in Diabetes Mellitus type 2 therapy. DPP4 inhibitors have been a large diverse group of drugs, divided into two narrow groups: peptidomimetics (e.g. saxagliptin and vildagliptin) and non-peptidomimetics (e.g. sitagliptin). Both of these groups act as competitive reversible inhibitors of DPP4, with peptidomimetics containing a nitrile group and forming a reversible covalent bond between drug and enzyme, the drug gradually and slowly dissociates from the complex to achieve long-term inhibitory activity, even after drug inactivation. On the other hand, non-peptidomimetics create non-covalent bonds with the catalytic domain of the enzyme, producing strong and immediate inhibition (11, 12). The difference in structure not only leads to a difference in the mechanism of action, but also to a difference in their metabolic pathways, as well as in the dosage range and dosage regimen (13). Given the fact that there are differences in daily fluctuations in glucose levels in patients using different DPP4 inhibitors, the question is whether the organism's response to oxidative stress in these patients is also different (14). In this regard, the aim of this study was to investigate the effect of administration of different drugs from the DPP4 inhibitor group on the parameters of oxidative stress and antioxidant protection in animals with type 2 diabetes.

MATERIALS AND METHODS

The material used in the study

Streptozotocin (MW=265.221), sitagliptin (MW= 523.32), saxagliptin (MW = 315.41) and vildagliptin (MW= 303.399) were purchased from Sigma-Aldrich Chemie GmbH Eschenstr. 5, 82024 Taufkircen, Germany.

Induction of Diabetes Mellitus Type 2

In the experimental protocol, 6-week-old animals were used (average body weight of 200 ± 20 grams). To induce type 2 diabetes, the animals were fed a high fat diet (HFd) for four weeks. After 4 weeks on HFd and after a 12-hour overnight fast, streptozotocin was administered at a single dose of 25 mg/kg. Three days (72 hours) later, glucose and insulin were measured. Animals with a fasting blood glucose level more than 7 mmol/L and the fasting insulin level above 6 mg/dL were included in the study and were considered as the rats with type 2 diabetes (15, 16).

The experimental treatment protocol for DPP4 inhibitors

Animals that developed diabetes type 2 (according to the previously described criteria), were divided into four experimental groups and one control group. Animals in the experimental groups (3 of 4) received DPP4 inhibitors intraperitoneally once daily for three weeks at the following doses: sitagliptin 0.6 mg/kg body weight, saxagliptin 0.45 mg/kg body weight, vildagliptin 9 mg/kg body weight. The last experimental group was not treated pharmacologically.

Experimental groups

Male Wistar albino rats, 60 animals (12 per group), were used for the study. Animals were housed in cages (four in each) in a vivarium with controlled conditions of humidity, temperature (22 ± 20 S) and light (12/12 hour light / dark cycle). At the time of completion of the experimental protocol, all animals were 13 weeks old. Animals were divided into five groups. The first group consisted of healthy animals using the standard diet (9% fat, 20% protein, 53% starch, 5% fiber), the remaining four groups consisted of type 2 diabetic animals using the high fat diet (25% fat, 15% protein, 51% starch, 5% fiber). In these four groups the animals differed in pharmacological treatment.

Groups:

1. Healthy Animals - Con
2. Animals with type 2 diabetes without pharmacological treatment - DM
3. Animals with type 2 diabetes and 0.6 mg/kg bodyweight sitagliptin therapy - DM + Sit
4. Animals with type 2 diabetes and 0.45 mg/kg body weight saxagliptin therapy - DM + Sak
5. Animals with type 2 diabetes and therapy and vildagliptin 9 mg/kg body weight - DM + Vld

Biochemical analyses

After completing experimental protocol, which lasted seven weeks, anesthetized animals were bled for analysis of parameters of oxidative stress and antioxidant protection. Citrate glass tubes were used for blood sampling. Immediately after collecting the sample, the sample is centrifuged to separate plasma, while the rest is rinsed in order to obtain a lysate of erythrocytes to determine the parameters of the antioxidative protection. The samples thus obtained were stored at -80^0 C until carrying out the predicted analyzes.

Plasma was used for the determination of oxidative stress markers; in these samples the concentrations of the tested parameters were determined as follows:

- the determination of superoxide anion radical (O2-) - is based on the reaction of O_2^- with nitro blue tetrazolium (Nitro Blue Tetrazolium - NBT) to nitroformazan blue. The measurement is performed at a wavelength λ = 550nm (17).
- hydrogen peroxide determination (H_2O_2) - is based on the oxidation of phenol red by hydrogen peroxide. This reaction is catalyzed by the horse radish peroxidase enzyme (HorserRadishPerOxidase - HRPO). This reaction results in the formation of a compound whose absorption maximum at a wavelength λ = 610nm (18).
- determination of nitrite (NO_2^-) - is based on the use of the Griess reagent, which builds with nitrites diazo-violet complex. After color stabilization at room temperature for 5-10 minutes approach to determining the concentration of nitrite liberated by spectrophotometry at a wavelength of λ = 550 nm (19).
- determination of lipid peroxidation index (TBARS) - is based on indirect determination by detection of the products of the lipid peroxidation reaction with thiobarbituric acid, hence the abbreviation TBARS (Thiobarbituric Acid Reactive Substances). The method is based on the determination of lipid peroxide levels based on the reaction of one of them, malonildialdehyde (MDA) with thiobarbituric acid (TBA). Determination is carried out spectrophotometrically at a wavelength of λ = 530nm (20).

For the determination of markers of antioxidant defense, a lysate of erythrocytes is used; in these samples the concentrations of test parameters were determined on the following way:

- determination of catalase activity (CAT) - after dilution of lysate with distilled water in a ratio of 1: 7 and addition of ethanol in a ratio of 0.6: 1, a further procedure was started. 50 µl of CAT buffer, 100 µl of sample and 1 ml of 10 mM H_2O_2 were placed in a test tube and measurement of samples at λ = 360 nm wavelength was started (21).
- determination of superoxide dismutase (SOD) activity - is carried out by the epinephrine method according to Beutler. Mixing 100 ml of lysate and 1 ml of carbonate buffer, initiating the process, followed by the addition of 100 ml of epinephrine. Measurements are made spectrophotometrically at a wavelength λ = 470nm (22).
- determination of reduced glutathione (GSH) - is based on the reaction of glutathione oxidation with 5,5-dithiobis-6,2-nitrobenzoic acid, by the Beutler method. The activity of the antioxidant molecule of reduced glutathione was measured by a spectrophotometric method at a wavelength λ = 450nm (23).

Statistical data processing

To test for differences between parameters, depending on their statistical normality, Student's t-test, Paired t-test, Mann-Whitney test, Fisher's absolute probability test, one-factor or two-factor analysis of variance will be used. When testing the difference between the parameters, in the case of the existence of multiple sub-groups, Bonferroni test is used. Statistical analysis was performed in the statistical package SPSS 20.0 for Windows (Statistical Package for the Social Sciences).

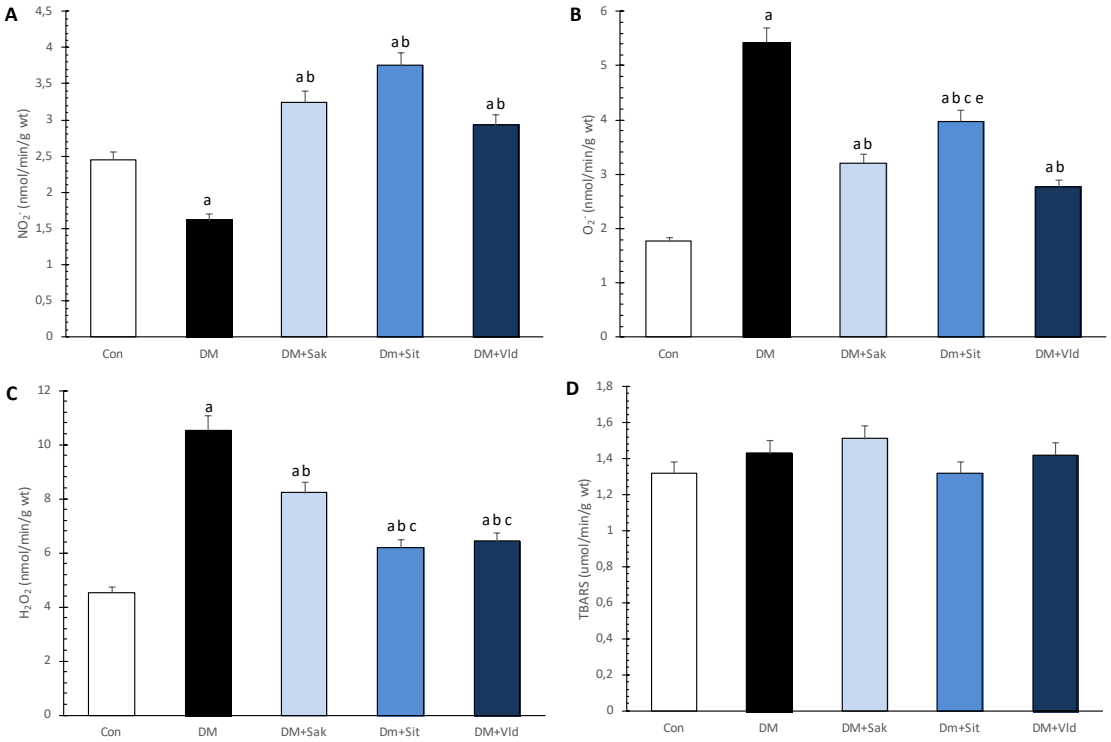
RESULTS

Effect of chronic DPP4 inhibitor administration on oxidative stress parameters

Induction of type 2 diabetes led to a decrease in the value of indirectly measured nitrite monoxide (Figure 1A). However, a four-week administration of all three drugs from the DPP4 inhibitor group resulted in an increase in nitrite values, which were statistically significantly higher than the control (Figure 1A). The highest nitrite value was observed in the rat group treated with sitagliptin. However, this value was not statistically higher than in the groups treated with the remaining two drugs (Figure 1A). Induction of type 2 diabetes led to a significant increase in the value of superoxide anion radical relative to healthy animals without diabetes (Figure 1B). Four weeks administration of all three drugs from the DPP4 inhibitor group resulted in a significant decrease in the superoxide anion radical value (Figure 1B). All three drugs reduced the levels of superoxide anion radicals, but this time there were differences in their effects, the greatest decrease was observed in the vildaglitin group, while sitagliptin showed the smallest effect on the decrease of these values, which was significant (Figure 1B). Although all three drugs reduced the levels of superoxide anion radicals, none of the

mentioned drugs managed to override these values sufficiently to bring them closer to the values measured in the group of healthy animals (Figure 1B). Induction of type 2 diabetes also led to an increase in hydrogen peroxide values relative to the values of this parameter in non-diabetic animals (Figure 1C). Four weeks administration of all three drugs in the DPP4 inhibitor group led to a significant decrease in the value of hydrogen peroxide (Figure 1C). There was a significant difference in the effects of saxagliptin relative to sitagliptin and vildagliptin; namely, saxagliptin led to the slightest decrease in hydrogen peroxide values (Figure 1C). None of these three applied DPP4 inhibitors succeeded to reduce the values of hydrogen peroxide to those measured in the control group (Figure 1C). Index of lipid peroxidation did not differ significantly between groups, regardless of disease induction and administration of DPP4 inhibitor values remained similar to those in healthy animals.

Figure 1. The effects of DPP-4 inhibitors on systemic oxidative stress parameters



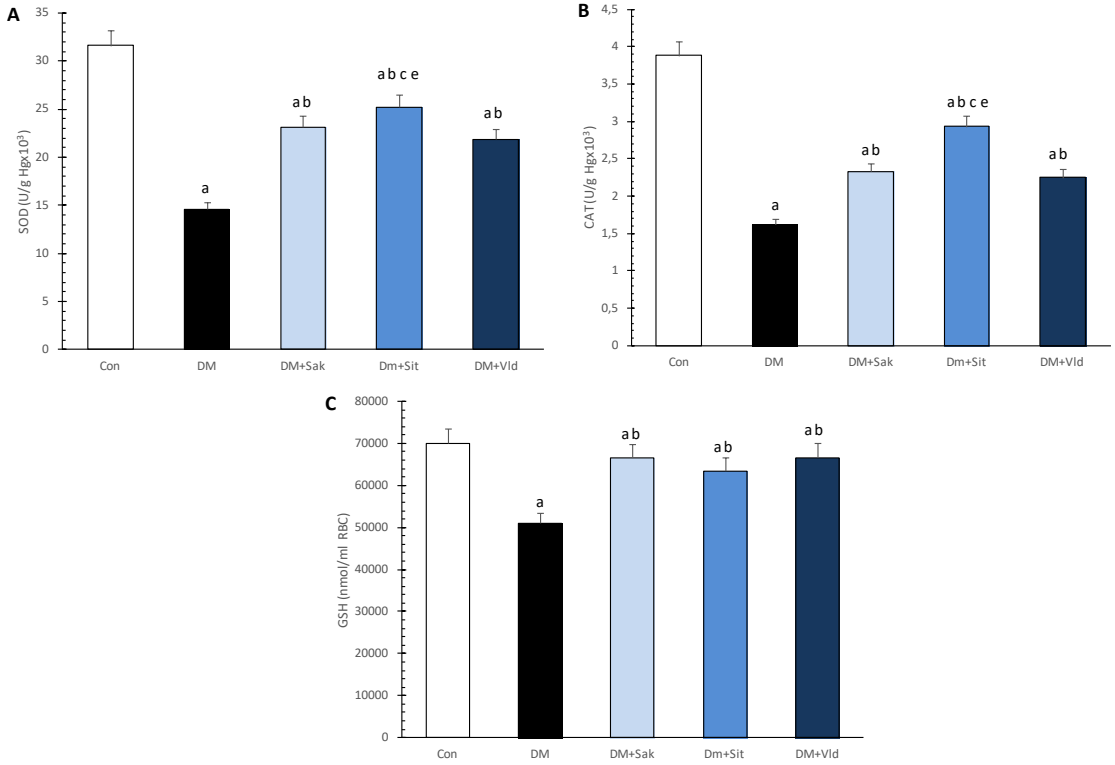
a - statistical significance of other groups in relation to control; b - statistical significance of other groups in relation to DM; c - statistical significance of other groups with respect to DM + sak; d - statistical significance of other groups with respect to DM + sit; e - statistical significance of other groups with respect to DM + vld. Results of mean ± SD, n = 12 per group are presented.

The effect of chronic administration of DPP4 inhibitors on antioxidant protection parameters

Induction of type 2 diabetes led to a significant decrease in the value of superoxide dismutase relative to the control group (Figure 2A). Administration of all three drugs from the DPP4 inhibitor group led to a statistically significant increase in superoxide dismutase (Figure 2A). However, it failed to completely nullify the effects resulting from type 2 diabetes, i.e. despite the administration of drugs, the levels of superoxide disumtase in these groups were significantly lower than the values of this enzyme in the control group (Figure 2A). Comparing the effects of DPP4 inhibitors, it was observed that administration of sitagliptin led to the greatest increase in superoxide dismutase values, while the remaining two DPP4 inhibitors had a similar effect on the production of this enzyme (Figure 2A). Almost identical trend was observed for catalase activity (Figure 2B). The induction of diabetes led to a decrease in catalase activity, and administration of a DPP4 inhibitor was able to increase catalase activity (Figure 2B). However, as with superoxide dismutase, the catalase values failed to return to baseline values, respectively, which were recorded in the control group where healthy animals were (Figure 2B). Induction of type 2 diabetes led to a decrease in the value of reduced glutathione (Figure 2V). Administration of all three drugs from the DPP4 inhibitor group resulted in a statistically significant increase in the reduced glutathione value (Figure 2V). However, similar to the two antioxidant

protection parameters described above, the reduced glutathione values were lower in these groups compared to the reduced glutathione values observed in the healthy rat group (Figure 2V). Comparison of the effects of DPP4 inhibitor on the reduced glutathione values did not show any significant difference between the drugs in this group (Figure 2V).

Figure 2. Changes in blood pressure and heart rate in healthy and rats with T2DM



a - statistical significance of other groups in relation to control; b - statistical significance of other groups in relation to DM; c - statistical significance of other groups with respect to DM + sak; d - statistical significance of other groups with respect to DM + sit, e - statistical significance of other groups with respect to DM + vld.
Results of mean ± SD, n = 12 per group are presented.

DISCUSSION

Type 2 diabetes (T2DM) is a complex multifactorial metabolic disorder characterized by abnormal metabolism of carbohydrates, fats and proteins leading to increased levels of glucose and lipids in the blood (24). Chronic exposure to elevated glucose and lipid levels triggers different pathways responsible for inducing decreased insulin secretion from pancreatic β-cells, insulin resistance in peripheral tissues, decreased glucose utilization in peripheral tissues, and pathophysiological production of glucose in the liver (25). In addition, there is evidence that T2DM is associated with numerous complications and strongly influences the development of hypertension and other cardiovascular diseases. Therefore, it is very important to identify the molecular mechanisms associated with the onset of diabetes in order to develop targeted and effective therapies to treat this complex disease and prevent potential complications (26).

The results of clinical studies indicate that oxidative stress is strongly associated with the prevalence of type 2 diabetes. Biomarkers of tissue damage such as oxidation of DNA molecule bases, 4-hydroxy-2-nonelane (HNE) protein, hydroperoxides, 8-hydroxy-deoxyguanine and 8-epi-prostaglandin F2α, were elevated in plasma and pancreatic samples of patients with type 2 diabetes (27, 28). Experimental studies have shown that total antioxidant potential (TAS) is significantly reduced in T2DM, while levels of peroxide and other biomarkers of oxidative stress are significantly increased (29). The results of our study showed that rats with type 2 diabetes had high levels of measured reactive oxygen species (ROS), superoxide anion radicals, and hydrogen

peroxide (Figure 1B and 1C). ROS are one of the main species that propagate the onset of oxidative stress and normally are created in the body as a by-product of metabolism. Specifically, the superoxide anion radical is formed during the cellular respiration process, where free electrons are released from the mithondria, which subsequently binds to molecular oxygen in the presence of the NADPH oxidase enzyme to form O_2^- (30, 31). Under physiological conditions, O_2^- does not exhibit toxic effects because the enzyme superoxide dismutase (SOD) converts it to the less toxic hydrogen peroxide H_2O_2 (31). The results of our study showed that in rats with T2DM there was also a strong increase in H_2O_2 (Figure 1C), with a dramatic decrease in the value of the SOD enzyme (Figure 2A). These results undoubtedly support the onset of oxidative stress due to the development of T2DM, as the activity of the SOD enzyme, which represents the first line of defense against the uncontrolled generation of reactive oxygen species was reduced, which results in elevated values of these markers. In addition to the decreased values of the SOD enzyme, a decrease in the activity of catalase (SAT), an enzyme representing the second line of defense against free radicals, was observed in our study in rats with T2DM (Figure 2B). Namely CAT converts hydrogen peroxide to water and molecular oxygen (32). Due to the reduced activity of the SOD enzyme and the consequent accumulation of O_2^-, it binds to nitrogen monoxide and produces even more toxic peroxynitrite (33). The results of our studies confirm this fact, because in rats with T2DM lower nitrite values were measured, representing an indirect measure of the released vasodilator molecule of nitrogen monoxide (Figure 1C). In our study, peroxynitrite values were not measurable, which may be one of the disadvantages to to accurately observe the whole situation; however, the reduced NO values with elevated O_2^- undoubtedly support the assumption that even more toxic peroxynitrite molecules were formed. With such increased ROS and reduced NO values, intensive lipid peroxidation would be expected (34); however, the results of our study did not show a statistically significant increase in index of lipid peroxidation, measured as TBARS (Figure 1D). A potential explanation for such TBARS values may be the insufficient duration of the disease (7 weeks) before sampling, and it is hypothesized that with progression of the disease these values would also increase. Glutathione (GSH), is the primary intracellular antioxidant and has been extensively studied in populations with T2DM. Murakami and coworkers have shown that in patients with T2DM there are low values of the reduced form of GSH, while the levels of the oxidized form of GSSG are very high (35). Our study confirmed the low values of the reduced GSH form (Figure 2C).

In patients with T2DM, therapy is traditionally initiated with oral hypoglycemic metformin, which is a biguanidine derivative. However, over time, the efficacy of this drug has declined, and over the years the proportion of patients with successful glycemic control has been shown to decrease. Namely, after three years of metformin treatment, 50% of patients manage to regulate glucose levels, while after 9 years the number of these patients further decreases dramatically and drops to 25% (36). For these reasons, combination therapy is required and most often for years as adjunct therapy some of the sulfonylurea derivatives were introduced (37). A major drawback of this group of drugs is that they lead to the closure of ATR-dependent potassium channels, which can cause numerous side effects to the heart and thus further increase the risk of developing cardiovascular complications in T2DM (38). In this connection, it is very important to choose the appropriate therapy that, in addition to influencing glucose regulation, will influence the modification of potential complications. As we have already shown, T2DM strongly induces oxidative stress and drastically reduces antioxidant protection, and is one of the mechanisms for further development of complications. As we have already shown, T2DM strongly induces oxidative stress and drastically reduces antioxidant protection and is one of the mechanisms for further development of complications. Usage of drugs that, in addition to glucose levels, would have the effect of reducing oxidative stress and increasing antioxidant protection, would result in lower risk of complications. In this study, a group of drugs called DPP4 inhibitors was used. These drugs work by inhibiting the dipeptidyl peptidase 4 enzyme, thereby increasing levels of intestinal hormones, incretin: glucagon-like peptide 1 (GLP1), and gastric inhibitory polypeptide (GIP) (39). An additional aim of this study was to investigate whether administration of a DPP4 inhibitor in a rat population with T2DM can reduce the disease-induced oxidative stress, and to compare the effects of three different drugs in this group on the values of oxidative stress parameter and antioxidant protection. Sitagliptin, saxagliptin and vildagliptin were used in the study because they are known to have varying degrees of DPP4 enzyme inhibition (40).

All applied DPP4 inhibitors showed a significant influence on the parameters of oxidative stress and antioxidant protection (Figures 1, 2). However, there was a difference in their effects at certain parameters. Of all three drugs used in this group, vildagliptin had the strongest effect on reactive oxygen species (Figure 1B, 1C). Four weeks of intraperitoneal administration of this drug to T2DM rats was sufficient to lead to a statistically significant decrease in the values of these parameters and to approximate them as much as possible to the control group, i.e. group of healthy rats. This effect can be explained by the activation of the first and second antioxidant defense lines, respectively, by the increase in SOD and CAT activities, which were also observed in this group (Figures 2A, 2B). *Sherif* et al. showed that vildagliptin may increase CAT activity in the liver in rats with ischemic/reperfusion injury to this organ (41). The remaining two drugs also showed statistically significant reductions in reactive oxygen species (Figure 1B, 1C) and an increase in SOD and CAT values (Figure 2A, 2B). These results are in agreement with previous studies that have addressed the antioxidant potential of these drugs (42-45). In addition to affecting these two enzymes, all three drugs also increased the concentration of reduced glutathione, another of the antioxidant enzymes (Figure 1C). Among all tested drugs, sitaglitin (Figure 2A-C) showed the most positive effect on the activation of antioxidant protection. A potential explanation for this effect of

sitagliptin might be that it belongs to a group of nonpeptidomimetics that form non-covalent bonds with the catalytic domain of DPP4 enzymes, producing strong and immediate inhibition (11, 12). *Refaat* and coworkers also demonstrated that administration of vildagliptin leads to an increase in the fraction of reduced glutathione in the liver in T2DM rats (46). Similar effects of sitagliptin on GSH in the liver have been demonstrated by El-Kashef et al (42). Saxagliptin and its effects on reduced glutathione have been studied so far in animal models at the kidney level (44). All three drugs resulted in increased synthesis of nitrogen monoxides; the value of this parameter was even higher in the T2DM-treated rat groups treated with any of the DPP4 inhibitors than in the healthy rat group. This is a very interesting finding because it indicates the great potential of these drugs to lead to vasodilation, which is a very important cardioprotective effect. It has already been mentioned that a large number of antidiabetics show negative effects on the cardiovascular system (37, 38).

To our knowledge, this is the first study concerning the systemic effect of DPP4 inhibitors on oxidative stress in T2DM rats. (a) As expected, rats with T2DM had elevated values of oxidative stress parameters and decreased antioxidant protection parameters values. (b) All investigated DPP4 inhibitors reduced oxidative stress and increased antioxidant protection. (c) Vildagliptin showed the highest reduction of oxidative stress, (d) while sitagliptin led to the greatest jump in antioxidant protection. (e) All three drugs showed great potential as vasodilators.

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ASSESSMENT OF THE TDCS INFLUENCE ON STRESS-INDUCED DISORDERS IN RATS WITH LOW STRESS SUSTAINABILITY AND ENDURANCE

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ISPITIVANJE UTICAJA TDCS-A NA STRESOM IZAZVANE POREMEĆAJE KOD PACOVA SA NISKOM TOLERANCIJOM NA STRES

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ABSTRACT

The aim of study is to analyze the tDCS influence on stress-induced disorders in rats with low stress sustainability and endurance. The animals with a low stress sustainability and endurance were divided into 3 groups: the comparison 1, the comparison 2 and the main. The control group consisted of intact rats. The rats of the comparison group 1 were subjected to orthostatic stress 24 hours after the 1st forced swimming test. The rats of the comparison group 2 and the main one were conducted the 2nd forced swimming test on the 7th day of the experiment, and 24 hours later they were subjected to the orthostatic stress. Rats of the main group got tDCS sessions after the 1st forced swimming test. The development of the orthostatic stress is accompanied by an increase in plasma content the following components: adrenaline by 88.9%, ACTH in 10.5 times, corticosterone by 70.1%, IL-1β by 178.2%, IL-6 in 6.7 times, IL-10 by 37.1% in comparison with intact animals. The usage of tDCS in rats with low stress sustainability and endurance increased the swimming duration by 47.7%. During the OS it was also accompanied by a decrease in plasma content: adrenaline in 1.4 times, ACTH in 8.2 times, corticosterone in 1.4 times, IL-1β in 1.5 times, IL-6 in 2.2 times, IL-10 in 1.2 times, relative to the comparison group 2. The obtained data showed the essential effect of tDCS on stress-related changes in the content of cytokines and hormones of blood.

Keywords: *stress, tDCS, rat, cytokines, corticosterone*

SAŽETAK

Cilj studije je da analizira uticaj tDCS na stresom izazvane poremećaje kod pacova sa niskom tolerancijom na stres. Životinje su bile svrstane u tri grupe: poredbena grupa 1, poredbena grupa 2 i glavna grupa. Kontrola grupa se sastojala od zdravih pacova. Pacovi iz poredbene grupe 1 su bili podvrgnuti ortostatskom stresu 24 sata nakon prvog testa forsiranog plivanja. Pacovi iz poredbene grupe 2 i iz glavne grupe su bili podvrgnuti 2. forsiranom testu plivanja 7. dana eskperimenta dok su 24 sata kasnije bili izloženi ortostatskom stresu. Pacovi iz glavne grupe su bili podvrgnuti tDCS sesiji nakon prvog testa forsiranog plivanja. Ortostatski stres je bio praćen povećanom koncentracijom sledećih komponenti plazme: adrenalina za 88,9%, ACTH za 10.5 puta, kortikosterona za 70,1%, IL-1b za 178,2%, IL-6 za 6,7 puta, IL-10 za 37,1% u poređenju sa zdravim životinjama. Korišćenje tDCS-a kod pacova je produžilo trajanje plivanja za 47,7%. Ortostatski stress je bio praćen smanjenjem plazma koncnetracije: adrenalina za 1,4 puta, ACTH za 8.2 puta, kortikosterona za 1,4 puta, IL-1b za 1,5 puta, IL-6 za 2,2 puta, IL-10 za 1,2 puta, u odnosu na grupu poređenja 2. Dobijeni podaci pokazuju značajni efekat tDCS-a na stresom izazvane promene u sadržaju citokina i hormona u krvi.

Ključne reči: *stres, tDCS, pacov, citokini, kortikosteron*

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INTRODUCTION

Stress is the organism's reply to various cognitive, emotional and somatic stressors, which realizes through the activation of a unified neuroimmune endocrine system and consists in the activation of a number of physiological and behavioral programs that facilitate survival (1, 2).

Stress-related changes in the functioning of sympathoadrenal (SAS), hypothalamic-pituitary-adrenal (GPAS) and immune systems are connected not only with the adaptation of the organism to new life conditions, but also with the risk of developing a variety of stress-associated diseases (3). In this case, the activation of non-specific inflammation takes a specific part in the development of adverse stress manifestations, which in particular reveals by an increase in the level of either pro-inflammatory or anti-inflammatory cytokines (4).

It is known that organisms with different coping strategies react differently to stressors and that this fact determines the stress outcome (5). In these circumstances, studying of the reaction to the stressor in animals with different stress sustainability turns into a theme of the especial interest. It opens the way to find methods for correcting stress sustainability and endurance in people who have active and passive coping strategies.

Opioid peptides take a significant function in controlling the growth of the stress reaction. The greatest amount of them is released in hypothalamus, pituitary and limbic structures of the brain during the pain or stress processes, as well as intense physical exercises (6, 7). The stress-limiting effect of opioid peptides is mediated by the activation of μ- and δ-opioid receptors, for which β-endorphin demonstrates high selectivity (7, 8).

The β-endorphin emitted during the stress growth mediates endocrine and behavioral reactions, aimed at adapting the organism to extreme conditions and conduces to restricting adverse effects of stress (9,10). β-endorphin stimulates the reward system, inhibits the activity of the GPAS, reduces the level of corticotropin-releasing hormone and adrenocorticotropic hormone (ACTH), and suppress pain and anxiety as well (7,11).

In connection with the expressed stress-limiting effect of opioid peptides, the research of methods which can stimulate the endogenous opioidergic system activity is an important task of modern medicine.

Transcranial direct current stimulation (cathodic tDCS or TES-therapy by Professor Lebedev) is a physiotherapeutic non-invasive method of electrical impact on the brain of humans and animals through the cranium tegument, selectively activating the defense mechanisms of the brain. Its main effects are due to the increased production of β-endorphin and the concomitant changes in the production of other neurotransmitters: dopamine, norepinephrine, serotonin, GABA and others (12, 13).

The main objective of this study was to explore the effectiveness of cathodic tDCS for correcting stress-induced disorders of hormonal and cytokine profile in rats with low stress sustainability and endurance.

MATERIAL AND METHODS

Characteristic of experimental animals

Experiments were performed on 182 males of white nonlinear rats weighing 195±15 g, which had no visible signs of disease. The animals were kept on a vivarium at a temperature of 22-24°C under the conditions of 5 animals in a cage, a 12/12 cycle of daylight in plastic cages with wood filings and free access to food (standard nutrition) and water. Such conditions excluded the impact of stress factors. The criteria for elimination from the experiment were external anatomical defects and signs of disease.

All experiments were carried out in accordance with the international rules of "Guide for the Care and Use of Laboratory Animals" (NAP, 2012).

The design of studying

At the beginning of the experiment the endurance and stress sustainability of animals were assessed with the help of the forced swimming test (FST) in the modification of the Federal Medical and Biological Agency of Russia (14). Rats with low stress sustainability and a swimming time not exceeding 184 seconds (n = 43) were selected in the research. Then the animals were divided into 3 groups: the comparison group 1 (n = 10), the comparison group 2 (n = 16) and the main group (n = 17). Intact rats (n = 10) selected from the general population using the random number method were used as control ones. The rats of the comparison group 1 were subjected to the orthostatic stress (OS) 24 hours after the 1st FST (on the 2nd day of the experiment), for which they were placed in the anti-orthostatic position at an angle of 90° to the horizontal surface in plexiglas fixators with a size of 210x65x65 mm (RPC Open Science Ltd.) for 45 minutes (15). The rats of the comparison group 2 and the main group were performed in the second FST on the 7th day of the experiment, and 24 hours later (on the 8th day of the experiment) they were subjected to the OS. Meanwhile, rats of the main group got one session of cathodic tDCS per day for 5 days after the 1st FST (from the 2nd to the 6th days) using a two-program electric stimulator "TRANSAIR-03" (TES CENTER Ltd., St. Petersburg) by the method in our own modification (16).

The rat's blood was sampled before the OS and 2 hours later it by the venepunction of jugular veins under the combined injection anesthesia: Zoletil 20 mg/kg IM (Virbac, France) and Xylanite 6 mg/kg IM (NITA-FARM CJST, Russia). Heparin (BELMEDPREPARATY RUE, Belarus) was used as an anticoagulant at a rate of 500 units per 1 ml of blood. Then the blood was centrifuged for 15 minutes at the acceleration of 1000g. Samples of the received plasma were stored in cryovials at a temperature of -80°C (Figure 1).

Table 1. The duration of swimming in rats with low stress sustainability and endurance in the forced swimming test on the 1st and the 7th days

Group	The duration swimming of the 1st FST, Me (Q_1-Q_3), sec.	The duration swimming of the 2nd FST, Me (Q_1-Q_3), sec.	p-value (W-test)
The comparison 2	161,5 (146,5-171,5)	166,5 (160-215,5)	0,05
The main	151 (122-166)	223 (168-274)	0,001

The method of enzyme-linked immunosorbent assay

Quantitative evaluation of the adrenaline and adrenocorticotropic hormone levels in plasma was realized by the method of enzyme-linked immunosorbent assay using Cloud-Clone Corp. (China) kits. Corticosterone level was evaluated by Immunodiagnostic Systems Limited (UK). IL-1β, IL-6 and IL-10 levels were evaluated by eBioscience (Bender MedSystems GmbH, Austria) using the vertical scanning photometer ANTHOS 2010 (Biochrom, Austria) with ADAP Software, version 2.0.

Statistical analysis

Statistical data analysis was carried out with the help of the software "MS Excel 2016" (Microsoft, the USA) and "Statistica 10.0" (StatSoft Inc., the USA). Verification of the normality of the quantitative attributes distribution in experimental groups was carried out using the Shapiro-Wilk test. Since the distribution of meanings differed from the normal one, nonparametric statistics were used for further research data processing. The results were expressed as Me (Q1-Q3), where Me is the median, Q1 is the lower and Q3 is the upper quartiles. To evaluate statistically significant differences in paired comparisons of dependent groups, the Wilcoxon test (W-test) was used, and for the intergroup differences between the two independent groups the Mann-Whitney (MW-test) criteria were applied. The critical level of significance (p-value) in the verification of statistical hypotheses was assumed to be 0.05.

RESULTS

The results of forced swimming test

Conducting 5 sessions of cathodic tDCS after the 1st FST in rats from the main group leaded to an increase in the swimming time according to the results of the 2nd FST by 47.7% (W-test, p = 0.001), whereas there were no significant differences in the comparison group 2 (W-test, p = 0.05) (Table 1).

Dynamics of indices of endocrine and cytokine profile

The level of adrenaline in the comparison group 1 before the OS was not significantly different from the control (p = 0.66). After modeling the OS the level of adrenaline increased by 126.2% in comparison with the control (p = 0.02) and by 82.1% compared to its level before the OS (p = 0.03). In comparison group 2 the level of adrenaline before the OS also did not differ significantly from the control (p = 0.70). After the OS the level of adrenaline was higher by 88.9% compared with the control (p = 0.02) and by 92.7%

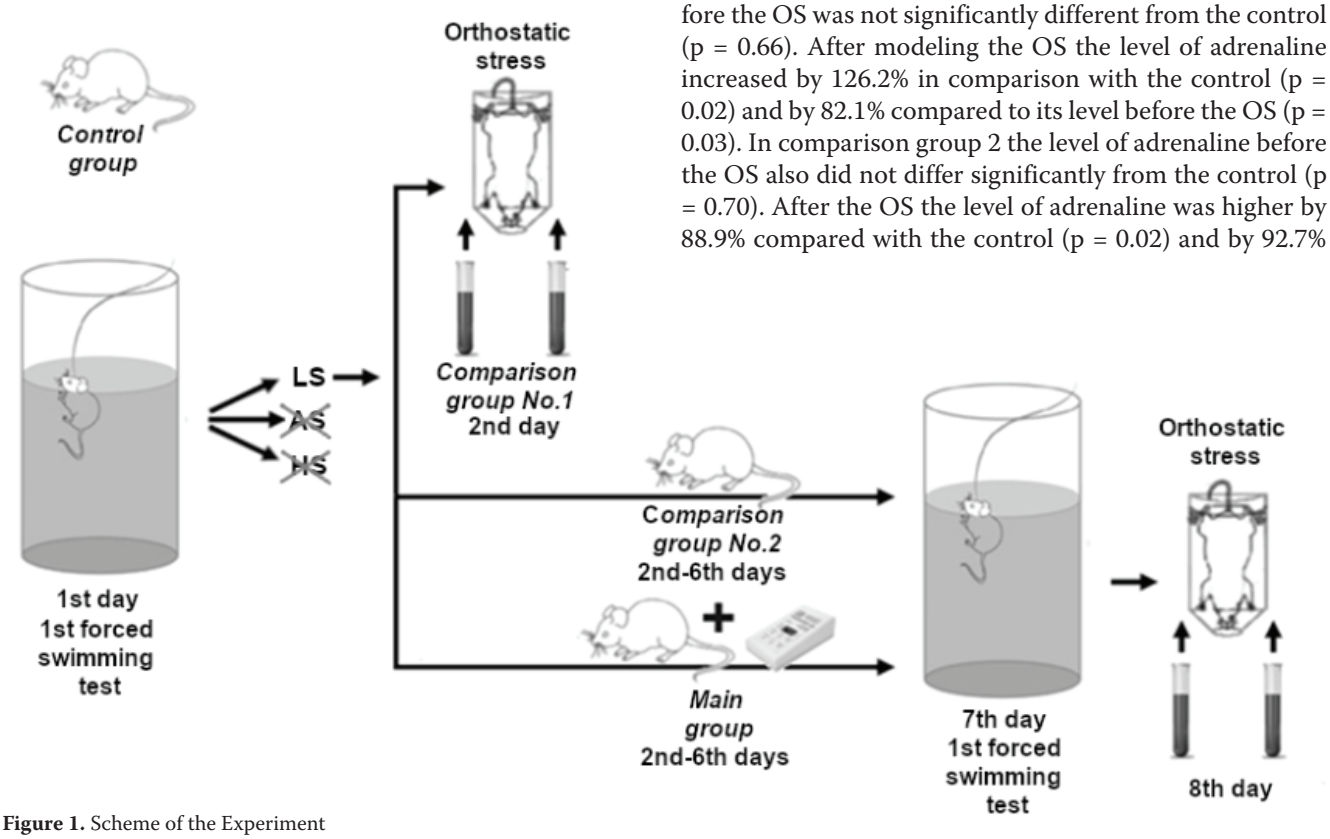

Figure 1. Scheme of the Experiment

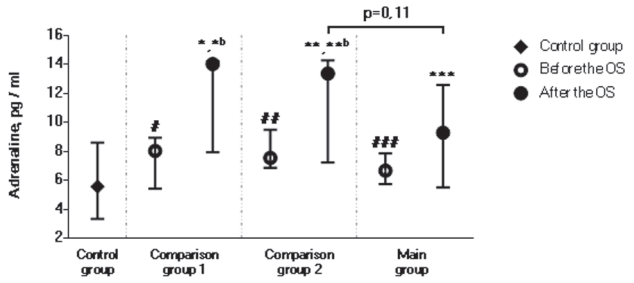

Figure 2. The level of adrenaline in blood plasma of rats

The level of adrenaline in blood plasma in comparison with the control group: # – p=0,66, ## – p=0,70, ### – p=0,82, * – p=0,02, ** – p=0,02, *** – p=0,40; the level of adrenaline in blood plasma in comparison with the level before the OS: *b – p=0,03, **b – p=0,04

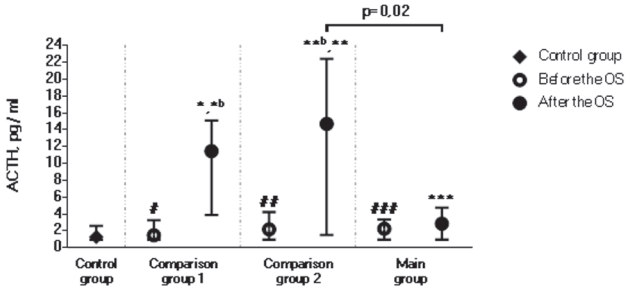

Figure 3. The level of ACTH in blood plasma of rats

The level of ACTH in blood plasma in comparison with the control group: # – p=0,25, ## – p=0,39, ### – p=0,82, * – p=0,004, ** – p=0,005, *** – p=0,46; the level of ACTH in blood plasma in comparison with the level before the OS: *b – p=0,008, **b – p=0,02

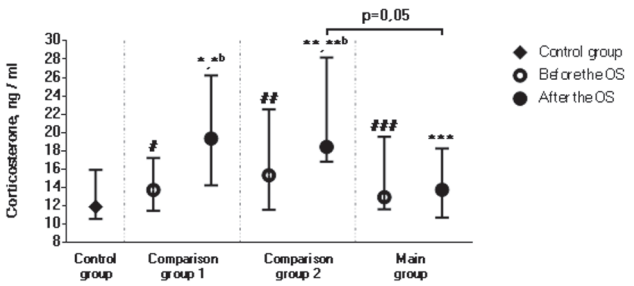

Figure 4. The level of corticosterone in blood plasma of rats

The level of corticosterone in blood plasma in comparison with the control group: # – p=0,66, ## – p=0,39, ### – p=0,59, * – p=0,02, ** – p= 0,02, *** – p=0,46; the level of corticosterone in blood plasma in comparison with the level before the OS: *b – p=0,22, **b – p=0,22

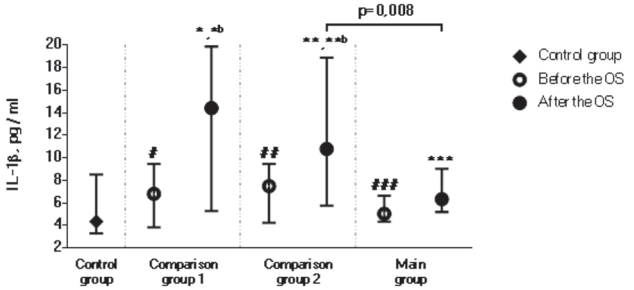

Figure 5. The level of IL-1β in blood plasma of rats

The level of IL-1β in blood plasma in comparison with the control group: # – p=0,79, ## – p=0,48, ### – p=0,94, * – p=0,03, ** – p =0,02, *** – p=0,22; the level of IL-1β in blood plasma in comparison with the level before the OS: *b – p=0,03, **b – p=0,02

compared to its level before the OS (p = 0.04). In the main group, both before and after the OS, there were no reliable differences with the control (p≥0.05). Meanwhile, the adrenaline level in the main group 2 hours after the OS was in 1.4 times lower than in the comparison group 2 (p = 0.11) (Figure 2).

The ACTH level in the comparison group 1 before the OS was not reliably different from the control (p = 0.25). After the OS the level of ACTH increased in 12.2 times in comparison with the control (p = 0.004) and in 5 times in comparison with the level before the OS (p = 0.008). In the comparison group 2 the level of ACTH before the OS was not reliably different from the control (p = 0.39). After the OS the level of ACTH was in 10.5 times higher in comparison with the control (p = 0.005) and in 5.5 times higher in comparison with the level before the OS (p = 0.02). In the main group of rats both before and after the OS there were no reliable differences with the control (p≥0.05). Meanwhile, the ACTH level 2 hours after the OS in the main group was in 8.2 times lower than in the comparison group 2 (p = 0.02) (Figure 3).

The level of corticosterone in the comparison group 1 before the OS was not reliably differ from the control (p = 0.66). After the OS the level of corticosterone increased by 105.1% compared with the control (p = 0.02). In the comparison group 2 corticosterone before the OS did not differ reliably from control (p = 0.39). After the OS the level of corticosterone was higher by 70.1% compared with the control (p = 0.02). In the main group of rats both before and after the OS there were no reliable differences with the control (p≥0.05). Meanwhile, the corticosterone level in the main group 2 hours after the OS was lower in 1.4 times than in the comparison group (p = 0.05) (Figure 4).

The level of IL-1β in the comparison group 1 before the OS was not reliably differ from the control (p = 0,79). After the OS the level of IL-1β increased by 275.7% in comparison with the control (p = 0.03) and by 136% compared with the level before the OS (p = 0.03). In the comparison group 2 the level of IL-1β before the OS was not reliably different from the control (p = 0.48). After the OS the level of IL-1β was higher by 178.2% compared with the control (p = 0.02) and by 59% compared with the level before the OS (p = 0.02). In the main group both before and after the OS, there were no reliable differences with the control (p≥0.05). In the main group the level of IL-1β 2 hours after the OS was in 1.5 times lower than in the comparison group (p = 0.008) (Figure 5).

The level of IL-6 in the comparison group 1 before the OS was not reliably different from the control (p = 0,13). After the OS the level of IL-6 increased in 7.2 times in comparison with the control (p = 0.004) and in 3 times in comparison with its level before the OS (p = 0.02). In the comparison group 2 the level of IL-6 before the OS was not reliably different from control (p = 0.10). After the OS, the level of IL-6 was in 6.7 times higher in comparison with the control (p = 0.005) and in 3 times in comparison with the level before the OS (p = 0.04). In the main group both

before and after the OS there were no reliable differences with the control (p≥0.05). Meanwhile, in the main group the level of IL-6 2 hours after the OS was in 2.2 times lower than in the comparison group (p = 0.05) (Figure 6).

Analysis of the IL-10 level in blood plasma revealed no reliable differences in any group of rats (p≥0.05) (Figure 7).

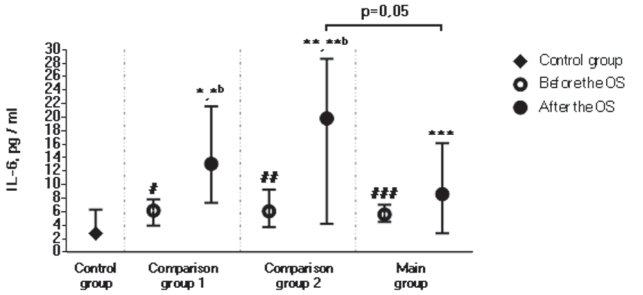

Figure 6. The level of IL-6 in blood plasma of rats

The level of IL-6 in blood plasma in comparison with the control group: # – p=0,13, ## – p=0,10, ### – p=0,18, * – p =0,004, ** – p=0,005, *** – p=0,10; the level of IL-6 in blood plasma in comparison with the level before the OS: *b – p=0,02, **b – p=0,04

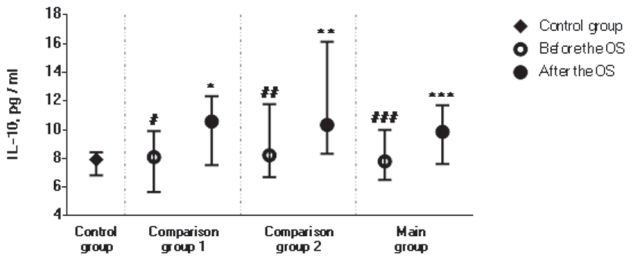

Figure 7. The level of IL-10 in blood plasma of rats

The level of IL-10 in blood plasma in comparison with the control group: # – p=0,93; the level of IL-10 in blood plasma in comparison with the level before the OS: ## – p=0,48. in relation to to the comparison group 2 two hours after the OS.### – p=0,82, * – p=0,13, – p=0,06, – p=0,52

DISCUSSION

The duration of swimming in the FST largely depends on the timely transition from active behavior (attempts to get out and active scrutiny of the aquarium) to passive one (immobilization on the water surface, saving style of swimming) (17).

According to the review of de Kloet and Molendijk, it is incorrectly, as was done before, to associate an increase in the duration of immobilization (passive coping) in the forced swimming test with depressive-like behavior. Oppositely, the passive coping strategy is associated with an increase in the swimming duration, and, consequently, this behavioral program facilitates to the survival of an animal (5).

Conducting the forced swimming test causes a significant reaction from the SAS and the GPAS, as well as from key neurotransmitter systems of the brain, primarily dopaminergic (18). An intent consideration to the dopaminergic system is due to its role in the pathogenesis of stress and the development of stress-induced diseases such as depression (19). It is the dopaminergic neurotransmission in the limbic brain system that plays a key function in the transition from active to passive behaviors in the forced swimming test (5,20). It is known that the cooperation of β-endorphin with μ- and δ-opioid receptors stimulates the production of dopamine (11).

Thus, it can be assumed that the increase in the swimming duration in rats receiving cathodic tDCS is due to β-endorphin-dependent stimulation of the dopaminergic system of the brain stem.

Adrenaline is a hormone that is released in a reaction to stress from the adrenal medulla in the bloodstream and mediates short-term response to stressors initiating a series of behavioral and physiological changes that allow the body to implement a "fight or flight" program (21). The catecholamines effects are mediated by the cooperation with central and peripheral α- and β-adrenergic receptors. Consequently, the result of increasing the level of circulating catecholamines (adrenaline and noradrenaline) is the activation of metabolism and energy mobilization from the depot, tachycardia, pupillary dilatation, bronchial dilatation, respiration strengthening, peripheral vasospasm and redistribution of circulating blood (21,22). According to the referenses, a high level of catecholamines in blood plasma is associated with aggressive behavior in rats (active-coping strategy) (23). In rats group applied tDCS were not reliable changes in adrenaline concentration compared to the control and the comparison groups. It was explained by the fact that blood sampling was carried out 2 hours later the orthostatic stress, and the released adrenaline was largely metabolized (24).

Moreover, according to the data of the references, rats with low swimming duration in the FST are characterized by a hypoactivity of the SAS on the background of a relative preponderance of the activity of the GPAS, which was observed during the comparison of corticosterone and adrenaline concentrations between comparison groups (rats with low stress sustainability and endurance) and the control (rats randomly selected from the general population) (21,25).

The tendency in the main group to reduce adrenaline concentration in 1.4 times in regard to the comparison group also can be explained by the limiting effect of the β-endorphin entering the systemic circulation on the release of catecholamines (26).

IL-1β is the central mediator of inflammation and takes an important part in the course of autoimmune, inflammatory, infectious and degenerative diseases. IL-1β increases the production of other cytokines involved in the development of inflammation and plays an important role in the depression pathogenesis (27,28). Today there is sufficient evidence that IL-1β plays an important role in neuroendocrine and behavioral reactions in stress. Receptors to IL-1β are found at all levels of the GPAS: hypothalamus, pituitary and adrenal glands. It stimulates the synthesis of glucocorticoids. At the same time high concentrations of glucocorticoids can suppress excessive synthesis of IL-1β. However, despite the

close interaction of the cytokines system and GPAS, a strict correlation between the levels of glucocorticoids and cytokines, in particular IL-1β, cannot be established (29). It has been established that the level of IL-1β increases at an acute and a chronic stress, while low (physiological) levels of IL-1β are adaptive. Thus, a blockade of IL-1β signaling can be considered as a method of prevention and treatment of stress-associated mental disorders (30,31).

IL-6 is a pro-inflammatory cytokine, an increase in the level of which accompanies the course of both acute and chronic stress (32). The main functions of IL-6 are an activation of T-lymphocyte proliferation and B-lymphocyte differentiation, a stimulation of leukocyte chemotaxis, an increase of fibroblasts and osteoclasts activity and an activation of the synthesis of acute phase proteins. It also takes part in a transition of acute inflammation to chronic and a control of body weight (33).

IL-6 participates in the modulation of behavior in the FST, mainly facilitating to the reduction of the swimming duration. Meanwhile, its effects are realized through the amygdala or hippocampus due to the activation of the Erk1/2 signaling track (34).

A distinct increase of the IL-6 concentration in blood is noted in anxiety disorders and depression (35). A number of studies show that, although regular physical activity reduces inflammation and production of cytokines, however, intensive exercises facilitate to the production and release of IL-6 by skeletal muscles. Meanwhile, IL-6 produced during physical activity inhibits production of TNF-α and induces production of IL-10 (32,36).

IL-10 is an important anti-inflammatory cytokine, whose key function is to protect tissues from damage caused by infectious agents and inflammation, scarring and preventing the development of autoimmune diseases as well (37). The absence of reliable intergroup differences in the plasma concentration of IL-10 supposedly indicates of its secondary role in the development of the OS. According to the references an increase of IL-10 in plasma concentration defers after an increase of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) in plasma (38). As studies show, at the same time acute stress has no significant effect on the level of IL-10 circulating (39).

In accordance with A.R. Mesquita, during the FST rats knocked out by the IL-10 gene showed an increase in the duration of immobilization, which was neutralized by injection administration of IL-10. In rats with increased IL-10 expression changes were opposite knocked-out. Meanwhile, in both cases the found regularity related to the female rats, and in male rats there also was no significant difference in the results of the FST (40).

CONCLUSIONS

According to the results of the 1st FST, the duration of swimming in rats with low stress sustainability and endurance was less than 184 seconds, and there were no significant differences with the 2nd FST. The development of the OS in rats with low stress sustainability and endurance is accompanied by an increase in plasma content of adrenaline, of ACTH, of corticosterone, of IL-1β, of IL-6, of IL-10. Based on the results of the 2nd FST, conducting 5 sessions of tDCS in rats with low stress stability increases the swimming duration by 47.7%, and, in this case, the OS is accompanied by a decrease in plasma content the following components: adrenaline, ACTH, corticosterone, IL-1β, IL-6, IL-10 towards the comparison group. There were no reliable differences in the IL-10 content between animal groups which indicate its secondary role in pathogenesis of acute stress.

The received data indicate a considerable potential of cathodic tDCS for further studies as a method of increasing stress sustainability and endurance, as well as preventing stress-associated diseases.

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ANTITUMOR EFFECT OF THE CHALCONE ANALOGUE, (E) -1- (4-ETHOXY-3-METHOXYPHENYL) -5- METHYLHEX-1-EN-3-ONE ON HELA CELL LINE

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ANTITUMORSKI EFEKAT ANALOGA HALKONA (E) -1- (4-ETOKSI-3-METOKSIFENIL) -5- METILHEKS-1-EN-3-ONA NA HELA TUMORSKE ĆELIJE

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ABSTRACT

Chalcones represent precursor compounds for flavonoids biosynthesis in plants. Chalcones, 1,3-diaryl-2-propen-1-ones, have unique chemical structure with conjugated double bonds and delocalized π-electron system on both aromatic rings. Various studies have shown that chemical structure of chalcone is responsible for their antitumor effect. In our study, we have examined the antitumor effect of chalcone analogue (E) -1- (4-ethoxy-3-methoxyphenyl) -5-methylhex-1-en-3-one (CH) on HeLa cells. The antitumor efficiency of different CH concentrations was compared to the antitumor effects of dehydrozingerone and cisplatin. The viability of the cells was evaluated using MTT assay; type of the cell death was evaluated by Annexin V-FITC/7-AAD staining using FACS analysis; morphology changes of treated cells were visualized and compared to untreated cells using phase contrast microscopy. The result of our research showed that CH have a stronger antitumor compared to the effect both of dehydrozingerone and cisplatin. Our results indicated that chalcone analogue induced cell death via activation of apoptosis more powerfully compared to the apoptosis induced with dehydrozingerone and cisplatin.

Keywords: *cervical cancer, cytotoxicity, apoptosis, morphology.*

SAŽETAK

Halkoni predstavljaju prekursore biosinteze flavonoida u biljkama. Halkoni, 1,3-diaril-2-propen-1-oni, poseduju jedinstvenu hemijsku strukturu sa konjugovanim dvogubim vezama i delokalizovanim π-elektronskim sistemom na oba aromatična prstena. Različite studije su pokazale da je hemijska struktura halkona odgovorna za njihov antitumorski efekat. U našem istraživanju ispitivan je antitumorski efekat analoga halkona (E) -1- (4-etoksi-3-metoksifenil) -5- metilheks-1-en-3-one (CH) na tumorskim HeLa ćelijama. Antitumorska efikasnost ispitivanog analoga halkona, u različitim dozama upoređivana je sa antitumorskim efektima dehidrozingerona i cisplatine. Vijabilnost tumorskih ćelija određivana je upotrebom MTT testa; tip ćelijske smrti upotrebom Annexin V-FITC/7-AAD bojenja pomoću FACS analize; morfološke promene tretiranih i kontrolnih ćelija vizualizovane su upotrebom faznog kontrastnog mikroskopa. Rezultati našeg istraživanja pokazali su da analozi halkona, CH ispoljavaju jači antitumorski efekat u odnosu na dehidrozingeron i cisplatinu. Naši rezultati ukazuju na to da analozi halkona efikasnije indukuju ćelijsku smrt u odnosu na dehidrozingeron i cisplatinu.

Ključne reči: *karcinom cerviksa, citotoksičnost, apoptoza, morfologija*

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INTRODUCTION

Cervical cancer (CC) represents leading cause of cancer related death in women worldwide influencing women during their reproductive age (Li, Bau, & Bao, 2018). As cervical squamous cell carcinoma this malig - nancy exhibiting high morbidity and mortality. Cervical cancer is the cause of approximately 200,000 deaths worldwide (Muñoz et al., 2003). Based on the information from 2012, Serbia is in the third place for the mortality from CC. In the period (1991-2011) the average age-standardized mortality rate was 7.03 per 100.000. The average age standardized mortality rate was from 6.05 in 1991 to 8.17 in 2008 (Naumovic, Miljus, Djoric, Zivkovic, & Perisic, 2015). More than 80% of early stages of CC can be cured by surgery and chemotherapy, with possibility of recurrence of CC (Ball & Madden, 2003). However, both chemotherapy and radiation cause serious adverse effects, therefore anti-cancer therapeutic efficiency of the new compounds is needed to treat cancer. By using anti-cancer therapeutics, cancer cell death can be caused by the induction of apoptosis, necrosis and autophagy.

Chalcones and their analogues represents natural or synthetic 1,3-diaryl-2-propenones with significant delocalization of the electron throughout the chalcone system, the combination of excellent charge-carrier mobility and a high stability structure. Chalcone molecules which are intermediate precursors to all flavonoid compounds along with their a π-conjugated system provides a large charge-transfer with appropriate substituent groups (Wu, Liu, & Zhu, 2010)(Mahapatra, Bharti, & Asati, 2015). This type of chemical structure of chalcones is responsible for their anti-tumor (Mahapatra et al., 2015)(Alibeiki, Jafari, Karimi, & Peeri Dogaheh, 2017), anti-inflammatory (K. Sahu, S. Balbhadra, Choudhary, & V. Kohli, 2012)(Kim et al., 2015) and anti-oxidant effects (Lahsasni, Al Korbi, & Aljaber, 2014)(Kim et al., 2015).

In our research, we used cisplatin and dehydrozingerone as reference substances. The cisplatin has a clinical application and is used to treatment malignant tumors of various tissues such as colon, lung, cervix, breast, ovaries and testicles (Taguchi, Nazneen, Abid, & Razzaque, 2005). The dehydrozingerone shows anti-tumor, anti-inflammatory and anti-oxidant effect on human tumor cells, e.g. HeLa, A549, LS174 и MDA-MB-231cell lines (Burmudžija et al., 2017)(Kubra, Bettadaiah, Murthy, & Rao, 2014).

In our study, we examined the antitumor effect of chalcone analogue (E) -1- (4-ethoxy-3-methoxyphenyl) -5- methylhex-1-en-3-one (**CH**) that was previously synthesized in the procedures described by Muškinja et al., (Muškinja, Ratkovi, Rankovi, & Kosani, 2016). Here, for the first time, we report the anti-tumor effect of CH on HeLa cell line.

MATERIAL AND METHODS

Cell line, compounds and reagents

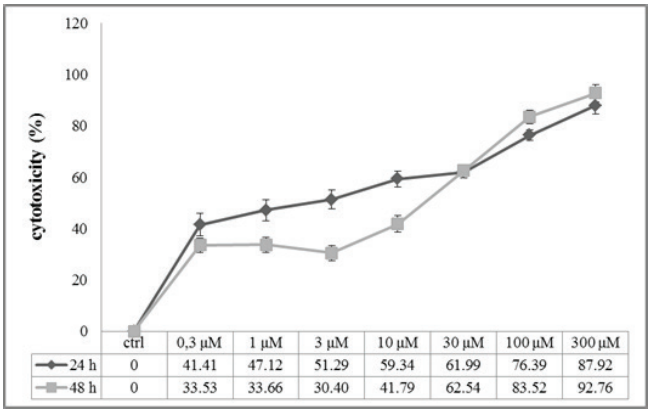
The cell line utilized in this study, HeLa (human cervical carcinoma cell line), was purchased from the American Type Culture Collection (ATCC® CCL-2™). The HeLa cell line was cultured in complete growth DMEM medium (Sigma Aldrich D5671). The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. Both, the synthesized chalcone analogue (CH) and dehydrozingerone were a gift from colleagues, Faculty of Chemistry, University of Kragujevac. Cisplatin was obtained from the Calbiochem (CAS 15663-27-1). Annexin V-FITC and 7-AAD (7-aminoactinomycin-D) was purchased from Abnova (KA3806). Chloroquine (obtained from the Sigma Aldrich, C6628) was dissolved in ultrapure water at a concentration of 100 μM, and then in DMEM at a final concentration of 20 μM. The chalcone analogue and referent substances (cisplatin, cysPt and dehydrozingerone, DHZ) were dissolved in DMSO (final concentration of DMSO was less than 0.5%).

MTT assay

Cells were seeded in a 96-well plate at $5x10^3$ cells/well, incubated for 24 and 48 h with different concentrations of CH (0.3, 1, 3, 10, 30, 100 and 300 μM) and referent substances – cysplatin and dehydrozingerone (tested concentrations were 3, 10, 30 and 100 μM) in triplicate. After treatment, the supernatant was removed and MTT was added to each (100 μl medium; 0.5mg/ml MTT). After incubation for 3 h MTT was discarded and 150μl DMSO was added to each well. The absorbance was measured at 595 nm using a micro-plate reader (Zenyth 3100, Anthos Labtec Instruments). The percentage of cytotoxic cells was calculated using the formula: Cytotoxicity (%) = (1 - (exp. group (ABS)) / (control group (ABS)) x 100). The IC50 values of investigated substances were calculated using Microsoft Office Excel, using the equation of linear dependence.

Annexin V-FITC/7-AAD staining

In order to determine the type of the cell death induced by CH, HeLa cells were seeded in 24-well plate ($1x10^5$ cells/well) and treated with IC_{50} values of CH during 48 h. The IC_{50} values both of cisplatin and dehydrozingerone were used as a positive control. To examine the influence of autophagy on the percentage of apoptotic cells, pretreatment with CQ was performed on experimental and control cell group. After treatment, cells were trypsinized, resuspended with Annexin binding buffer and incubated with 10μl of Annexin V-FITC and 20 μL of 7-AAD for 15 min in the dark. Following incubation and addition of 400 μl binding buffer, the cells were analyzed by flow cytometer Cytomics FC500 (Beckman Coulter, USA) and expressed as percentage (%) of apoptotic cells.



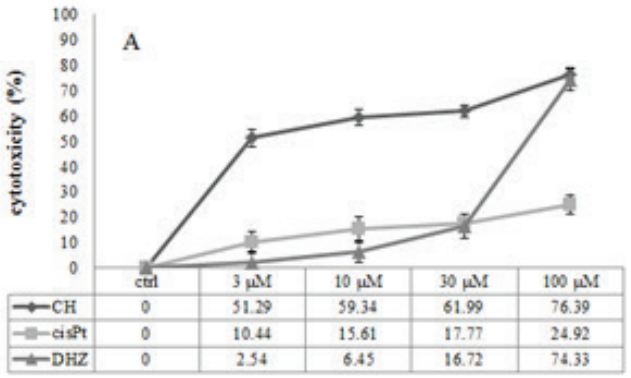
	ctrl	0,3 μM	1 μM	3 μM	10 μM	30 μM	100 μM	300 μM
24 h	0	41.41	47.12	51.29	59.34	61.99	76.39	87.92
48 h	0	33.53	33.66	30.40	41.79	62.54	83.52	92.76

Figure 1. Sensitivity of cervical cancer cell line (HeLa) on CH treatment after 24 and 48 h. The percentage of cytotoxicity cells was determined with MTT assay following treatment of cells with different concentrations of CH for 24 and 48 h. The highest dose of CH 300 μM resulted in nearly 90% of cytotoxic HeLa cells for 24 and 48 h, respectively. The lowest dose of CH 0.3 μM resulted in 41.41% and 33.53% of cytotoxic HeLa cells for 24 and 48 h, respectively.

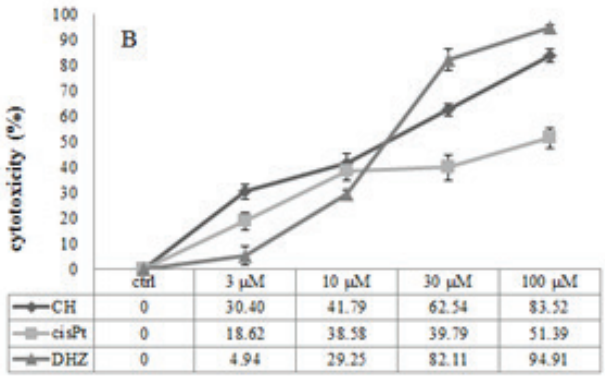
RESULTS

In our experiment, first, we evaluated the cytotoxic effect of CH on HeLa cells for 24 and 48 h using MTT assay at various concentrations of the substances. The results of MTT assay are shown in Figure 1. The data indicate that CH showed the concentration-dependent cytotoxic effect on HeLa cells after 24 and 48 h treatment. Application of the lowest investigated dose of CH resulted in 41.41% (after 24 h) and 33.53% (after 48 h), while the highest dose resulted in 87.92% (after 24) and 92.76% (after 48 h) of cytotoxic HeLa cells, respectively. 48 h treatment with lower concentrations of CH (0.3-10 μM) resulted in a lower percentage of cytotoxic HeLa cells, while higher CH doses (from 30-300 μM) increased the percentage of cytotoxic HeLa cells.

A part of our research was the comparison of the cytotoxic effect of CH to the cytotoxic effects of both cisplatin and dehydrozingerone on the HeLa cells after 24 and 48 h treatment (Figure 2). Calculated IC_{50} values showed that



	ctrl	3 μM	10 μM	30 μM	100 μM
CH	0	51.29	59.34	61.99	76.39
cisPt	0	10.44	15.61	17.77	24.92
DHZ	0	2.54	6.45	16.72	74.33



	ctrl	3 μM	10 μM	30 μM	100 μM
CH	0	30.40	41.79	62.54	83.52
cisPt	0	18.62	38.58	39.79	51.39
DHZ	0	4.94	29.25	82.11	94.91

Figure 2. Comparison of cytotoxic effects of CH and referent substances (cisPt and DHZ) on cervical cancer cell line (HeLa) after 24h (A) and 48 h (B) treatments. The dose of CH 100 μM resulted in 76.39 % and 83.52% of cytotoxic HeLa cells for 24 and 48 h treatment, respectively.

Morphological analysis

In order to both investigate and compare the cytotoxic effect of tested substances (CH, cisplatin and dehydrozingerone) on the morphology of treated and untreated HeLa cells, we have used contrast microscope. The HeLa cells were seeded in a 24-well plate and incubated for 48 h with CH, dehydrozingerone and cisplatin (3, 10, 30 and 100 μM). Morphological changes of both experimental and control HeLa cells were visualized with phase contrast microscopy under 100 X magnification on Olympus microscope (model BX51).

Statistical analysis

The significant differences between two groups were determined using paired t-test or by analysis of variance (ANOVA). All data are presented as mean ± standard deviation and each experiment was repeated at least three times independently.

all investigated agents exhibited statistically significant cytotoxic effects on HeLa cells after 24 and 48 hours of treatment (Table 1). Our results showed that treatment of the cells with CH (IC_{50} = 2.55μM) during 24h had more efficient cytotoxic effect compared to the cytotoxic effects of both cisplatin (IC50 = 9.70μM) and dehydrozingerone (IC_{50}= 3.61μM). However, our results clearly showed that difference in the cytotoxicity between CH (IC_{50}= 3.64μM) and cisplatin (IC_{50} = 3.80μM) was insignificant, while dehydrozingerone (IC_{50}=2.41μM) exhibited more efficient cytotoxic effect on the HeLa cells after 48 hour treatment (Table 1).

Table 1. Calculated IC_{50} values for (E) -1- (4-ethoxy-3-methoxyphenyl) -5- methylhex-1-en-3-one and referent substances.

HeLa IC_{50}	CH	cisPt	DHZ
24h	2.55 ± 1.23	9.70 ± 1.22	3.61 ± 2.16
48h	3.64 ± 0.86	3.80 ± 0.4	2.41 ± 0.57

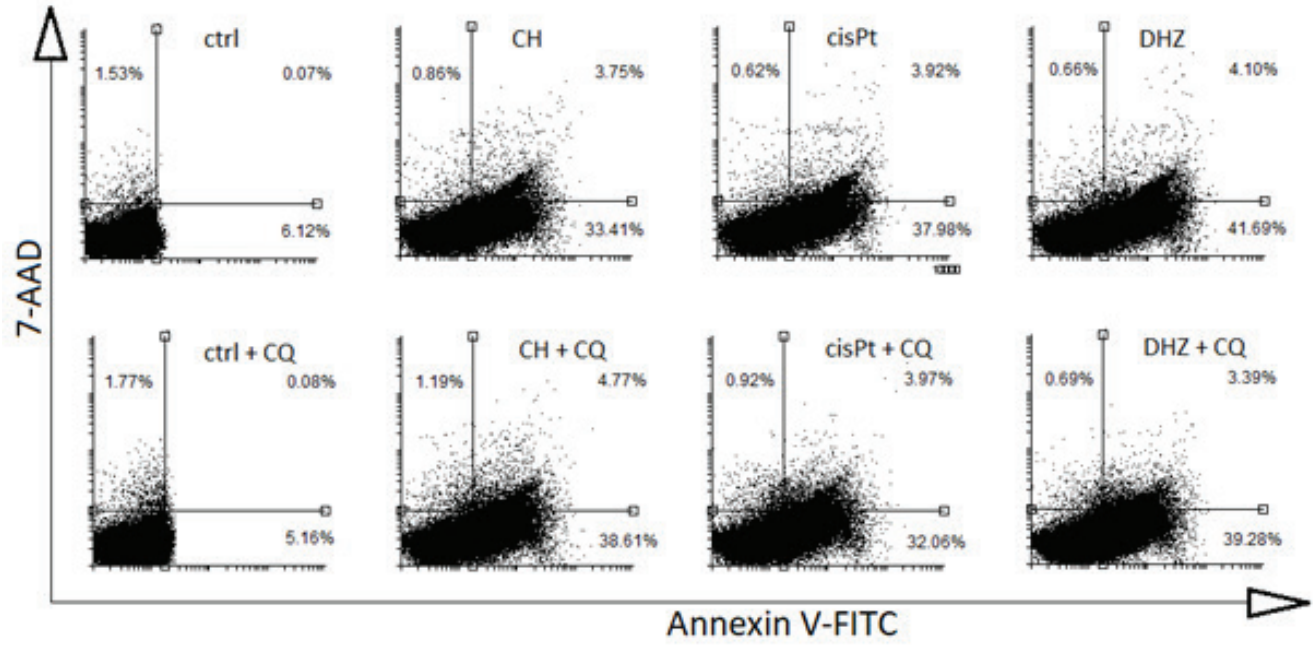


Figure 3. (E) -1- (4-ethoxy-3-methoxyphenyl) -5- methylhex-1-en-3-one induces apoptosis in HeLa cells. Flow cytometry analysis was used to determine the cell apoptosis and autophagy in HeLa cells after 48 h treatment with IC_{50} values of CH, cisplatin and dehydrozingerone, as well as co-treatment with CQ (20 μM) by Annexin V-FITC/7-AAD. Upper left square – necrosis (%); bottom right square – early apoptosis (%); upper right square – late apoptosis.

After determining the cytotoxic effect of CH and referent substances, we next examined the type of the cell death induced by all tested substances using FACS analysis. The HeLa cells were treated with IC_{50} values of investigated substances during 48 hour period (Figure 3). Our results showed that CH induced apoptosis in the HeLa cells. After 48 h treatment on the HeLa cells with CH (IC_{50}=3.64 μM), cisplatin (IC_{50}=3.80 μM) and DHZ (IC_{50}=2.41 μM) we demonstrated that 33.41, 41.69 and 37.98% of cells were in early apoptosis; 3.75, 4.10 and 3.92% of cells were in late apoptosis; 0.86, 0.66 and 0.62 of cells were in necrosis, respectively (Figure 3).

In order to determine the type of cell death, we assessed influence of autophagy on percentage of apoptotic HeLa cells after the treatment with CH. Co-treatment with CH (3.64 μM) and chloroquine (CQ) (20 μM) was per-

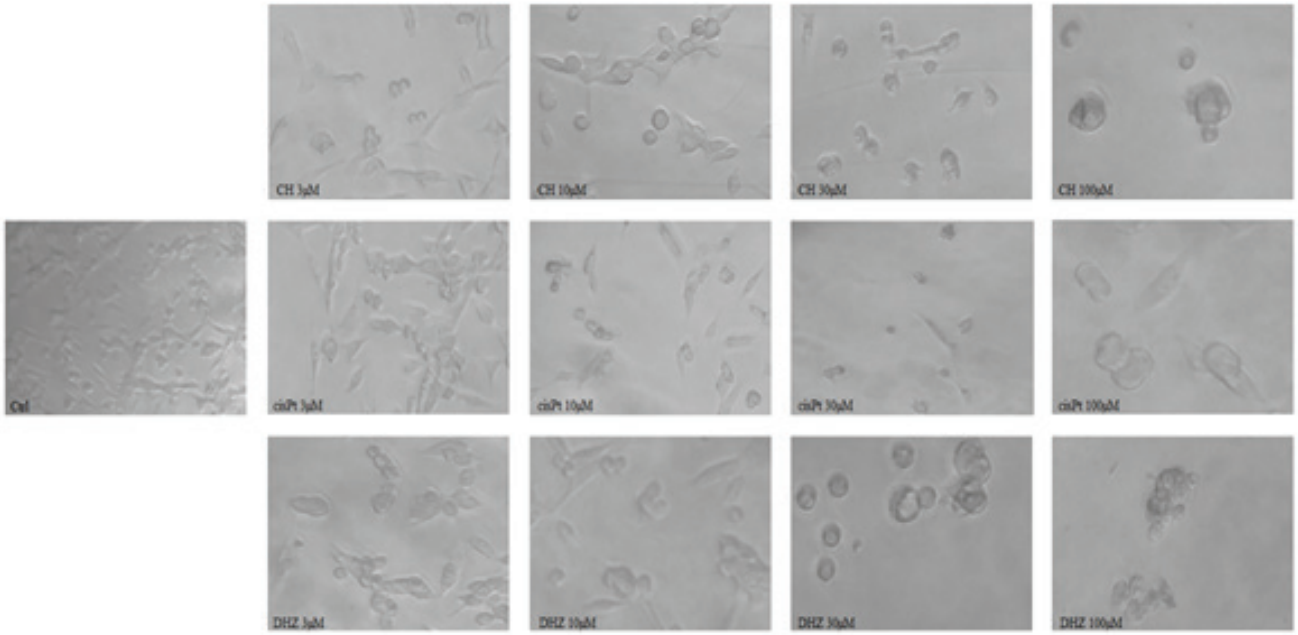


Figure 4. CH induces morphological changes of the HeLa cells in a dose-dependent manner that is consistent with MTT assay and FACS analysis. The HeLa cells were plated in 24 well plates and allowed to attach for 24 h. Cells were exposed different concentrations of CH, DHZ and cisplatin during 48 h period and morphology of the cells was analysed on microscope. After 48 h treatment with different concentrations of all investigated agents the morphology of the treated HeLa cells was significantly impaired compared to the untreated HeLa cells.

formed on experimental HeLa cells for 48 h period. The percentage of apoptotic HeLa cells (early and late apoptosis) treated with CH was 37.16%, while after co-treatment with chloroquine this percentage was 43.38% (Figure 3). Results indicated that addition of chloroquine was not significant for change in percentage of the apoptotic cells, and that the autophagy was most probably not involved in the mechanism of cytotoxic action of the CH and referent substances (Figure 3).

Changes in the morphology of the treated HeLa cells compared to control HeLa cells was evaluated after 48 h treatment with different doses of investigated substances. Our results showed that CH induced morphological changes of HeLa cells in dose dependent manner compared to the control group (Figure 4). These morphological changes were characterized in cell shrinkage cells, loss of shape cells and decrease in the number cells. Increasing the concentration of the CH from 3 μM to 100 μM resulted in a complete loss of cell morphology, which was in direct correlation with the results obtained by MTT assay and Annexin V-FITC/7-AAD staining.

DISCUSSION

Although today there are many options in cancer therapy, including surgery, radiotherapy and immunotherapy, treatment with anti-cancer drugs due to their non-selective cytotoxicity remains the primary treatment option. However, currently available anti-cancer drugs are cytotoxic for the normal cells resulting in a number of adverse effects. Therefore, it is necessary to synthesize new compounds with more efficient antitumor effect and highly selective antitumor. Chalcones are precursor compounds for flavonoids biosynthesis in plants. Changes in their chemical structure have offered a high degree of biological diversity that has proven to be useful for the development and synthesis of new medicinal agents. These medicinal agents exhibit lesser toxicity on normal cells and greater selectivity towards tumor cells compared to natural agents. For example, twelve structural Millepachine analogues (especially (3-hydroxy-4-methoxyphenyl)(5-methoxy-2,2-dimethyl-2H-chromen-8-yl) methanone) showed more effective anti-proliferative activity on five human cancer cell lines (A549, HeLa, HCT 116, A2780 and MGC803) compared to natural compound, Millepachine (An, Zhang, Yan, Huang, & Li, 2017).

Our research provides information of the antitumor effect of chalcone (E) -1- (4-ethoxy-3-methoxyphenyl) -5- methylhex-1-en-3-one (CH) and its potential for development in anti-cancer therapy. The CH represents the structural analogue of dehydrozingerone which is a natural chalcone isolated from ginger (Kuo et al., 2005). The results of our study that of both the newly synthesized chalcone (CH) and natural chalcone (DHZ) showed more effective cytotoxic effect on the HeLa cells compared to cisplatin, as shown in previously reported research (Kolundžija et al., 2014)(Ahmed, Abd El-Hafeez, Abbas, Abdelhamid, & Abdel-Aziz, 2018). Also, our results are in correlation with previous studies that reported the cytotoxic effect of various chalcone analogues on the HeLa cells (Kolundžija et al., 2014)(L. Zhao, Mao, Hong, Yang, & Liu, 2015)(Zhang et al., 2015). Results of the study performed by Zhang et al. indicated that different chalcone analogues exhibited effective cytotoxic effect on HeLa cells with IC_{50} values ranging from 1.4 to 6.8 μM (Zhang et al., 2015). Calculated IC_{50}values for our CH substance was from 2.55 and 3.64 μM, respectively.

Following MTT assay and determination of cytotoxic effects of CH analogues on the HeLa cells, the type of the cell death was evaluated using FACS analysis. Literary data from previous studies have shown that apoptosis was the dominant type of cell death induced with CH analogues (Ramirez-Tagle et al., 2016)(Fogaça et al., 2017)(Takac et al., 2018)(Abdelwahab, Abdul, Zain, & Hadi, 2012). Apoptosis occurs as a defense mechanism in cells during immune reactions or in cells that are damaged by disease (Norbury & Hickson, 2001). Apoptosis include morphological and biochemical changes of affected cells. These changes are characterized with cytoplasmic shrinkage, membrane blebbing, chromatin condensation, externalization of phosphatidylserine activation of caspases and breakdown of proteins. Apoptosis can be initiated via two major signaling pathways: the intrinsic (mitochondrial pathway) through the release of mitochondrial proteins such as cytochrome c into the cytosol; the extrinsic (death receptor pathway) through binding of death receptor ligands to corresponding death receptors such as TRAIL to TRAIL receptors. (Tamm, Schriever, & Dörken, 2001)(Malhotra & Kaufman, 2009). The results of the research by Abdelwahab correlated with our results. Namely, Abdelwahab et al. determined the type of cell death with IC_{50} values of the *Zerumbone* and cisplatin on HeLa cells after 48 h treatment (Abdelwahab et al., 2012). Their results showed that the percentage of apoptotic HeLa cells was significantly higher after 48 h treatment with *Zerumbone*and cisplatin compared with untreated HeLa cells. However, the literature data showed that chalcones induced both apoptosis and autophagy in tumor cells (S. Zhao et al., 2012)(Gao et al., 2016). As part of the research, we examined impact of autophagy on the percentage of apoptotic HeLa cells. The results of our study have shown that Co-treatment with CQ did not induced changes in the percentage of apoptotic cells and that the autophagy was not included in the mechanism of cytotoxicity of the investigated agents. The research by Abdelwahab et al., showed that *Zerumbone* induced different morphological changes in the HeLa cells (Abdelwahab et al., 2012). The chalcones induced apoptosis of the HeLa cells with morphological changes including shrinkage of the cells, loss of the shape cells and decrease in the number cells. The incubation with different concentrations of the CH results in a complete loss of the morphology of treated cells while untreated cells remain confluent throughout the incubation period. We herein reported for

the first time anti-tumor effect CH and we showed that our previously synthesized chalcone had more effective anti-tumor effect compared to many other anti-tumor drugs.

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SYNERGISM OF PDL/PD1 AND IL33/ST2 AXIS IN TUMOR IMMUNOLOGY

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SINERGISTIČKI EFEKAT PDL/PD1 I IL-33/ST2 SIGNALNIH PUTEVA U TUMORSKOJ IMUNOLOGIJI

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ABSTRACT

When it comes to tumor immunology, understanding of molecular pathways is rather important. During oncogenesis, many molecules should be taken in consideration altogether in context of a single malignancy. It is of a great significance to determine whether these molecules act synergistically or contrary, whether to understand a malignant disease more thoroughly, or even more important, to reveal new approaches of therapy. In this review, we discuss whether and how IL-33/ST2 and PD-1/PDL axis involve in antitumor immunity.

Keywords: *IL-33, PD-1, tumor, antitumor immunity*

SAŽETAK

U tumorskoj imunologiji, molekularni signalni putevi mogu biti veoma važni. U toku nastajanja tumora, treba razmatrati veliki broj molekula u okviru jedne maligne bolesti. Od velike je važnosti utvrditi da li ti molekuli deluju sinergistički ili ne, bilo da se radi o boljem poznavanju biologije određenog tumora, ili, još važnije, otkrivanju novih terapisjkih pristupa. U ovom preglednom članku, mi razmatramo da li i kako IL-33/ST2 i PD-1/PDL signalni putevi utiču na antitumorsku imunost.

Ključne reči: *IL-33, PD-1, tumor, antitumorska imunost*

INTRODUCTION

In terms of tumor immunology (oncology), there are myriad of molecules that participate in process of malignant transformation of a cell. Many of these intertwine to breed or to restrain oncogenesis. In order to find better and new treatment of a malignant disease, it is important to understand how cancers grow, metastasize and triumph over immune response. During this process, many molecules should be taken in consideration altogether in context of a single malignancy. It is of a great significance to determine whether these molecules act synergistically or contrary – especially when it comes to new approaches of therapy. In this review, we will try to summarize current data on two molecules that are well known in tumor immunology - IL-33 and PD-1.

INTERLEUKIN-33

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. They share similar signaling molecules, like MyD88 adaptor, IL-1 receptor (IL-1R1) and IL-1 receptor accessory protein (IL-1RAcP) (1,2). 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 (3, 4). Expression of IL-33 has been found in a variety of tissues, including stomach, lung and prostate; however, in contrast to other family members that are expressed predominantly in immune cells, IL-33 can be expressed in endothelial cells, epithelial cells and fibroblasts (5, 6). IL-33 plays an important role in tissue repair, allergy,

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autoimmune disease, infectious disease, and cancer. These roles are carried out through its receptor, Suppressor of Tumorigenicity 2 (ST2), which is broadly expressed on immune cells, such as regulatory T cells (Treg), group 2 innate lymphoid cells (ILC2s), myeloid cells, cytotoxic NK cells, Th2 cells, Th1 cells, and $CD8^+$ T cells (7).

ROLE OF INTERLEUKIN 33 IN BIOLOGY OF TUMOR AND ANTITUMOR IMMUNITY

Having in mind these multiple functions and its versatility of expression, the role of IL-33 has been investigated in many areas of immune response, particularly in tumor immunology. IL-33 can either stimulate or inhibit antitumor immune response, depending on the type of tumor, or tumor microenviroment (summarized in table 1).

It has been shown that exogenous IL-33 enhanced the infiltration of $CD4^+$, $CD8^+$ T cells and tumor antigen- specific $CD8^+$ T cells in established melanoma. Moreover, there is significantly increased IFN-γ production and KLRG1 expression in tumor-infiltrating $CD8^+$ T cells after applying exogenous IL-33 (8-10). Main outcome of these changes is enhancement of antitumor immune response. Also, in a study conducted by Yang et al, it is shown that IL-33 expression is significantly lower in tumor tissue than adjacent healthy pulmonary tissue. The expression was verified by immunohistochemistry and PCR. IL-33 and its receptor, ST2, are significantly down-regulated in pulmonary adenocarcinoma compared to adjacent healthy lung tissues. In addition, the level of IL-33 protein in adenocarcinoma inversely correlates with tumor grade and size. Moreover, higher expression of IL-33 mRNA in tumor tissue correlates with longer overall survival of patients suffering from adenocarcinoma (11). In pulmonary adenocarcinoma, IL -33 restores dendritic cells activation and maturation by interacting with ST2 receptor on dendritic cells, induces co-stimulatory molecule expression and in that way triggers the type I antitumor immune responses by having a direct effect on $CD8^+$ T cells and NK cells (12).

Other studies suggest protumorigenic role of IL-33. In a mouse model of 4T1 mammary carcinoma, in $ST2^{-/-}$ mice decreased metastasis was observed. In our study, we have shown that in $ST2^{-/-}$ mice, tumor appearance is delayed and the growth of primary tumor is slower. Moreover, ST2 deletion significantly decreased number of pulmonary metastasis in $ST2^{-/-}$ mice compared to WT mice. Molecular mechanisms underlying these differences are attributable to higher numbers and efficacy of effector cells. Firstly, ST2 deletion increased total numbers of $CD4^+$ and $CD8^+$ T cells. Secondly, cytotoxicity of both NK and $CD8^+$ T cells is constitutively higher in ST-/- compared to WT mice (13).

In a mouse model of metastatic colorectal carcinoma, similar results were obtained. When IL-33 was given along with MC38 tumor cell line, higher incidence of liver metastasis was detected, compared to MC38 tumors only. However, there were no differences concerning matrix metalloproteinases (MMPs) or lysil-oxidases (LOX), enzymes that are important for tumor metastasis. As no morphological differences were observed, authors concluded that increased metastatic potential is not attributed to cancers cells only and its changed properties and it is rather a result of other processes like enhanced angiogenesis. Also, there are increased numbers of $CD11b^+F4/80^+$ macrophages and $CD11b^+Gr1^+$ MDSCs (14). In presence of signaling of IL-33/ST2 axis, number of immunosuppressive cells in tumor stroma increases (15, 16).

IL-33 promotes tumor growth through recruiting immunosuppressive cells, such as myeloid-derived suppressor cells (MDSC) or Tregs. Also, IL-33 can stimulate type 2 of innate lymphoid cells, mast cells or alternatively activated macrophages (M2), favoring in that way type 2 immune response, which is inefficient in tumor elimination (17, 18). Some authors speculate that it is possible that the nuclear localization of full-length IL-33 also have important role in tumor genesis (19).

IL-33, as mentioned, can have both, antitumorigenic or protumorigenic role. Dual and opponent role in genesis of malignancies of a single molecule is yet to be clarified. It has been speculated that downregulation of IL-33 in epithelial cells can lead to development of a malignancy. In the same way, upregulation of IL-33 in the tumor stroma and serum can lead to inefficient tumor immune response. IL-33 expression on tumor cell promotes type I antitumor immune response through $CD8^+$ T cells and NK cells, and thus makes tumor more immunogenic. On contrary, IL-

Table 1. IL-33/ST2 axis in antitumor immunity

	Cells	Markers	Study
Effects of blocking IL33/ST2 signaling	↑ $CD8^+$ T cell	↑↑ IFN-γ	*Jovanovic et al (2011)*
	↑ NK cells	↑↑ IFN-γ ↑↑ CD11b ↑↑ CD27	*Jovanovic et al (2011)*
	Tumor cells (mammary carcinoma)	↓↓VEGF	*Milosavljevic et al (2015)*
Effects of IL33/ST2 signaling	MDSCs	↓ Accumulation in tumor microenviroment	*Xiao et al (2015)*
	ILC2	↑IL-13 ↑Th2 response	*Li et al (2014)*
	Tregs	↑IL-10 ↑TGFβ	*Siede et al (2016)*

33 expression in tumor stroma facilitates immune suppression via Tregs and MDSCs, so the tumor is less immunogenic and more prone to faster growth and higher incidence of metastasis (20).

PROGRAMMED DEATH 1 MOLECULE

PD-1 (CD279) molecule was first discovered in 1992, when it was thought to play important role in the process of programmed death (hence the name - programmed *death 1, PD 1*). It was thought that all immune cells that enter apoptosis synthesize de novo this protein, express it on the outer membrane and undergo apoptosis (21). Since then, blocking of PD-1 was studied regarding all aspects of immune response, maybe most thoroughly in tumor immunology.

PD-1 molecule, along with CTLA-4, LAG-3, TIGIT, TIM-3, inhibits activation of immune cells. These molecules are referred to as checkpoint inhibitors (22). Many cells express PD-1, such as $CD4^+$ T cells, $CD8^+$ T cells, B cells, natural killer T cells, activated monocytes, dendritic cells, macrophages. In physiological circumstances, it works as molecule that limits immune response and therefore prevents excessive tissue damage and protects from autoimmunity (23-25). Also, PD-1 is highly expressed on T cells that are exposed to antigens for a long time, which makes it a marker of exhausted T cells (26, 27). PD-1 molecule structurally belongs to immunoglobulin family. It contains one extracellular immunoglobulin domain and two intracellular tyrosine-based signaling motifs- ITSM (Immunoreceptor tyrosine-based Switch Motif) and ITIM (Immunoreceptor tyrosine-based Inhibitory Motif). Upon activation, these motifs dephosphorylate and inhibit many signaling molecules and adaptor proteins that are crucial for activation of T cells (28).

Expression of PD-1 is mainly induced by signals from antigenic receptors of T cells (TCR) or B cells (BCR) during immune cell activation, but many cytokines also induce PD-1 activation, especially IL-2, IL-7, IL-15 and interferons (29, 30).

There are two known ligands for PD1: PD-L1 and PD-L2. Both of them contain immunoglobulin-like extracellular domains, similarly like B7 family of costimulators. Although they have same function, PD-L1 and PD-L2 differ to some extent. PD-L1 inhibits T-cell function in peripheral tissues, whereas PD-L2 suppresses immune T-cell activation in lymphoid organs. PD-L2 has a higher affinity for PD-1. Also, unlike PD-L2, PD-L1 can bind to a costimulator molecule, B7-1 (31). PD-L1 is constitutively expressed on many cell types - dendritic cells, macrophages, myeloid cells, T cells, B cells, and few non - hematopoietic cells (vascular endothelial cells, fibroblasts, epithelial cells, neurons and even on some cells at sites of immune privilege). PD-L2 is expressed on dendritic cells, macrophages, peritoneal B1 B, memory B cells (32, 33). In spite of these differs in sites of expression, both can be induced by cytokines, such as IL-2, IL-7, IL-15, TNF-α and interferons, especially IFN-γ (34, 35, 36).

ROLE OF PD1/PDL AXIS IN ANTITUMOR IMMUNITY

PD1/PDL axis suppresses immune responses, by blocking mainly T cells in effector phase. In that way, this axis inhibits T cell proliferation and promotes anergy and apoptosis of activated T cells (37). When PD1/PDL axis is activated, it stops the function of $CD8^+$ and NK cells that can be crucial for immune responses to tumors. It also polarizes $CD4^+$ lymphocytes to forming regulatory T cells, and therefore creates immunosuppressive environment suitable for arising all kinds of diseases, especially malignant. Upon activation, this axis increases activation of a indoleamine-2,3-dioxygenase (IDO), enzyme that degrades tryptophan, amino acid essential for normal function of immune cells. When deprived of tryptophan, T cells tend to become regulatory cells or remain inactivated (38). Even if PD-1 or PDL expression is high on non – hematopoietic cells, it can create immunosuppressive conditions, whether by forming Tregs or inactivating effector cells. It is worth mentioning that Tregs constitutively express higher levels of PD-1; hence, once created, they tend to maintain immunosuppressive conditions. Data from a study by Chen et al suggest that $CD4^+$ T cells without PD-1 molecule have significantly lower predisposition to become regulatory cells, thus showing that formation of immunosuppressive cells specific for tumor antigens is one of the main mechanisms of PD1/PDL axis (39). Tumor cells can be induced to express ligands for PD1, depending on tumor microenvironment. If anti tumor immune response is strong and there are a lot of cytokines involved, such as IFN-γ, TNF-α, PDL expression increases. In that way, tumors acquire immune resistance (40, 41).

ANTI PD-1/PD-L THERAPY

Having in mind all of the above mentioned consequences of activation PD1/PDL axis, it is no wonder that blocking of PD-1 or PDL is currently being considered as a new, promising treatment for autoimmune diseases and malignancies (42). Up to this day, there are five FDA approved monoclonal antibodies that block PD1 or PDL - nivolumab, pembrolizumab, atezolizumab, duvalumab, avelumab. These are used for treatment of some malignancies (renal cell carcinoma, urothelial carcinoma, Hodgkin lymphoma, hepatocellular carcinoma, head and neck carcinoma, mismatch-repair colorectal carcinoma) since 2013 and are currently being investigated in many more types of tumors (43).

Anti PD-1 or anti PD-L therapy improves anti tumor immune response by enhancing life span and performance of effector CD 8^+ cells, NK cells, NKT cells and even den-

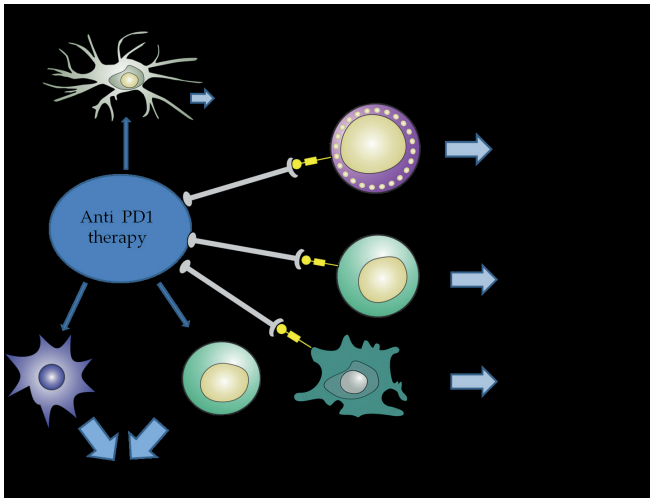

Figure 1. Effect of anti-PD1 therapy. By blocking PDL/PD1 axis, effector cells (cytotoxic T cells, helper T cells, macrophages, etc) become more efficient, have longer half life. Also, immunosuppressive cells are less generated. Overall result of these effects is enhanced antitumor response.

dritic cells (Figure 1). Result of these actions is instantly restored adequate tumor immune response and its overall augmentation (44). One of the good sides of anti PDL/PD1 therapy is certainly its possibility to be combined with other therapeutic drugs, especially other checkpoint inhibitors, or even radiotherapy and surgery. These modalities of treatment of a malignancy, when combined with anti PDL/PD1 therapy show beneficial outcomes. (45-48).

COMBINED BLOCKADE OF IL33/ST2 AND PDL/PD1

Emphasis in treating malignancies is nowadays, without doubt, immunotherapy. To define new or redefine old protocols for treating a malignant disease, it would be convenient to discover potential molecular pathways that could act simultaneously in order to advance anti tumor immune response or suppress tumor invasiveness. Data on combining blockade of IL33/ST2 and PDL/PD1 are very modest. Until now, there is only one study that examines combined effect of these signaling pathways. In a mouse model of acute myeloid leukemia, an immunogenic hematological malignancy, application of exogenous IL-33 itself delayed the onset of leukemia. IL-33 treatment increases production of IFN-γ and IFN-γ producing $CD8^+$ T cells resulting with more efficient immune response against leukemia. Also, in these conditions, there are greater numbers of active antigen-specific $CD8^+$ T cells. Unfortunately, with higher production of IFN-γ, it is possible and expected to have higher expression of PDL1 or even PD1. So, even if IL-33 overcomes peripheral tolerance to malignancy, it also can form ideal circumstances for blocking immune response through PDL/PD1 axis. When a checkpoint inhibitor is added to this therapy, peripheral tolerance is more thoroughly deprived. As expected, survival of mice that received combined therapy was significantly higher (49).

But what about non – immunogenic tumors? As mentioned before, IL-33 has diverce roles in development of these tumors. Some authors agree that every tumor can be immunogenic, i.e. there is always immune response to tumors; but, in non-immunogenic tumors it is rather impaired (50). Overcoming tolerance to tumors by blocking PDL/PD1 axis on the other hand and minimizing immunosuppressive circumstances via blocking IL33/ST2 axis could potentially reveal new, individualized and smarter approach to non – immunogenic malignant diseases.

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CONFLICT OF INTEREST

The authors declare no financial or commercial conflict of interest.

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LAPAROSCOPIC SPLENECTOMY IN THE TREATMENT OF HEMATOLOGICAL DISEASES OF THE SPLEEN

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LAPAROSKOPSKA SPLENEKTOMIJA U LEČENJU HEMATOLOŠKIH BOLESTI SLEZINE

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ABSTRACT

Methods of surgical treatment of hematological diseases of the spleen have changed significantly in the past decade. The introduction of laparoscopic and minimally invasiveprocedures as standard for solving a significant number of conditions in abdominal surgery, has led surgeons toincreasingly use laparoscopic surgery of the spleen. However, some unique anatomical characteristics of the spleen can lead to limitation in the application of laparoscopy. In this study, we investigated the application of laparoscopic splenectomy in the treatment of haematological disorders of the spleen, intraoperative and postoperative characteristics, the presentation ofoperational technique and the evaluation of the success of this procedure.In the treatment of benign hematological diseases, the effectiveness and efficiency of laparoscopy has been proven. The speculation of medical professionals is that laparoscopic splenectomy is an equal, if not the superior way of treating benign hematological diseases of the spleen in relation to the open procedure, and that there is a chance that laparoscopy might completely replace the classical surgery in most of it's indications.

Keywords: *laparoscopy, spleen, laparoscopic splenectomy, hematological diseases, ITP*

SAŽETAK

Modaliteti hirurškog lečenja hematoloških oboljenja slezine značajno su se promenili tokom poslednjih desetak godina. Uvođenje laparoskopskih i minimalno invazivnih procedura kao standardnih za rešavanje značajnog broja stanja u abdominalnoj hirurgiji dovela je do toga da hirurzi sve više primenjuju laparoskopske operacije slezine. Međutim, neke jedinstvene anatomske karakteristike slezine mogu dovesti do ograničenja u primeni laparoskopije. U ovoj studiji smo analizirali primenu laparoskopske splenektomije u lečenju hematoloških oboljenja slezine, intraoperativne i postoperativne karakteristike, prikaz operativne tehnike i procenu uspešnosti ove procedure. U lečenju benignih hematoloških oboljenja uspešnost i efikasnost laparoskopije je dokazana te je stav današnje stručne javnosti da je laparoskopska splenektomija ravnopravan, ako ne i superioran način lečenja benignih hematoloških oboljenja slezine u odnosu na otvorenu proceduru, te da postoji šansa da u budućnosti laparoskopija u potpunosti zameni klasičnu hirurgiju u većini njenih indikacija.

Ključne reči: *laparoskopija, slezina, laparoskopska splenektomija, hematološka oboljenja, ITP*

INTRODUCTION

The first splenectomy was performed by A. Zaccarelli in 1549. in Naples on a young woman due to splenomegaly (1). Quittenbaum did the first planned splenectomy in 1826. on a woman with liver cirrhosis and ascites (2). Hermann Schloffer, on the proposal of his then medical student, Kaznelson, in 1916. successfully performed splenectomy for idiopathic thrombocytopenicpurpura (3). The first laparoscopic splenectomy in adults was performed by Delaitre and Maignen in 1991 (4), and in children, it was done by Tulman in 1993 (5). In 2007. it was Prof. Slavko Matić at the First Surgical Clinic CCS, who first performed laparoscopic splenectomy in Serbia (6).

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The most common indications for laparoscopic splenectomy are idiopathic thrombocytopenic purpura (immunological thrombocytopenic purpura - ITP), (60-80% of all operated patients), followed by hereditary spherocytosis, autoimmune hemolytic anemia (AIHA), benign tumors and cysts, thrombotic thrombocytopenic purpura (TTP), Hodgkin's disease with malignant diseases at the end (7).

Idiopathic thrombocytopenia purport is an autoimmune hematological disorder, characterized by different degrees of thrombocytopenia (7). Surgical removal of the spleen (splenectomy) was the primary form of treatment for ITP until just over 50 years ago when the era of immunomodulatory therapy started (8). The significance of splenectomy is reflected in the fact that the spleen is not just the site of the most intensive degradation of damaged platelets, but also the site of antibody formation that opens the plateletmembrane, and prepares them for degradation (9). Since 1996. laparoscopic splenectomy has again become one of the main modalities of ITP treatment, while surgical treatment is considered in case of failed initialmedication therapy. Today, main indications for splenectomy in ITP are symptomatic splenectomy refractory to medical treatment, the need for high doses of corticosteroids to maintain remission, and relapses after initial response to steroids and the existence of contraindications to their use (10, 11).

So far, numerous advantages of laparoscopy have been demonstrated in relation to open surgery. Bettervisualization of the operating field is of particular importance since the spleen is a richly vascular organ, with numerous vascular relationships with surrounding structures (11). Clinical studies show a lower rate of mortality in laparoscopic splenectomy compared to open (12). Byimproving the technique and increasing the experience of the surgical team, the duration of the surgery is shortened (12, 13). Laparoscopy reduces thelikelihood of serious complications and therefore the need for reintervention (12, 13). Benefits of laparoscopy are also decreased intraoperative blood loss, fasterestablishing ofperistalsis, and lower need for postoperative analgesics, shorter recovery, and better cosmetic outcome (12, 13). The duration of postoperative hospitalization is also shorter, which reduces the overall cost of the treatment (11-13).

The goal of our study is to present a series of patients in whom we performed laparoscopic splenectomy for hematological diseases with the analysis of patient characteristics and outcomes.

MATERIALS AND METHODS

The first surgical clinic began to practice laparoscopic splenectomy began in 2007. Our study was conducted with 82 consecutive patients undergoing surgery at the IX Department of the Clinic for Digestive Surgery of the KCS - First Surgical Clinic in the period from 2007. to 2015. with alaparoscopic splenectomy for benign hematological diseases of the spleen only. All thepatients with malignancy were excluded, as well as those who had any kind of contraindications for laparoscopic surgery, such as prior abdominal surgery, extreme obesity or associated cardiovascular comorbidities. The study was performed as a retrospective case-series analysis of data obtained from thehistory of the disease. In all patients, the haematological diagnosis and primary treatment were provided by the hematologist.

All patients included in this study were primarily treated with medication therapy. Operative treatment decision, based on the above-mentioned demands, was obtained consilliary by the surgeon and hematologists. On the immediate preoperative preparation at the hematologist, there were 37 (45%) patients with an aim to increase the platelet count to>$50x10^9$/L, and they were given pronison (prednisolon), IVIg, danazol, imuran (azithioprine) or some of the combinations of the drugs mentioned. Also, very important role in preoperative preparation, has vaccination in order to reduce the risk of infection by encapsulated bacteria (Streptococcus pneumoniae, Neisseria meningitidis and Haemophylus influenza).

Laparoscopic splenectomy is performed under conditions of general endotracheal anesthesia (OETA). After the patient is introduced into general anesthesia, a nasogastric tube is placed in order to decompress the stomach (11). The choice of approach depends on the operator, the size of the spleen, the anatomical characteristics of the patient and the eventual need for performing another procedure in the same act, for example, Cholecystectomy (13). More commonly, the right side (lateral) approach is practiced. This is theso-called hanging spleen technique (a technique of a hanging spleen), which implies that the patient lies on the right side of the operating table, while the body of the patient with the surface of the operating fieldcovers the of 45 to 75 degrees. The operating table is also adjusted so that the patient's legs are lower than the head, in the so-called, reversed Trendelenburgposition (11,12).

At the beginning of the operation, CO_2 insufflation is performed, creating pneumoperitoneum, which is maintained at 13-15 mmHG. Pneumoperitoneum exceeding 15mmHg can hemodynamically compromise the patient. Pressure inside is maintained <t constant levelsautomatically through the trocar throughout the procedure (7). There are four ports through which various laparoscopicinstruments are placed during the operation. The 12-mm diameter port extends left laterally from the anterior axillary line above the anterior superior iliac spine, since the stapler for dissection of the hylum is placed through it, and also discharges the splenic material at the end of the surgery. The camera is placed through a second port placed on the left and laterally from the umbilicus. The third is subxyphoid, and the fourth in the middle or posterior axially line under the left costal margin (11).

Placing the first trocar requires special attention, as it is the only part of the procedure that is performed blindly, before inserting the camera. When examining

the abdomen (liver, lymph nodes, detection of eventual presence of accessory spleens), we begin by dividing the ligaments that hold the spleen in its position and in relationships with surrounding structures. The dissection proceeds with the division of the splenocolic ligament, which is cut by a laparoscopic ultrasound (Ultracission®), which provides us with access to the inferior pole of the spleen. We continue by cutting short gastric blood vessels, to approach the hylum of the spleen. Hilar arterial and venous blood vessels are most often handled with endovascular stapler, Hemolock® clips, titanium clips, and intracorporal ligatures, after which it is possible to relieve the spleen from other remaining ligaments (splenorenal, splenophrenic and splenogastric ligament) and thus completely separate it from its attachments. The left lateral trocar is then removed and through the same site we introduce polyethylene bag into which spleen is later placed. Inside the bag the spleen is fragmented withintention for easier extraction through trocar of the largest dimension. After rinsing the operative field and aspiration, it is necessary to re-examine the abdomen and insert surgical drain (3). Some authors believe that the drain is needed only in case of pancreatic injury, while others put it inroutinely at the end of each surgery (14). Nasogastric tube, placed before the surgical intervention, is removed after the establishment of intestinal peristalsis.

For classification of postoperative complications, the Clavien-Dindo Classification was used (15). Patients platelets were analyzed daily before surgery, as well as after surgery from the first postoperative day until the day of discharge. They were treated with low molecular weight heparin, with the addition of antiaggregation therapy in the case of an increase in the platelet count to over 500x10^9/L. Corticosteroid therapy was gradually tapered until discontinuation.

The data was processed in the SPSS 20.0 program. The results are presented in the form of an arithmetic mean and standard deviation if they meet the criteria of normal distribution, otherwise they are represented by the median and the range of values. Categorical data are presented with absolute and percentage values. An X^2test was used to analyze categorical type data. P values ≤ 0.05 are determined as significant.

RESULTS

In the group of 82 patients operated by the laparoscopic method there were 15 (18.3%) men and 67 (81.7%) women. The mean age of the of our patients was (38.8 ± 11.79) years (16-74). The average duration of hematologic treatment was 33.3 months (3 - 179). Most of the operatedpatients had a diagnosis of ITP-a 72 (87.8%), eight patients (9.8%) were diagnosed withspherocytosis, and two (2.4%) had diagnosis of AIHA. The average BMI was 24.3 kg/m^2 (18-30). The average platelet count was 81.8 (25-240) x 10^9/L.

Table 1: Types of used haemostatic techniques

Endo GIA	48	58.5%	<0.001
Hemolock clips	27	32.9%	
Intracorporal ligatures	4	4.9%	
Titaniumclips	3	3.7%	

19 accessory spleens were detected intraoperatively, indicating their presence in 23.2% of the operated patients. For mobolization of the blood vessels of the hills of the spleen a. i v. lienalis (haemostatic technique), Endo GIA stapler with vascular filling, Hemolock® clips, intracorporal ligatures and titanium clips were used. (Table 1)

Intraoperatively, the average blood loss was of 36.8ml (10-200) and average mass of the spleen fragmentsof 211.48 ± 32.73g (151-270). Of the total number of patients undergoing surgery, in 4 patients (4.9%) a conversion into an open procedure was performed, due to bleeding, in one patient due to an instrumental fracture of the spleen, in two due to a poorly placed Endo GIAstapler and one due to aninjury to the arteries for the inferior pole of the spleen. The average duration of the surgery was 83.6 minutes (48-135). In our group of patients, we postoperatively observed complications of (Grade I) in four of ourpatients, (Grade II) in six, one patient had complications of (Grade III), one of (Grade IV) and only one was found to have complications of (Grade V).In our group of operated patients, none of them had undergone reintervention. Median length of postoperative hospitalization was of 5.7 days (3-14).In one patient (1.2%), fatal outcome was recorded due to fulminant sepsis, after a series of non-surgical complications. The therapeutic response was complete in 60 (74.1%) patients. In 18 of them, additionaltherapy was required, and in 3 patients, the therapeutic response proved to be poor. (Table 2)

Table 2: Perioperative characteristics and patient outcomes

Characteristics	average	min	max
Intraoperative blood loss in ml	36.8	10	200
Duration of surgery in min	83.6	48	135
Length of postoperative hospital stay in days	5.7	3	14
Complications, n (%)			
I	4 (30.8)		
II	6 (46.1)		
III	1 (7.7)		
IV	1 (7.7)		
V	1 (7.7)		
Therapeutic response, n (%)			
Complete response	60 (74.1)		
Additional therapy	18 (22.2)		
Poor therapeutic response	3 (3.7)		
Death	1(1.2)		

DISCUSSION

The objectives of laparoscopic splenectomydefined in 1991. when the first intervention was made, were: Maintain results identical to open surgery in terms of efficacy and safety, while reducing the trauma of the abdominal wall, easier postoperative recovery, shorter duration of hospitalization (16).

Laparoscopic splenectomy was quickly accepted by medical professionals and became widely applicable worldwide. This enabled analyzing the results obtained from different studies, that follow an increasing number of these interventions, easier. With the purpose of answering the question of whether the application of laparoscopic splenectomy is really justified?

Delaitre et al. analyzed 209 patients who were treated almost exclusively by laparoscopy in a study conducted at the level of 12 surgical centers in France (12). The Japanese study of Wu and associates analyzed 10 patients over a five-year period and the aim of the study was to examine the effectiveness of laparoscopic splenectomy, as well as the safety and benefits of using this technique in the treatment of ITP in patients with an extremely low platelet count (<1x10^9/L), which explains the small number of subjects involved in the study (17).

The results of our study show that hematological disorders of the spleen were more common in female subjects with incidence of 81.7% compared to men with 18.3%. The average age of patients in our group was 38.8 years (16-74). Based on the analysis of demographic characteristics, which include the age and gender of patients, we can say that the results we obtained do not differ significantly from the data found in literature.

Patients included in our study were cared for by their hematologist before being subjected to surgical intervention. The period of medication treatment ranged on average 23.5 months (3-420). Wu et al. calculated that in their patients, the average age of patients at the time of diagnosis was 21.5 (9-43) years, while at the time of surgical intervention they were 32.6 (15-62) years (7). The authors do not state that the time from diagnosis to surgery could be significant for the possibly different outcome of treatment, but we may conclude on the basis of the results that show that all patients, even at the last checkup performed after 36 months, had a value of platelets >100x10^9/L (in one patient the value was 126x10^9/L, in one 154x10^9/L, and for the remaining eight patients, the value was over 200x10^9 /L) (7).

In the study of Elezović and colleagues, the patients age at the time diagnosis ranged from an average of 35 years (17-74), and splenectomy was commonly performed within 12 months (2-160). The results showed that the occurrence of remission did not depend on the time elapsed from diagnosis to surgical intervention (8). The majority of our patients of interest received corticosteroids, mainly prednisone, and less commonly urbason and methylprednisolone, while other drug options such as, combinations of prednisone and IVIg, danazol or immuran,werereservedfor refractory forms of the disease. According to The American and British society of Hematology ITP Treatment Guide, Corticosteroids and ivIg should be an initial treatment option, or should have priority over splenectomy (13). A study by Dolan and associates analyzed some of the attempts to avoidsplenectomy as a treatment option in patients who did not have an adequate response to steroids, by using new therapeutic options like anti-D antibodies and anti-CD20 antibodies. Ultimately such treatment turned out to be inferior to splenectomy (5). In our group of patients, the platelet number on the admission was 81.8 x 10^9/L (25-240) on average. Thrombocyte values of over 100 x 10^9/L have been seen in some cases and are the consequence of the use of medication therapy prescribed by the hematologist with an intention to prepare patients for the surgical intervention. In the French study, Delaitre and Associates, the mean platelet count was 92.7 x 10^9/L, ranging between 3 and 444 x 10^9/L. In this study, as many as 178 out of 209 patients underwent preoperative preparation, which explains this high value of platelets before surgery (3).

The first intraoperative characteristic analyzed in our study was the detection of accessory spleens, which were observed in 19 patients. They were most commonly found (in cases where there were more than one) in the hylum, gastrocolic ligament, omentum, the tail of the pancreas. The first major study on the effectiveness of the detection of accessory spleens in patients operated with classic open technique and a laparoscopic approach was published by Samphat and Associates (2007) (9). Today's impression of laparoscopy is that it does not lag open surgery regarding the possibility of intraoperative detection of accessory spleens.

According to our study, mean intraoperative blood loss during laparoscopic splenectomy was 36.8 ml (10-200), whereas in the study of Park and associates, the mean blood loss was 162 ml (5-1400) (17). Amongst many data found in literature, maybe the best indicator of the safety and benefits of laparoscopyare the study by Wu and associates, which states that the blood loss in patients operated by the laparoscopic technique ranged on average 44 ml (10-100) (7). Adding to thisthe fact that this was a group of vulnerable patients, in which even the lowest blood loss could be fatal, we can see the real significance in their treatment.

In the majority of patients, hilar vessels are divided with Endo GIA stapler with vascular filling at 48. In 27 patients, Hemolock® clips were used, in four, intracorporeal ligatures and in three patients, we used titanium clips. Habermaltz and associates cite bleeding as the most significant intraoperative complication and the most common reason for conversion to an open procedure. They also recommend the use of endovascular staplers for the treatment of blood vessels of the hylum of the spleen, instead of ligatures or clips, on the basis of studies that have shown that this can prevent bleeding during and after intervention (14). Dolan and associates listed the use of an endovascular stapler as

a standard in the treatment of hilar spleen vessels (5). Park and associates expressed an opinion, that the application of endovascular stapler should be given priority over the clips. This paper states that the bag for the extraction of the spleen, is an integral part of the equipment for the laparoscopic splenectomy, and is to be used whenever possible (17). In our study, we used these bags in all the patients, except for when conversion to an open procedure was done.

In our study, the conversion to open procedure was performed in 4 patients (4.9%) due to bleeding, which was also the only type of complication recorded. In one patient, due to instrumental rupture of the spleen, a conversion was made, in one due to an injury to the arteries for the inferior pole of the spleen, and in two of them due to a poorly placed Endo GIA stapler (the stapler was caught in the clips previously placed on surrounding smaller blood vessels). In France, the study of Delatrie and associates, conversions were performed in 17.2% of the operated patient, and the cause was, in all cases, bleeding (3). There were no conversions in the Japanese study of Wu and associates in 10 operated patients (7). Park and associates published a study in 2001. which also included results oflaparoscopic splenectomies in the period 1995-1999, performed by 14 surgical teams, each of which had a sample of at least 24 patients. Only one sample of 49 patients did not make a conversion. All other authors recorded a certain percentage of conversions ranging from 3 to 19% (17). What is to be expected, is that the percentage of conversions into an open procedure is reduced by gaining greater experience and skills, but one should not forget that the type of pathology in question and the size of the spleen are of great importance when considering this problem. In the US National Registry, from 2005 to 2010, 37006 splenectomies were recorded, showing a worrying 22.5-33.9% of conversion, with only 13.3% of patients being treated with laparoscopic technique (18).

According to our results, the average duration of surgery was (83.60 ± 14.75) minutes (48 - 135). In the previous years, the length of laparoscopic splenectomy was much longer. That can be seen from manypublished studies, amongst which is the one published by Delaitre and associates, where the average duration of surgery was 144 minutes. The authors of this study concluded that the experience of the surgical team was one of the most important factors. It greatly influenced the duration of laparoscopic intervention and predicted its shorter duration with the acquisition of the necessary experience (3).

In the study of Delaitre and associates, 209 patients were treated laparoscopically, and three patients required reinterventions. In two of them, because of bleeding and in one the cause was pancreatitis (3). Unlike theirs, our study and the study of Sampath and associates did not have a single reintervention (9), but we should bear in mind that Delaitre had many more patients, so it is expected that the number will be higher.

For the evaluation of postoperative complications, in our group of operated patients, Clavien-Dindo classification was used (15). In one patient, fatal outcome was recorded due to fulminant sepsis after a series of non-surgical complications (Grade I). In one patient, myocardial infarction (Grade IV) was recorded. Percutaneous drainage for necessary collection after surgically removed spleen (Grade III) was performed in one. In six patients, additional antibiotic therapy was required (Grade II), and four patients needed additionaltherapy in the form of antiemetics, analgesics and antipyretics (Grade I). Literature indicates a significantly lower percentage of postoperative complications in patients undergoing laparoscopic surgery compared to an open one. Winslow and Brunt together with collaborators worked on a meta-analysis of 51 series with a total of 2940 patients (2119 of them were laparoscopic splenectomies and 821 open procedures) and showed a statistically lower percentage of complications in the laparoscopic group, which was 15.5% compared to 26.6% in the open procedure group (19). Postoperative complications in the study of Delaitre and associates occurred with a frequency of 8.7%, while in the case of an open procedure, due to bleeding during the operation, it increased to as much as 19.4% (3).

The average duration of postoperative hospitalization was, in our patients, 5.7 days (3-14), in the study by Delaitre and associates, it was found to be 5.1 days (12), and in the study by Wu and associates, 6.8 days (4-9) (7).

In our study, the drain was placed at the end of all surgical procedures. Delaitre and associates placed the drain in 72.3% of the patients and they also recorded a higher morbidity of 13.7% in that group, compared to the group of 5% of patients where drain was not inserted. But no statisticalsignificance was proven (3). Habermalz and associates state that there is still no clear position regarding the use of drainage after laparoscopy and that it is the decision on the operator, except in cases of suspicion of pancreatic injury when drainage is certainly required to be placed (14).

According to our results, a good therapeutic response was found in 60 patients (74.1%), moderate response, ie the need for additional therapy, in 18 patients (22.2%) and 3 patients had poor therapeutic response (3.7%). In 2008. The European Association for Endoscopic Surgery clinical practice guideline published that the authors of certain studies (Trias et al. Cordera et al. Lozano-Salazar et al.) managed to prove that long-term results were identical in laparoscopic and classical surgical patients (11). Dolan and associates classified the results of 5 studies, which compared patients with laparoscopic surgery and a classic approach and concluded that there was no statistically significant difference in either of these studies (5). The experts think that laparoscopy is at least as effective, if not more effective, in the treatment of ITP and other benign hematological diseases of the spleen, as well as open surgery.

As our study is presented as a case-series of 82 patients, we still should be cautious about drawing conclusions, and further prospective randomized analyses should be performed. Possible other limitations that should be noted are in regard of multiple hematological diseases for which the patients were operated, andrelatively short follow-up period.

Today, 26 years after the first laparoscopic splenectomy in adult patient was presented to the medical public, the chances of laparoscopy, completely replacing conventional surgery in the future in most of its indications, are high. Even so, this does not exclude the need for new research papers to compare the outcomes of the laparoscopic and classic procedures, however there is a problem related to laparoscopic treatment of malignant diseases of the spleen in the presence of splenomegaly and problems with manipulation of the spleen in the operative field. Even if laparoscopic splenectomy will not become broadly accepted in the surgery of malignant diseases of the spleen, there remains a wide spectrum of benign hematological diseases where the effectiveness and efficiency of laparoscopy is proven. It can help this large group of patients not only by treating their illness, but also, by reducing the trauma of the body, which will result in faster recovery, and all along with a better quality of life.

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USE OF PERFLUOROCARBON BASED BLOOD SUBSTITUTE PERFTORAN IN CORRECTION OF HYPOXIA DURING ACUTE ANEMIA IN ANIMALS

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UPOTREBA ZEMENE ZA KRV ZASNOVANE NA PERFLUOROKARBONU ZA KORIGOVANJE HIPOKSIJE TOKOM AKUTNE ANEMIJE KOD SISARA

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ABSTRACT

The cause of acute and severe hypoxia of the organism is acute posthemorrhagic anemia. To eliminate posthemorrhagic anemia in animals, the perfluorocarbon blood substitute Perftoran (Russia) with a gas-transporting function was used. The aim of this study was to determine the clinical effectiveness of the perfluorocarbon based blood substitute Perftoran with a gas-carrying function in acute posthemorrhagic anemia in animals and reveal possible side effect of the blood substitute and remove them. In the study conducted in the Clinic of Veterinary Medicine of Pushchino Research Center (Russia) participated 20 cats of both sexes, who were admitted with internal bleeding as a result of injuries. The animals were divided into two groups: the control and the treatment groups (10 per group). All animals with anemia were examined according to the standard scheme: anamnesis vitae and anamnesis morbi, physical examination (basic methods of research were used), additional methods that were used: complete blood count (CBC) and biochemical analysis of blood (BA), microscopy of blood smears, abdominal ultrasonography. Based on the obtained results, we can conclude that the use of the gas-carrying substitute for donor blood Perftoran in the treatment group of animals with posthemorrhagic anemia, which resulted from polytrauma, eliminated tissue hypoxia; the treatment of the animals in the control group with standard solutions (by infusing Stabisol) without gas transport correction led to the development of persistent hypoxia, which persisted to the stage of reticulocyte crisis.

Key words: *perfluorocarbon blood substitute, hypoxia, cats*

SAŽETAK

Uzrok akutne i teške hipoksije organizma je akutna posthemoragična anemija. U cilju sprečavanja posledica posthemoragične anemije kod životinja, korišćena je zamena za krv na bazi perfluorokarbona Perftoran (Rusija) koji ima ulogu transportera gasova. Cilj ovog istraživanja je bio utvrđivanje kliničke efikasnosti zamene za krv na bazi perfluorokarbona (Perftoran) sa funkcijom nosača gasova u akutnoj posthemoragijskoj anemiji kod životinja i ispitivanju mogućih neželjenih efekata i njihovog uklanjanja. U istraživanje sprovedeno na Klinici za veterinarsku medicinu istraživačkog centra Pushchino (Rusija) je uključeno 20 mačaka oba pola, koje su primljeni sa unutrašnjim krvarenjem kao posledica povreda. Životinje su podeljene u dve grupe: kontrola i grupe za lečenje (10 po grupi). Sve životinje sa anemijom ispitane su u skladu sa standardnom šemom: anamnesis vitae i anamnesis morbi, fizički pregled (korištene su osnovne metode istraživanja), a kao dodatne metode korišćene su: potpuna krvna slika (CBC) i biohemijski analiza krvi (BA), mikroskopija krvnih mrlja, abdominalna ultrasonografija. Na osnovu dobijenih rezultata, zaključuje se da upotreba Perftorana, kao zamene za donorsku krv sa svojstvima nosača gasova, u lečenju životinja sa posthemoragijskom anemijom nastalom usled politraume, uspešno sprečava hipoksiju tkiva. Lečenje životinja standardnim rastvorima u kontrolnoj grupi sa standardnim rastvorima (rastvor Stabisol) bez poboljšanja transporta gasova uzrokovala je nastajanje uporne hipoksije, koja je perzistirala na stadijumu retikulocitne krize.

Ključne reči: *zamena krvi na bazi perfluorokarbona, hipoksija, mačke*

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INTRODUCTION

The pathological condition of organism with a decrease in the total amount of red blood cells (RBCs) and hemoglobin in the volume of blood is called anemia. The etiological factors of anemia are diverse; they can be congenital, acquired, but regardless of the cause and pathogenesis, the common result of anemia is the decrease in the total amount of hemoglobin and RBCs in the volume of blood and as a consequence – the decrease of oxygen-carrying function of RBCs and the development of hypoxemia and hypoxia (1-4).

The most frequent cause of acute and severe hypoxia in both human and veterinary medicine is acute posthemorrhagic anemia. Acute posthemorrhagic anemia develops during the massive blood loss of 20% of the total circulating blood volume (CBV) (2, 3). Usually this pathological process is the consequence of injuries. There are four stages in the dynamics of acute posthemorrhagic anemia, as indicated by Alexeev et al. in his work (1).

The first is a stage of collapse and it occurs immediately after the blood loss and lasts approximately one day after the bleeding ceases. At this stage the clinical picture is dominated by the symptoms of collapse, whereas the picture of peripheral blood has almost no deviation from the norm, because with rapid massive blood loss the decrease in hemoglobin and RBCs is only due to the decrease of CBV (1-5). Compensatory factor in this stage is peripheral vascular spasm. The second is a hydremic stage in which decrease in CBV leads to the activation of mechanisms directed towards restoring the amount of fluid circulating in the vascular system: the tissue fluid passes into the vessels; the resulting thirst stimulates the flow of water into the body, which, along with a decrease in dieresis, which develops as a result of both the spasm of kidney vessels and the delay of sodium in organism under the influence of aldosterone released by adrenal glands, leads to an increase in the amount of water in vascular bed (2-7). At the same time the release of RBCs from depots to blood occurs. Hypoxia, which occurs immediately after the blood loss, activates the secretion of erythropoietin by the kidney and stimulates erythropoiesis in the bone marrow. But this process takes time, thus its first signs are observed only on the 4th-5th day after the blood loss. By that time the second stage ends and the third stage begins (2-7). The final stage is a stage of reticulocyte crisis and stage of recovery in which erythropoiesis intensifies, which is indicated by the significant increase of reticulocytes, immature RBCs, in the volume of blood (9). As a result of the processes developed in the stage of reticulocyte crisis the hemoglobinization of RBCs is normalized, the color index is restored. Together with erythropoiesis, leukopoiesis is also stimulated, which is manifested in a slight leukocytosis (3-8, 10).

The greatest effect on the animal's organism hypoxia has is in the hydremic stage, since all RBCs reserves are consumed, and the number of RBCs may continue to decrease as a result of autoimmune hemolysis (5, 6). Cats' RBCs have their own features: they are more spherical and smaller in size (40-50 μm) than dogs' RBCs. Their lifespan is relatively small: from 60 to 80 days. Cats have two types of hemoglobin: HbA and HbB; they are present in different ratios in different individuals and have less affinity for oxygen, than hemoglobin of other animals (7, 8). Considering the morpho-physiological features of cats and stages of posthemorrhagic anemia, it is necessary to immediately carry out therapy aimed at eliminating hypoxia of the organism.

The best effect on elimination of systematic hypoxia can be achieved through blood transfusion, but in cats, unlike dogs, blood transfusion can lead to adverse reactions even with the first perfusion; furthermore, it is impossible to collect a clinically significant amount of blood from a donor cat (5-9).

It is known that cats have three blood groups: A, B and AB. It is necessary to perform cross-matching, since if the donor's and recipient's blood is incompatible, the severe hemolytic reaction will occur due to the appearance of alloantibodies. The most severe reaction occurs when the RBCs of the blood group A is administered to the recipient of blood group B (7, 10). However, knowledge of the blood groups of the donor and recipient does not guarantee prevention of hemolytic reaction; antibodies can be present, bonded with other factors of the blood group, which have not yet been well defined in cats (9-12). Furthermore, with transfusion of incompatible blood in cats, the lifespan of donor RBCs is only several hours, therefore the therapeutic effect cannot be achieved. On the 15th day of the blood storage RBCs significantly lose elasticity of the membrane and deformability, which also reduces their therapeutic effect (13).

Well, in severe forms of anemia the use of donor RBCs in cats is limited and poses a serious threat to the recipient. Therefore, the question arises, whether the use of an artificial blood substitute, which has a gas-carrying function, for anemia in cats is advisable. The aim of this study was to determine the clinical effectiveness of the perfluorocarbon based blood substitute Perftoran with a gas-carrying function in acute posthemorrhagic anemia in animals and reveal possible side effect of the blood substitute and remove them.

MATERIALS AND METHODS

Ethical approval

All of the experimental procedures and were carried out in accordance and with the permission of the Institutional ethical committee for the welfare of laboratory animals.

Protocol of study and animals

In the studies conducted in the Clinic of Veterinary Medicine of Pushchino Research Center (Russia) partici-

pated 20 cats of both sexes, who were admitted with internal bleeding as a result of injuries. The animals were divided into two groups: the control and the treatment groups with n=10 in each.

In the first, control group, traditional infusion therapy was used, which included the use of a colloidal solution Stabisol in a dose of 20 ml/kg with the oxygenation by medical oxygen with a flow saturation of O2 = 87-95,5 %, productivity 5 l/min (oxygen concentrator Armed 7F-5L).

According to the instruction for use, Stabisol is a volume expander (plasma-substituting agent) and a 6% isotonic solution of synthetic colloid hydroxyethyl starch (HES) with an average molecular mass of 450000 Da. Stabisol HES 6% has a volemic effect within the 85-100% of the administered volume, which lasts for 6-8 hours, which is due to the ability of the drug to bind and retain water in the intravascular space. Stabisol HES 6% improves the rheological properties of blood and microcirculation, as well as cerebral and feto-placental blood flow (including by reducing the hematocrit). This leads to improved blood supply to tissues, a decrease in plasma viscosity and platelet aggregation and prevents RBCs from aggregating.

In the second, treatment group, in addition to the colloidal solution of Stabisol, perfluorocarbon blood substitute Perftoran was used. Perfluorocarbon blood substitute Perftoran was developed in USSR-Russia in the period 1979-1996. Main works were carried out at the Institute of Biophysics of the USSR Academy of Sciences and continued at the Institute of Theoretical and Experimental Biophysics of the Russian Academy of Sciences; from 1979 to 1985 under the supervision of F.F. Beloyartsev and G.R. Ivanitsky; from 1986 to 1997 under the supervision of S.I. Vorobyev.

Synthetic blood substitute Perftoran is a 20% emulsion of perfluorinated compounds (PFCs) nanoparticles, emulsified to an average size of 100 nm, and a gas-carrying substitute for donor blood, and, in terms of its functions, it is best suited for eliminating hypoxia in animals that has developed as a result of anemia. Most effectively the drug realizes its properties in the first 6 hours after infusion, when breathing pure oxygen or air, enriched with oxygen to 60-70%. Due to the presence of chemically inert perfluorinated compounds in the drug, it is capable of dissolving O2 and CO2 up to 7 vol% (with pO2=760 mmHg) and 60 vol% (with pCO2=760 mmHg), respectively. Admitted into a clinic animals with anemia were prescribed emulsion Perftoran in a dose of 10 ml/kg (this dose was recommended by the manufacturer in case of the blood loss). Infusion was carried out on animals on the 1st, 2nd, 3rd, 5th and 7th day after injury. Drug administration started with a bioassay (a gradually controlled administration): 0,1 ml of the drug was diluted with 0,9% NaCl to 5 ml, injected slowly intravenously, and then drip with pauses of 60 s – 3, 5, 10, 30 drops, gradually increasing the rate of infusion to 1 drop per sec.

In admitted sick animals viral infections were excluded: viral leukemia of cats and viral immunodeficiency using rapid tests (Vet-Expert, Poland) through chromatographic immunochemical analysis. All animals with anemia were examined according to the standard scheme: anamnesis vitae and anamnesis morbi, clinical examination (basic methods of research were used), additional methods that were used: complete blood count (CBC) and biochemical analysis of blood (BA), microscopy of blood smears, abdominal ultrasonography.

For the CBC, hematological analyzer (Mindray BC-2800 vet, China) was used, which made it possible to monitor the gas composition of peripheral blood. The degree of anemia was determined by the hematocrit level.

For the pulse oximetry of injured animals, during the surgical intervention the device Mindray iPM 10 vet (China) was used to determine the peripheral oxygen saturation - SpO2.

BA of blood was performed using the biochemical blood analyzer IDEXX VetTest 8008 (USA) and Reflovet Plus (Switzerland); the lactate level was determined, which showed the degree of hypoxia at the cellular level, as well as the content of creatinine, urea, K+, Ca2+, bilirubin, which gave information about the functional capability the of kidneys and liver.

Ultrasonography was performed immediately after the admission of the animals to clinic. To visualize the structure of the parenchymal organs, tumor changes and the presence of free fluid in the abdominal cavity, the system with the color doppler Mindray Z6 (China) was used.

Statistical analyses

Statistical data processing was carried out in the MatLab program. During the analysis, the arithmetic mean values and standard deviations were calculated. The hypothesis of equality of means in two groups was tested using one-tailed Student t-test for independent samples.

RESULTS

Effects of colloidal solution Stabisol (Control group)

By admission the clinical picture of the animals in the control group was dominated by symptoms associated with a decrease in total CBV: a sharp drop in blood pressure, paleness of skin and visible mucous membranes, tachycardia and tachypnea; hematological and clinical signs corresponded to the first stage of posthemorrhagic anemia (stage of collapse). At the same time, the hematocrit and the lactate level remained within the normal range, since the tissue hypoxia had not developed yet, and the proportional loss of plasma and blood cells did not affect the hematocrit. After the intravenous infusion of Stabisol in a dose of 20 ml/kg, the animals underwent surgery to eliminate the internal bleeding. The peripheral oxygen saturation SpO2 in animals under general anesthesia was 89-93%, and only with intensive oxygenation SpO2 increased to 95%, which indicated acute hypoxemia. The

animals heavily recovered from general anesthesia, the condition of three patients were critical.

Thus, in cats of the control group, posthemorrhagic anemia in the stage of collapse had a sufficiently clear clinical picture, but without the characteristic hematological changes.

On the 2nd day after the blood loss, distinct hematological signs of anemia showed in the animals: the hematocrit decreased to 19%, the lactate level increased to 6 mmol/l. The clinical picture was manifested in the form of "porcelain" mucosa, shortness of breath, oppression, feed refusal and tachycardia. These hematological changes characterized the second stage of posthemorrhagic anemia (hydremic stage). The animals were given infusion of Stabisol 10 ml/kg and intensive oxygen inhalation.

On the 3rd day after the bleeding, the animals continued to be in a serious condition, distinct signs of hypoxia were evident: tachycardia, tachypnea, diaphragmatic breathing, anemic mucosa, increased thirst, loss of appetite. The animals were given another infusion of Stabisol in a dose of 10 ml/kg and intensive oxygen inhalation. The hematocrit was at the level of 20%. The lactate level increased to a critical value of 6,9 mmol/l. There was a lethal outcome in one animal.

On the 5th day of the animal observation, the general state stabilization was noticeable: appetite improved, the hematocrit increased to 25%, which was reflected in an increase in activity, the mucous membranes became pale pink, and tachycardia disappeared. The lactate level was reduced to 5,8 mmol/l, but with an increase in activity animals had shortness of breath. Clinical picture corresponded to the third stage of posthemorrhagic anemia (stage of reticulocyte crisis). The animals were given another infusion of Stabisol in a dose of 10 ml/kg and intensive oxygen inhalation.

On the 7th day of the animal observation, hematocrit of the animals in control group increased to 30,8%, the level of lactate decreased to 2,7 mmol/l; with increased activity, shortness of breath was absent, appetite improved, mucosa was pale pink. The animals were given another infusion of Stabisol in a dose of 10 ml/kg and intensive oxygen inhalation.

Effects of colloidal solution Stabisol in combination with perfluorocarbon blood substitute Perftoran (Treatment group)

The clinical picture of the animals in the treatment group was the same as in the control group at admission. On the 1st day, as studies showed, hematological indices in animals did not change, hematocrit 38%, lactate level 2,6 mmol/l.Hypoxia of the tissues had not developed yet, and the proportional loss of plasma and blood cells did not affect the hematocrit. The clinical picture was dominated by symptoms associated with a decrease in total CBV: a sharp drop in blood pressure, paleness of skin and visible mucous membranes, tachycardia and tachypnea; hematological and clinical signs corresponded to the first stage of posthemorrhagic anemia (stage of collapse). To replenish CBV Stabisol in a dose of 10 ml/kg was used according to the same scheme, as in the control group. To correct the gas composition of blood Perftoran in a dose of 10 ml/kg was used. During the infusion patients were given an oxygen inhalation. Then the animals underwent the surgery to eliminate internal bleeding.

SpO2 in animals under general anesthesia was 95-97%, and even without intensive oxygenation SpO2 did not fall below 95%, which indicated the absence of hypoxemia, despite the acute blood. The animals adequately recovered from general anesthesia, the patients' condition was stable.

On the 2nd day after the blood loss in animals, despite the decreasing level of the hematocrit to 16% and the distinct signs of anemia in the form of anemic mucosa, shortness of breath, oppression, feed refusal and tachycardia, the lactate level remained within the reference values. To replenish CBV and gas composition of blood patients were given another infusion of Stabisol in a dose of 10 ml/kg and Perftoran in a dose of 10 ml/kg, combined with an oxygen inhalation.

On the 3rd day after the bleeding, the cats were in a satisfactory condition, the lactate level decreased to 2,5 mmol/l; despite a low level of the hematocrit of 18%, the signs of hypoxia were noticeable: tachycardia, tachypnea, diaphragmatic breathing, anemic mucosa, increased thirst, loss of appetite. As a curative care patients were given infusion of Stabisol and Perftoran in the same dose, combined with an oxygen inhalation.

On the 5th day of the animal observation, the state stabilization in patients was noticeable: appetite improved, the hematocrit increased to 21%, which was reflected in an increased activity, the mucous membranes became pale pink, and tachycardia disappeared. Lactate did not tend to increase and was 2,0 mmol/l. To fixate a positive clinical picture, the animals were infused with Stabisol and Perftoran in the same dose, combined with an oxygen inhalation.

On the 7th day of the animal observation, the hematocrit increased to 31%, whereas lactate – to 2,7 mmol/l, with increased activity, shortness of breath was absent, appetite improved, mucosa was pale pink. Despite the obvious improvement of clinical and biochemical parameters, cats were given another infusion of Stabisol and Perftoran in the same dose, combined with an oxygen inhalation.

DISCUSSIONS

The aim of this study was to determine the clinical effectiveness of the perfluorocarbon based blood substitute Perftoran with a gas-carrying function in acute posthemorrhagic anemia in animals and reveal possible side effect of the blood substitute and remove them.

Prevoius studies have shown, that infusion of the gas-carrying drug Perftoran, which provides an oxygen supply and helps the organism of the patient, which has received severe polytrauma, to go through the general anesthesia, surgical intervention and postoperative period, is necessary to be carried out on injured animals (14-16).

Table 1. Dynamic of changes in hematocrit and lactate level in animals with posthemorrhagic anemia in control and treatment groups with statistical significances. *p<0.05 represent significant differences between control and treatment groups

Parameter	Groups	Time of research (days)				
		1	2	3	5	7
Hematocrit (%)	*Control*	38.00±3.65	19.00± 1.76*	20.00± 1.4*	25.00± 1.7*	30.8±3.12
	Treatment	38.00± 5.33	16.00±1.87	18.00±2.21	21.00±1.76	31.00± 3.89
Lactate (mmol/l)	*Control*	2.5±0.25	6.00±0.93*	6.9±0.65*	5.8±0.28*	2.7±0.27*
	Treatment	2.5± 0.22	2.7± 0.3	2.5± 0.28	2.0± 0.37	1.5± 0.45

Hematological indices of cats in both groups. Using pulse oximetry during the surgical intervention in animals with acute blood loss made it possible to compare the SpO2 of the patients in the treatment and control groups on the 1st day of admission. It is clearly seen that the SpO2 level of the animals in the treatment group after the administration of Perftoran, despite the severe blood loss, has not fallen below 95%, which indicates the absence of hypoxemia. On the contrary, in the control group the SpO2 level dropped to 79%, indicating a life-threatening hypoxemia.

During anesthesia saturation must always remain within 95-100%. If the saturation is 94% or lower, the patient develops hypoxemia, and emergency measures must be carried out. Saturation below 90% requires urgent care (6, 17, 18).

Our results have shown, that hematological parameters of the cats in the control group in all stages were almost identical to the parameters of the cats in the treatment group, however, the lactate level in the hydremic stage, i.e. 48 hours after the blood loss, increased above the reference values and tended to increase, which indicated the development of the tissue hypoxia of the injured animal, and only 7 days later after the injury the lactate level was reduced to reference values, which was associated with the increase in RBCs and decrease in tissue hypoxia. As a result of developing posthemorrhagic anemia of moderate severity, in injured animals their own endogenous compensation mechanisms activated, which allowed the body to replenish the lost volume of RBCs and maintain its viability.

In the treatment group of animals, in comparison with the control group, the lactate level remained within the reference values for 7 days, despite the low level of RBCs, which indicated the absence of tissue hypoxia (Table 1, Figs. 1 and 2).

It is known that lactate in the animals is formed as a result of anaerobic metabolism. Its level increases due to the poor blood supply and tissue hypoxia. The range of normal lactate levels in cats is 0,5-2,8 mmol/l (5, 18, 19). Studies have shown that at injection of gas-carrying blood substitute Perftoran the lactate level has been significantly better in the treatment group. This is due to the gas-carrying properties of the perfluorocarbon emulsion – the basis of Perftoran (20).

The perfluorocarbon emulsion increases the mass transfer of O_2 due to the following effects: an increase in level of physically dissolved O_2 in the plasma (21); accelerated diffusion of O_2 in perfluorocarbons; increase in saturation speed of O_2 in perfluorocarbons; large surface area for gas exchange; intensification of O_2 extraction by emulsion particles from the RBCs' hemoglobin; formation of a "pearl-thread" structure of perfluorocarbons in the bloodstream, which are oxygen channels, through which oxygen conductivity is 20-25 times higher than in the plasma (21, 22).

Perfluorocarbon emulsion reduces the viscosity of the blood and improves the vascular bed, which facilitates the passage of RBCs through the capillaries, thus increases the delivery of O_2; improves the parameters of gas composition and acid-base balance of blood, reduces acidosis.

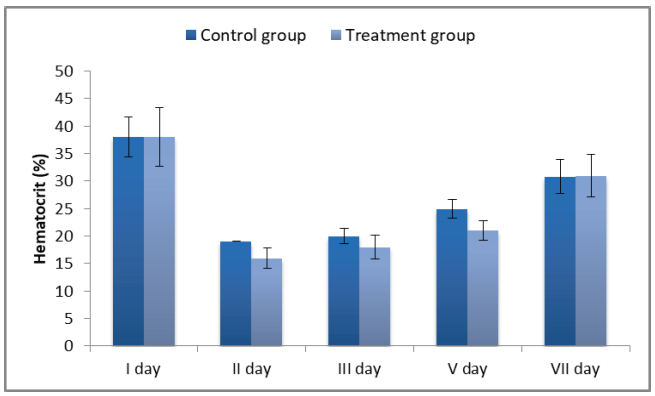

Figure 1. Hematocrit (%) in control and experimental group during the experimental period. Values are presented as mean ± standard deviations.

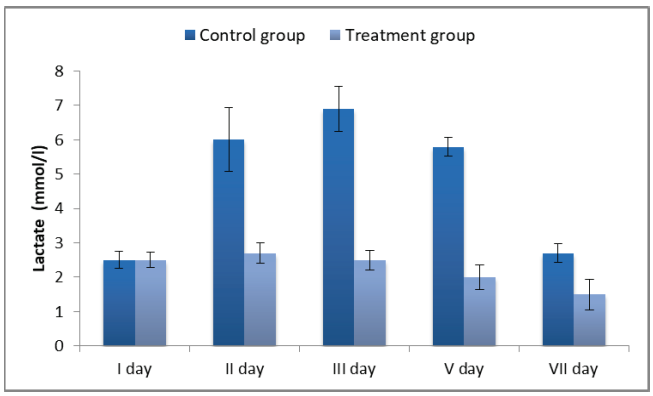

Figure 2. Lactate levels (mmol/l) in control and experimental group during the experimental period. Values are presented as mean ± standard deviations.

CONCLUSIONS

Based on the obtained results, the following conclusions can be drawn. The use of the gas-carrying substitute for donor blood Perftoran in the treatment group of animals with posthemorrhagic anemia, which resulted from polytrauma, eliminated tissue hypoxia. Also, the treatment of the animals in the control group with standard solutions (by infusing Stabisol) without gas transport correction led to the development of persistent hypoxia, which persisted to the stage of reticulocyte crisis. We can conclude that the use of Perftoran in correction of massive blood loss allows maintaining the level of gas transport properties of blood (SpO2) at the physiological level, which does not allow the hypoxemia to develop. Finally the use of Perftoran with strict adherence to the rules did not lead to adverse allergic reactions in animals. In the future, the clinical experience in treating animals with polytrauma allows the recommendation of perfluorocarbon based blood substitute with gas-carrying function Perftoran as an alternative substitute for donor blood.

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THE EFFECT OF SOCIODEMOGRAPHIC FACTORS ON THE PATIENT SATISFACTION WITH HEALTH CARE SYSTEM

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UTICAJ SOCIODEMOGRAFSKIH FAKTORA O ZADOVOLJSTVU PACIJENTA SISTEMOM ZDRAVSTVENE ZAŠTITE

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ABSTRACT

The goal of this paper is to determine the level of patient satisfaction with health care among adults in the Republic of Serbia and to analyze the correlation between the satisfaction and socio-demographic characteristics of the interviewees. The paper is based on the data provided by the National health survey of the Republic of Serbia. For the purposes of this paper, we used data on age and household of the people aged 19 and more. By eliminating the interviewees who were neither satisfied nor dissatisfied with the health care services, we obtained the sample containing 18.206 interviewees. Demographic characteristics and well-being index represented independent variables in the research. Dependent variable of the patient satisfaction was transformed into a binary variable by categorizing satisfied and very satisfied interviewees into one group and by placing dissatisfied and very dissatisfied interviewees into group of dissatisfied patients. The connection between satisfaction and predictors was examined using Chi-Square test and logistic regression. The percentage of the satisfied patients with health care was 72.9%. The satisfaction level was directly connected to age, gender, marital status, employment, region the interviewee comes from and well-being index. Patients who were more satisfied included older people, women, as well as married people, the unemployed and those living in the cities. The analysis of the financial situation shows that the poorest interviewees were the most satisfied with health care.

Keywords: *health care, patient satisfaction, socio-demographic characteristics*

SAŽETAK

Cilj ovog rada je da se utvrdi stepen zadovoljstva pacijenata sa zdravstvenom zaštitom među odraslima u Republici Srbiji i da se analizira korelacija između zadovoljstva i socio-demografskih karakteristika ispitanika. Rad je zasnovan na podacima Nacionalnog zdravstvenog istraživanja Republike Srbije. Za potrebe ovog rada koristili smo podatke o starosti i domaćinstvu ljudi starijih od 19 godina. Eliminacijom ispitanika koji nisu bili zadovoljni niti nezadovoljni zdravstvenim uslugama, dobili smo uzorak sa 18. 206 ispitanika. Demografske karakteristike i indeks blagostanja predstavljaju nezavisne varijable u istraživanju. Zavisna varijabla zadovoljstva pacijenta pretvorena je u binarnu varijablu kategorizacijom zadovoljnih i veoma zadovoljnih ispitanika u jednu grupu i postavljanjem nezadovoljnih i veoma nezadovoljnih ispitanika u grupu nezadovoljnih pacijenata. Veza između zadovoljstva i prediktora ispitana je korišćenjem testa Chi-Skuare i logističke regresije. Procenat zadovoljnih pacijenata sa zdravstvenom zaštitom iznosio je 72,9%. Nivo zadovoljstva bio je direktno povezan sa starošću, polom, bračnim statusom, zaposlenjem, regionom od kojeg ispitanik dolazi i indeksom blagostanja. Pacijenti koji su bili zadovoljniji uključivali su starije ljude, žene, kao i venčane, nezaposlene i one koji žive u gradovima. Analiza finansijske situacije pokazuje da su najsiromašniji ispitanici bili najviše zadovoljni zdravstvenom zaštitom.

Ključne reči: *zdravstvena zaštita, zadovoljstvo pacijenta, socio-demografske karakteristike*

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INTRODUCTION

Health care represents an organized and wide activity within one society which has a goal to protect and improve the heath of people. Making an available and comprehensive health care system represents a major challenge for every country (1). The old, but quite comprehensive divison of the health care model into Bismarck's, Siemaszko's and Beveridge's models has been abandoned resulting in mixed models in many countries, containing elements of the two or even all three models. Unsustainability of Bismarck's model in Serbia has forced the health policy creators to start a reform of the health care system during the 1990s. The reform included both financial and organizational changes (2).

The quality of health care, according to the definition by the World Health Organization, reflects the level of achieved goals of the health care system aimed at protecting and improving the health of people (3). Improving the quality of health care is conducted in three steps. First step includes defining the quality itself, the second includes defining the assessment indicators of it and the third includes designing of the improvement program (4). The biggest contribution to the process of defining indicators for the quality assessment of the given services was given by Avedis Donabedian. According to him, three major components which define the quality of health care are: structure, process and the result (5). The structure requires human resources, technical resources and clinical working guidelines. The process includes the percentage of patients who received the necesarry and, at the time, the best treatment in relation to the total number of patients (e.g. the percetange of patients with myocardial infarction who received thrombolytic therapy). The result is connected to the type of the disease and it includes the following: mortality and morbidity rate, functional and working status, the quality of life and patient satisfaction with the given health care service (6).

Subjective quality assessment of the given health care services mostly uses the following two indicators: patient satisfaction with health care and self-assessment of the health condition (7). Patient satisfaction with health care is reflected in a general and optimal quality of the given service which meets the needs of patients at the given moment (8). The level of patient satisfaction with health care services is used in the analysis of the health care reforms in a certain country as well as for comparison between different countries. Therefore, the World Health Organization, in cooperation with the Organization for Economic Cooperation and Development (OECD) started a project in 2002 in order to improve the quality of the given services across many countries around the world (9).

THE GOAL OF THE PAPER

The aim of this paper is to conduct an analysis of the sociodemographic factors and their effect on the patient satisfaction with the given health care services.

MATERIAL AND METHODS

The retrospective study which represents the part of the National research of public health in the Republic of Serbia was conducted in 2013 and funded by the Serbian Ministry of Health. The research was conducted as a cross-study research and the obtained sample was a stratified two-phase sample, without repetition. The stratification was conducted in such a way that all 4 geographical areas (Vojvodina, Belgrade, Sumadija and West Serbia, South and East Serbia) represented one and the main stratum in the sample. Following that, each stratum was divided into cities and other regions. The total number of strata was 8. Two-phase sampling includes municipalities as the units of the first phase and households as the units of the second phase. The questions and indicators in the questionnaires were standardized according to the questions used in EU, while the indicators are included in the database "Health for all" in World Health Organization.

In order to meet the requirements of the research, adult population aged 19 and more was analyzed (excluding Kosovo and Metohija). Dependent variable of the patient satisfaction, measured using Likert-type scale, was transformed into binary variable in the following way: the interviewees who were very satisfied or satisfied were categorized into the group of the satisfied patients and the interviewees who were dissatisfied or very dissatisfied were categorized into the group of patients who were dissatisfied with health care system. The interviewees who weren't either satisfied or dissatisfied were eliminated from the research.

STATISTIC METHODS

The data were described using descriptive statistical methods and analyzed using univariate or multivariate techniques of data analysis. The descriptive statistical method which was used was the patient ratio with the certain outcome. χ^2 test was used to test the significance of the differences in frequency, using contingency tables. The correlation of the dependent variables with independent predicators was studied using logistic regression. The risk was estimated using OR value (odds ratio), with 95% of confidence interval.

The results are shown in tables. The results show the values (p) and the more important values are those which are $p \leq 0.05$.

The data will be processed in SPSS (Statistical Package for the Social Sciences) 19.0 program.

RESULTS

The average age of the interviewees was 50.49±17.59. Patients who were satisfied were 4 years older on average (53.13±17.75, as opposed to 49.38±16.52, $p<0.001$). Gen-

der analysis shows that women are more satisfied. Two thirds of married people and one third of those who are not married were satisfied with health care, 69.4% of the unemployed and 30.6% of the employed were satisfied with health care. The region analysis shows that the inhabitants of Sumadija and West Serbia are satisfied the most while people living in Vojvodina are the least satisfied, namely, every third person is dissatisfied with health care. People who live in cities are more satisfied that those who don't and half of the total number of interviewees graduated from high school. The analysis of the financial situation of the patients' households, measured using well-being index shows that the richest people are the least satisfied and the poorest are satisfied the most (Table 1).

The results of the binary logistic regression show that there is a statistically significant correlation between the patient satisfaction with health care and variables such as age, gender, marital or working status, region or financial situation of the interviewee.

Patient satisfaction reduces with age. Men are almost 1.2 times less satisfied with health care in comparison to women, and the same situation repeats with married and employed people. The region analysis shows that the interviewees coming from Vojvodina and Belgrade are 1.5 and 1.2 times less satisfied with health care respectively in comparison to people coming from South and East Serbia. The richest interviewees are 1.3 times less satisfied with health care in comparison to the poorest members of our society (Table 2).

DISCUSSION

Patients' experience and satisfaction represents an important indicator of the quality of the provided services (10). Based on that, one can notice and define the problems and realize which areas could be improved (11). The researches show that patient satisfaction influences the amount of hos-

Table 1. Satisfaction and sociodemographic characteristics of patients, univariate analysis

Variable	Satisfaction		Total n	χ^2	p
	Satisfacted n (%)	Unsatisfacted n (%)			
Gentle					
Men	5715 (43.1)	2358 (47.8)	8073	32.59	<0.001
Women	7558 (56.9)	2575 (52.2)	10133		
Marital status					
Married	8829 (66.6)	3374 (68.5)	12203	5.27	<0.05
Other	4420 (33.4)	1555 (31.5)	5975		
Employment					
Employed	4054 (30.6)	1879 (38.1)	5933	92.71	<0.001
Unemployed	9213 (69.4)	3053 (61.9)	12266		
Region					
Vojvodina	2938 (22.1)	1484 (30.1)	4422	229.7	<0.001
Belgrad	2458 (18.5)	1112 (22.5)	3570		
Sumadija and West Serbia	4538 (34.2)	1236 (25.1)	5774		
Southern and Eastern Serbia	3339 (25.2)	1101 (22.3)	4440		
Residence					
Urban	7040 (53)	2793 (56.6)	9833	18.4	<0.001
Rural	6233 (47)	2140 (43.4)	8373		
Education					
Elementary	4919 (37.1)	1511 (30.6)	6430	74.79	<0.001
Second	6527 (49.2)	2585 (52.4)	9112		
High	1827 (13.8)	837 (17)	2664		
Well-being index					
Poor	3106 (23.4)	946 (19.2)	4052	88.68	<0.001
Second	2872 (21.6)	1062 (21.5)	3934		
Third	2826 (21.3)	960 (19.5)	3786		
Fourth	2430 (18.3)	968 (19.6)	3398		
Rich	2039 (15.4)	997 (20.2)	3036		

Variable	Values	OR (95% CI)	p
Age		0.989 (0.986-0.991)	<0.001
Gentle			
	Women	1	
	Men	1.197 (1.118-1.281)	<0.001
Education			
	Elementary	1	
	Second	0.945 (0.866-1.032)	>0.05
	High	1.067 (0.947-1.201)	>0.05
Marital status			
	Other	1	
	Married	1.105 (1.028-1.189)	<0.05
Employment			
	Unemployed	1	
	Employed	1.087 (1.004-1.176)	<0.05
Region			
	Southern and Eastern Serbia	1	
	Vojvodina	1.536 (1.399-1.686)	<0.001
	Beograd	1.255 (1.128-1.395)	<0.001
	Sumadija and West Serbia	0.816 (0.743-0.896)	<0.001
Residence			
	Urban	1	
	Rural	1.030 (0.951-1.116)	>0.05
Well-being index			
	Poor	1	
	Second	1.144 (1.030-1.272)	<0.05
	Third	1.036 (0.926-1.160)	>0.05
	Fourth	1.154 (1.021-1.305)	<0.05
	Rich	1.313 (1.145-1.507)	<0.001

pital days, the results of the treatments and the degree of doctors' mistakes and it is one of the indicators of the successful work performed by doctors and medical institutions (12, 13). Patient satisfaction with health care is extensively used while analyzing reforms of the health care system all around Europe, Asia and America (14, 15).

The comparison of patient satisfaction among 4000 interviewees from Great Britain and USA shows that there is a significant difference between these two countries as well as between the regions themselves within one country.

The reform analysis of the health care system in China clearly shows the rise of patient satisfaction with health care among the interviewees coming from rural places following the reforms of the health care system.

The influence of age and gender factors on the satisfaction level varies. Some researches show that the older the patients are, the more satisfied they are (16-18) while some other researches deny the correlation between age and the level of satisfaction (19, 20). Some researches argue that men are more satisfied whereas some argue the opposite (21-23), while marital status generally does not affect the level of patient satisfaction (24). However, patient satisfaction is affected by a well-being index. Namely, patients whose financial situation is poor and those who are less educated are more satisfied with health care than those with better financial conditions (25, 26).

Communication between medical personnel and patients, along with socio-demographic factors, greatly influences the level of patient satisfaction with health care. The level of trust in the chosen doctor is directly correlated with the successful communication, especially among women and highly educated patients. So, by providing patients with detailed information about their condition prior to surgery, not only the patient satisfaction increases but the frequency of postsurgical complications reduces and the length of stay in the hospital decreases.

CONCLUSION

Analysis of the impact of socio-demographic characteristics of respondents to the satisfaction of health care is the way to better evaluate and understand the expectations of patients. Socio-demographic characteristics of the users of health care services significantly affect the satisfaction

of health care. These facts health policy makers and health care providers should take into account when analyzing the health services.

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CLINICAL USEFULNESS OF ^{99M}TC-HYNIC-TOC AND ^{131}I-MIBG SCINTIGRAPHY IN THE EVALUATION OF ADRENAL TUMORS

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KLINIČKI ZNAČAJ SCINTIGRAFIJE SA ^{99M}TC-HYNIC-TOC I ^{131}I-MIBG U EVALUACIJI TUMORA NADBUBREŽNIH ŽLEZDA

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ABSTRACT

Disorders and morphological abnormalities affecting the adrenal gland, could lead to profound clinical consequences, owing to its biochemical structure-activity and morphological characteristics.

The recent focus on theranostic approach has led to a need for tumors characterization and early diagnosis at the molecular level. Many radiotracers have been developed with specific imaging characteristics for the adrenal tumors, by exploiting different physiological mechanisms of uptake and metabolism.

The aim of present study is to provide a prospective confirmation of ^{131}I-MIBG and ^{99m}Tc-HYNIC-TOC scintigraphy, for the evaluation of patients with known or suspected tumors of the adrenal region.

The research is designed as a cross-sectional observational study of the clinical correlates and diagnostic accuracy of radionuclide-based imaging methods in relation to in vitro analysis, clinical manifestations and morphological characteristics of these tumors. Furthermore, the present study also evaluates the usefulness and the clinical impact of each radiopharmaceutical for the detection and management of tumors, and functional imaging modality as well.

Visual scintigraphic appearance of an increased focal tracer uptake in the suspected tumor site revealed that ^{99m}Tc-HYNIC-TOC is highly sensitive and reliable tumor-seeking radiotracer for adrenal tumors, but does not distinguish between adenoma and pheochromocytoma, and the existence of hormone secreting adrenocortical tumor cells. However, ^{131}I-MIBG scintigraphy is highly sensitive and specific method only in differentiating catecholamine-secreting adrenal tumors.

Clinical significance of this research is in the accurate localization of adrenal tumors, and is of paramount importance for an algorithmic diagnostic approach and management, and provide the rationale to different therapeutic possibilities.

Key words: *^{131}I-MIBG, ^{99m}Tc-HYNIC-TOC, adrenal tumors*

SAŽETAK

Ekspanzivni procesi nadbubrežnih žlezda manifestuju se kao poremećaji funkcije i morfologije, i odlikuju izraženom raznolikošću kliničke slike.

Prateći tokove savremene medicine, dijagnostička procedura navedenih poremećaja se sve više fokusira na morfo-funkcionalnu evaluaciju na ćelijskom nivou, i različiti tumorotropni radiofarmaceutici su razvijeni sa ciljem da se potvrdi postojanje i odredi funkcijski status tumora.

Osnovni cilj istraživanja je da se ispitaju parametri dijagnostičke pouzdanosti scintigrafije sa ^{131}I-MIBG i ^{99m}Tc-HYNIC-TOC u evaluaciji ispitanika sa ekspazivnim procesima nadbubrežnih žlezda.

Istraživanje je dizajnirano kao klinička, opservaciona, studija preseka, u cilju ispitivanja parametara dijagnostičke pouzdanosti scintigrafskih metoda, njihove dijagnostičke tačnosti u odnosu na kliničke, laboratorijske i morfološke dijagnostičke parametre. Ispitivane su scintigrafske karakteristike svakog radiofarmaceutika ponaosob, u odnosu na postojanje tumora i njihovu sekretornu aktivnost, kao i utvrđivanje njihove kombinovane prediktivne vrednosti.

Studija je pokazala da se kvalitativnom analizom scintigrama sa ^{99m}Tc-HYNIC-TOC mogu uspešno prepoznati bolesnici sa i bez prisustva nadbubrežnih žlezda, mada ovaj radiofarmacetik ne pokazuje moć distinkcije u smislu adrenalne i medularne propagacije, kao ni za procenu sekretorne sposobnosti adrenokortikalnih tumora. Međutim scintigrafija sa ^{131}I-MIBG ima dijagnostičku korist, i to u diferencijaciji tumora hromafinog tkiva.

Sa kliničkog aspekta, značaj ovog istraživanja je u preciznoj proceni lokalizacije i proširenosti ekspazivnih procesa nadbubrežnih žlezda, što može imati praktični značaj u kreiranju dijagnostičkog algoritma ovih oboljenja, kao i u odabiru adekvatne terapijske opcije.

Ključne reči: *^{131}I-MIBG, ^{99m}Tc-HYNIC-TOC, nadbubrežne žlezde, tumori*

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INTRODUCTION

Primary adrenal tumours encountered in clinical practice comprise a broad spectrum of clinical presentations due to their biochemical structure-activity and morphological characteristics. All neoplasms derived from the adrenal medulla and cortex can give rise to benign or malignant tumours that can be hyperfunctioning or non-functioning (1-3).

While the overall prevalence of adrenal masses are estimated to occur in approximately 9% of the general population (1), the incidence of adrenal nodules at autopsy is between 8.7% and 32% of patients without suspicion of adrenal disease (2, 4-6). Ageing is associated with an increased frequency, the prevalence being <1% among individuals less than 30 years of age to approximately 6% in those over 60 years of age (1, 7, 8). These masses are more frequently unilateral, but in about approximately 15% of cases, they present as bilateral (5, 7-9).

Adrenal adenomas are the most commonly encountered adrenal masses at up to 80%, with myelolipoma accounting for 6% and pheochromocytoma for 3%. (2, 4, 5, 7, 8, 10). The vast majority of these lesions are benign, non-hyperfunctioning adrenocortical adenomas and require no treatment (60-80%), 5–47% secrete cortisol, and 1.1–10% secrete mineralocorticoids, while androgen or oestrogen secreting masses and primary malignancies of the adrenal gland are extremely rare (1, 5, 7, 10, 11). There are no current screening recommendations for adrenal tumours in the general population, except for patients with known or suspected familiar syndromes (1).

Determining the nature of the adrenal mass is often a clinical challenge. Early diagnosis and an appropriate assessment of an adrenal mass is an essential prerequisite prior to its definitive treatment (2, 7, 12)

The initial diagnostic approach includes a clinical and biochemical assessment of cortical and medullary adrenal function and allows for the identification of hypersecreting adrenal lesions. However, a tumour mass may not cause adrenal hyperfunction since it may be non-hypersecreting or secrete non-active products (2, 6, 7, 12, 13).

Improvements in imaging modalities and their interpretation have increased dramatically over the past few years and can now offer a considerable amount of material to help inform clinical decision making (6, 13, 14). Computed tomography (CT) scans and magnetic resonance imaging (MRI) can provide anatomic details of adrenal tumours and often allow malignancy to be ruled out, although a significant portion of patients have indeterminate tumours (7, 8, 13-16). Furthermore, in hormonally non-functioning tumours, differentiating adrenocortical lesions from other lesions is a major diagnostic challenge (5, 10, 17, 18).

Nuclear imaging techniques performed with specifically radiolabelled agents that display unique biological behaviour and target elements of adrenal function may provide specific information for tumour characterization and an estimate of the functional status of the adrenals (19-22). It is non-invasive and complements the imaging data obtained by CT or MRI to further characterize the lesion. (13, 20, 23).

Although $^{131I-}$ and ^{123}I-metaiodobenzylguanidine (MIBG), a norepinephrine analogue whose uptake is proportional to the number of neurosecretory granules within the tumour, is the most common functional imaging technique used in the assessment of pheochromocytomas (21, 22, 24, 25), the scintigraphic evaluation of patients with adrenocortical tumours is currently limited (13, 19, 20, 23).

In recent years, radiolabelled somatostatin analogues that were proposed in the diagnostic evaluation of malignant neuroendocrine tumours reflecting the presence of somatostatin receptors have now been proposed in the diagnostic evaluation of patients with adrenal abnormalities (19, 20, 26-29)

In this research, we describe the role of nuclear medicine imaging using radiolabelled peptide ^{99m}Tc-hydrazinonicotinylacid-d-phenylalanyl^{1}-tyrosine^{3}-octreotide (^{99m}Tc-HYNIC-TOC) and ^{131}I- MIBG in the diagnostic evaluation of patients with adrenal tumours in order to perform lesion characterization and determine the functional status of these tumours.

MATERIAL AND METHODS

Study population

This cross-sectional study was conducted during the year 2016-2017, at the Centre for Nuclear Medicine and the Centre for Endocrinology, Diabetes and Metabolism Diseases, Clinical Centre Kragujevac. The research was conducted in accordance with the Declaration of Helsinki (2005) of the World Medical Association and was approved by the Ethics Committee of the Clinical Centre Kragujevac. After being informed of the study's purpose, risks and benefits, all patients provided written informed consent to participate in the study.

We analysed 27 male and female consecutive patients older than 18 years, who had a documented clinical diagnosis of an adrenal tumour. The control group was comprised 19 patients with clinically diagnosed pituitary adenoma without clinical characteristics of adrenal involvement.

All patients underwent a standardized diagnostic evaluation of hypothalamic–pituitary–adrenal tumours based on biochemical and clinical parameters and imaging criteria (2, 6, 7, 12, 13, 30).

The exclusion criteria were defined in the protocol study. Therefore, some patients were excluded after randomization according to these protocols. The important exclusion criteria were: pregnancy, breast feeding, diseases and administration of drugs influencing hormonal secretion, disorders with a similar clinical presentation, amyloidosis or infiltrative disease potentially affecting the adrenal glands, history of malignant disease and other severe life-threatening diseases (pre-existing coronary and other atherosclerotic vascular disease), and no consent given.

Demographic characteristics collected for patients were: sex, age, socio-demographic characteristics, and data from each patient's medical record and clinical course.

Determination of biochemical parameters

In relation to endocrine functionality, all patients underwent hormone tests related to pheocromocytoma, subclinical Cushing's syndrome, Conn's syndrome or androgen-secreting adrenal tumours. Hormonal studies were performed in all cases, which included plasma cortisol measurement with an overnight dexamethasone suppression test (DST) (screening, low-dose and high-dose), plasma prolactin level, which was determined immunoradiometrically (IRMA Cis-Biointernational, France) measured on a Wallac Wizard 1470 Automatic gamma counter (PerkinElmer Life Sciences, Wallac Oy, 2005, Finland). Plasma adrenocorticotropic hormone (ACTH), serum progesterone, testosterone and β-estradiol, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), plasma free metanephrine and catecholamines, serum aldosterone and plasma renin activity (PRA), which were measured using commercially available enzymatic reagents (Makler d.o.o, Belgrade, Serbia) adapted to an autoanalyser (Olympus AU 400).

Diagnostic imaging

Radiological imaging

In the imaging evaluation, the conventional morphological characteristics was assessed with a 64-row multidetector CT (MDCT) scanner (Aquilion™, Toshiba, Japan). All scans were performed in the axial plane with subsequent multiplanar reconstruction. The standard examination protocol was comprised of a pre-contrast CT to provide density measurements of the lesions. Two post-contrast scans, wash in (WI) at 60 sec and wash out (WO) at 15 min after iodinated contrast agent injection began, can quantify the percentage of absolute or relative contrast enhancement washout and show the vessels in the region of the adrenal glands. We also evaluated Hounsfield Units (HU) before and after contrast media administration in all lesions using a CT examination.

All MRI imaging studies were performed on 1.5-T closed magnet (Magnetom Symphony™, Siemens, Germany). Imaging of adrenal glands included T1- and T2-weighted images, plus chemical shift imaging (CSI) (in-phase and out-of-phase imaging) and/or dynamic-gadolinium sequences. The CSI signal loss on MRI can be quantitatively calculated by measuring the signal intensity index (ASII) using the formula: (SIIP-SIOP)/SIIP x 100% (IP=in phase; OP=opposed phase; S =signal intensity).

Nuclear Medicine Imaging

All patients underwent ^{131}I-metaiodobenzylguanidine (MIBG) whole-body scintigraphy, on dual-head Gamma camera (Syngo-E.cam™, Siemens, Germany), equipped with high energy collimators. After the administration of a thyroid blockade with Lugol solution (1 day before and 3-7 days after) all patients received 370 MBq of ^{131}I-MIBG. 24-48 h after administration, the whole-body planar (anterior and posterior) images were acquired using a dual-head Gamma camera with a window setting of 364 keV. Qualitative uptake intensity was rated according to the following method: 0 if no uptake was present, and 1, 2, and 3 represent tumour uptake less than, equal to, and more than activity in the liver, respectively (30).

Somatostatin receptor scintigraphy (SRS) was also performed in both subject groups, using the commercially available somatostatin analogue ^{99m}Tc-HYNIC-TOC. Whole body scintigraphy was performed 2 h after i.v. administration of 740 MBq in the anterior and posterior projections (256x1024 matrix, 12 cm/min), with a two headed large field of view gamma camera equipped with low energy high resolution collimators at a window setting of 140 keV. The investigation was followed by single-photon emission computed tomography (SPECT) scan of a particular region with the following parameters: 360° noncircular orbit (body contour mode) step and shoot mode, at 30 s per view, 1.23 zoom. The acquired data were collected in a 128x128 image matrix and reconstructed using an iterative ordered subset expectation algorithm. The scoring of the visual uptake (qualitative evaluation) was based on a five-point scale: 0, no uptake; 1, very low/equivocal uptake; 2, clear but faint uptake (less than or equal to liver uptake); 3, moderate uptake (higher than liver uptake); 4, very intense uptake (31).

^{131}I-MIBG and ^{99m}Tc-HYNIC-TOC scintigraphy were performed within at least a 4-week interval.

The images were interpreted qualitatively and independently by 2 experienced nuclear medicine physicians, who were unaware of the other imaging findings and/or other clinical information.

Standard of Reference

For hormone hypersecreting adrenal tumours with typical symptoms and in patients with adrenal incidentaloma larger than 4 cm, a definitive diagnosis was established by pathologic examination (gross pathology, light microscopy and immunohistochemistry evaluation) after surgical resection. For clinically silent adrenal masses with diameters less than 4 cm, a serial clinical follow-up was planned, with clinical, biochemical and CT evaluations, for at least 2 years to ensure a benign diagnosis.

Statistical analysis was performed using SPSS for Windows 20.0 (*SPSS Inc., USA).* Continuous variables are summed as arithmetic means, medians and standard deviations, and categorical variables as proportions (percentages of categories). The estimates of sensitivity, specificity, positive and negative predictive values, and accuracy were obtained with the use of 2x2 contingency tables. The mean±standard deviation were used for continuous vari-

Table 1. Demographic and clinical features of patients with adrenal tumors.

	all adrenal tumors	non-secreting adenomas	hormonally functioning tumors		
			cortisol	aldosterone	catecholamines
no. of adrenal masses	27	13	6	4	4
gender					
male	7	4	0	2	1
female	20	9	6	2	3
age	53,66±11,58	52,38±9,06	54,90±14,59		
diameter mean[a] (cm)	3.45±1.35	4.2±1.6	2.7±1.1		
site					
right	9	5	1	1	2
left	17	8	4	3	2
bilateral	1	0	1	0	0
exams					
CT	20	9	5	4	2
MRI	8	4	1	1	2
CT & MRI	6	3	1	1	1
Scintigraphy[b]	27	13	6	4	4

a. the largest diameters of adrenal lesions was used for analysis
b. ^{131}I-MIBG and ^{99m}Tc-HYNIC-TOC scintigraphy

ables, whereas the number and percentage were used for nominal variables. The alpha level for significance was set to $p<0.05$.

RESULTS

This study included 46 patients. Of those, 27 were diagnosed with an adrenal tumour (AT), 7 were males and 20 were females. The mean age of the patients were 53.66±11.58 years, and the median age was 54.00 years (range 31-71 years). The control group (CG) were a consecutive series of nineteen patients with pituitary tumours without clinical proof of adrenal involvement (2 women and 21 men; average age 47.78±12.78, median age 48.00 with range 24-69 years).

Distribution of clinical parameters in patients with adrenal tumours are reported in Table 1. Androgen-secreting adrenal tumours was been detected in the study population.

All the secreting forms (SF) of AT (13 cases) underwent surgical resection of the adrenal mass (adrenalectomy); 9 patients (64.3%) by laparoscopy and 4 patients (35.7%) by open surgery. Ultrasound or CT-guided fine needle aspiration percutaneous biopsy was performed in 1 case of non-functioning (NF) AT, while in 2 cases with tumours larger than 4 cm adrenalectomy was performed. For clinically silent adrenal masses with diameters less than 4 cm (n=10), a serial clinical follow-up was planned, with clinical, biochemical and CT evaluations, for at least 2 years to ensure a benign diagnosis. One tumour was rated as being most likely benign but could not be classified as adrenocortical or nonadrenocortical because the hormonal assessment did not reveal hormonal activity, and a histopathological analysis was not available because the patient refused both surgery and biopsy.

The biochemical features of AT patients and CG are reported in Table 2. The prevalence of altered parameters of cortisol secretion and DST tests was similar in CG vs NFAT but elevated in cortisol secreting forms. A similar trend was found for the prevalence of catecholamines and aldosterone secreting AT and their concomitant hormones and metabolites.

The location, size and shape of the lesions were determined with MDCT and MRI and it was found that 23 of the 27 subjects had features of adrenal tumours. Twelve of these were NF of AT, with 4.2±1.6 cm for the largest diameter. Measurement by nonenhanced MDCT revealed a mean value of -13.5±7.6 HU. A semiquantitative study of pre and postcontrast media injection on MDCT at 60 s WI and 15 min WO revealed an adenoma-like appearance with less than 20 HU.

Cushing syndrome was detected in 4 patients, Conn's syndrome in 3 and pheochromocitoma in 3 cases, with a non-enhanced MDCT mean value of -4.5±2.1 HU, and between 20 and 30 HU on postcontrast media injection. Conversely, measurements less than 10 HU were observed in one patient with pheochromocitoma.

Based on findings from conventional MRI imaging, qualitative and quantitative analyses of chemical-shift techniques, the diagnosis of adenoma was made in 6 of 8 patients. Two adrenal masses were diagnosed as pheochromocytomas and 4 as NF adrenal lesions.

The chemical shift in MRI with a cut-off of 16.5% did not demonstrated substantial differences between these two groups of AT. The small number of cases diagnosed with MRI in our study did not allow for the determination of a reliable threshold value in the signal intensity ratio between secreting and non-secreting forms of adrenal tumours.

Despite the sharp anatomic detail of MDCT or MRI, the evaluation with adrenal scintigraphy in conjunction

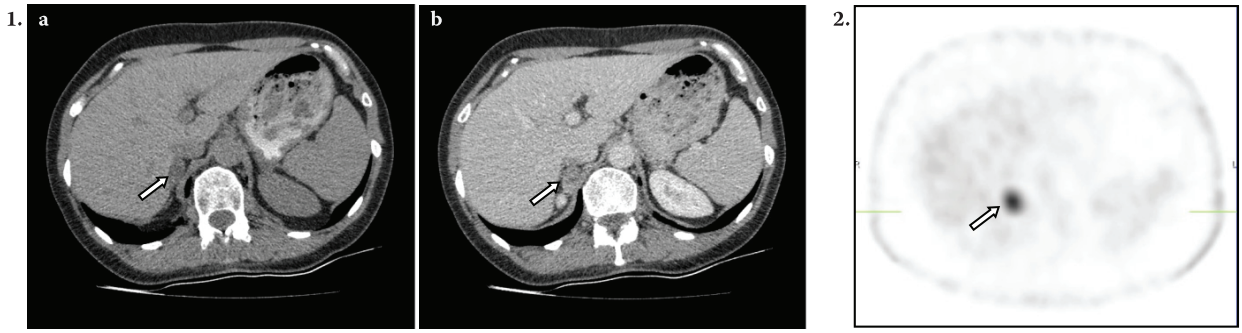
Table 2. Patients results of biochemical parameters

	control group (n=19)	non-secreting adenomas (n=13)	hormonally functioning tumors (n=14)			
			all tumors	catecholamines	aldosterone	cortisol
	median (range)	median (range)	median (range)	median (range)	median (range)	median (range)
cortisol 8h. (154-638nmol/L)	449,50 (305-881)	418,50 (171-790)	534,00 (305-1281)	416,50 (305-591)	468,50 (331-606)	792,00 (534-1281)
cortisol 16-20h. (80-388nmol/L)	275,00 (106-722)	132,50 (92-189)	256,00 (198-556)	152,50 (142-163)	176,60 (50-303)	227,00 (198-556)
cortisol 24h. (50-200nmol/L)	165,52 (133-204)	46,95 (21-85)	194,00 (49-214)	58,40 (21-204)	122,00 (49-195)	161,00 (108-214)
DST "screening" (<150nmol/L)	39,00 (20-620)	39,00 (11-703)	147,00 (28-1455)	95,60 (30-101)	46,40 (28-65)	375,00 (193-1455)
DST "low-dose" (<150nmol/L)	391,50 (38-451)	40,50 (8-495)	175,00 (37-1381)	56,55 (37-75)	-	396,00 (175-1381)
DST "high-dose" (<50% базалног)	172,65 (21-449)	28,00 (9-96)	190,00 (25-1423)	50,10 (24-75)	-	376,00 (190-1423)
ACTH (7,2-63,3pg/mL)	21,32 (5-175)	21,75 (5-39)	5,00 (1,7-63,8)	-	-	5,0 (1,7-63,8)
aldosterone-rest (1,76-23,20ng/dL)	6,02 (5,2-8,5)	5,61 (4,3-8,5)	10,55 (3,8-79,3)	14,1 (4,4-32,1)	41,55 (3,8-79,3)	-
PRA-rest (2,8-39,9μIU/mL)	12,6 (2,1-40,4)	10,00 (2,1-40,4)	1,30 (0,9-6,3)	-	1,30 (0,9-6,3)	-
aldosterone-stress (2,52-39,2ng/dL)	33,15 (9,3-57,0)	15,95 (2,3-29,6)	33,15 (9,3-57,0)	-	33,15 (9,3-57,0)	-
PRA- stress (4,4-46,1μIU/mL)	12,41 (5,5-19,3)	12,41 (5,5-19,3)	1,60 (0,8-2,4)	-	1,60 (0,8-2,4)	-
β-estradiol 8h. (28-156pmol/L)	39,00 (4-249)	39,00 (7-90)	13,00 (4-249)	-	-	13,00 (4-249)
progesterone 8h. (0,7-4,3nmol/L)	0,50 (0,2-2,4)	0,50 (0,2-1,5)	1,45 (0,2-4,0)	-	-	1,45 (0,2-4,0)
testosterone 8h. (1,73-7,74ng/mL)	0,62 (0,1-3,9)	0,90 (0,3-3,9)	0,39 (0,2-45,0)	-	-	0,39 (0,2-45,0)
FSH 8h. (1,27-19,2mIU/L)	8,29 (1-157)	9,74 (3,3-157,0)	7,00 (2,1-54,2	-	-	7,00 (2,1-54,2)
LH 8h. (1,1-8,6mIU/L)	7,05 (1-60)	7,05 (2,3-89,8)	12,00 (1,2-17,8)	-	-	12,00 (1,2-17,8)
epinephrine (0-27μg/dU)	17,14 (3,98-39,0)	10,29 (3,9-39,0)	5,30 (3,8-88,0)	46,65 (5,3-88)	-	8,64 (3,8-13,5)
norepinephrine (0-97μg/dU)	122,16 (8-305)	88,30 (72-305)	72,80 (19-1169)	642,25 (115-1169)	-	45,86 (19-73)
f-metanefrine (<90pg/ml)	31,60 (17,0-80,5)	25,37 (0,5-80,5)	89,85 (18,3-473,8)	164,80 (121,1-473,8)	22,20 (17,2-27,2)	38,30 (18,0-58,6)

Table 3. Accuracy of ^{99m}Tc-HYNIC-TOC scintigraphy in characterization of adrenal tumors

	sensitivity (%) (95% CI[a])	specificity (%) (95% CI[a])	accuracy (%)	predictive value	
				positive (%) (95% CI[a])	negative (%) (95% CI[a])
all adrenal tumors (n=46)	77.78 (57.74-91.38)	89.47 (66.86-98.70)	82.60	91.30 (71.96-98.93)	73.91 (51.59-89.77)
hormonally functioning tumors (n=27)	57.14 (35.14-87.24)	38.46 (13.86-68.42)	51.85	52.94 (27.81-77.02)	50.00 (17.71-81.29)

a. confidence interval



1. A transaxial MDCT image: a) without contrast enhancement shows a homogenous circumscribed well delineated tumor in the right adrenal gland (arrow) with HU of 7. b) At 60 seconds post contrast, the HU measures 43, and at 15 minutes demonstrates a HU of 18.
2. ^{99m}Tc-HYNIC-TOC SPECT transaxial image at the same patient: solitary extremely somatostatin-avid tracer uptake in the right adrenal gland (arrow) confirming the diagnosis of an adenoma.

with hormonal analysis was used not only in defining the function of adrenal lesions but also in the diagnosis and staging of tumours of adrenal origin.

The ^{131}I-MIBG uptake was positive in all 4 cases of pheochromocytomas (grade 3 uptake was found in 3 and grade 4 in one case of these tumours). Only in one case of adrenocortical tumour was grade 1 uptake noticed.

SRS with ^{99m}Tc-HYNIC-TOC was performed in 27 patients with adrenal tumours and 19 in the control group. The qualitative uptake of the tracer was compared with clinical and biochemical assessments of adrenal function, radiological imaging modalities and histopathologic examination after surgery and/or biopsy. A concordant scintigraphic pattern, defined as an increased radiotracer uptake at the side of the detected mass, has been proposed as a typical pattern of an adrenal tumour (Figure 1). In contrast, a discordant pattern with absent or decreased uptake by the adrenal mass may indicate physiologic accumulation. Table 3 summarizes the diagnostic potential of ^{99m}Tc-HYNIC-TOC scintigraphy for localization of the primary adrenal tumour and for assessment of adrenal function. Interestingly, ^{99m}Tc-HYNIC-TOC scintigraphy identified all cases of the 4 adrenal pheochromocytomas, which is similar to ^{131}I-MIBG imaging.

With histology as the gold standard, the correct diagnosis was missed with radiological imaging in 5 (18.5%) of 27 patients. All these cases were scintigraphy–positive (Table 4).

Patients with <4 cm diameter adrenal lesions, with endocrine negative tests, were sent to six months laboratory follow-up, with CT after 12 months then every year for three years, and an annual endocrine re-evaluation.

DISCUSSION

The detection of an adrenal tumour requires a multidisciplinary approach. Initial clinical and biochemical work-up is usually performed by an endocrinologist because primary tumours in the adrenals can be hyperfunctioning and produce excess hormones from the cortex or the medulla and are accompanied by clinical symptoms (2, 7, 12).

The gender distribution among patients with adrenal tumours appears to vary in different series, but females are still commonly affected, which was the case in our study (80.4%). They occur at all ages but are most common in the fourth to sixth decade of life (5, 6, 8, 10, 11,

Table 4. Discordant result of MDCT and MRI imaging and pathology reports of adrenal masses

Patient No.	Age	Sex	Size (cm)[a]	Hystopathology diagnosis	MRI	MDCT	^{99m}Tc-HYNIC-TOC	^{131}I-MIBG
1	54	female	3.6	adrenocortical adenoma	inconclusive/ metastatic	-	positive (grade 3)	negative
2	65	female	3.8	adrenocortical adenoma	carcinoma		positive (grade 4)	negative
3	59	male	4.0	pheochromocitoma	-	adenoma	Positive (grade 3)	positive (grade 3)
4	32	female	2.8	adrenocortical adenoma		negative	positive (grade 2)	negative
5	63	female	2.5	adrenocortical adenoma		hypodense structure	positive (grade 2)	negative

a. the largest diameters of adrenal lesions

13). The recorded mean age in our study of 53.66±11.58 (range 31-72 years) is in accordance with results reported in our region (32, 33). Several large series reports have found the majority of adrenal adenomas are less than 4 cm in largest diameter (5, 8, 10, 11, 13). Our data correspond to these results, with a median diameter of 3.45±1.35 cm (range, 1.0–15.0 cm) in clinically diagnosed tumours.

The results of the present study demonstrate that adrenal cortical tumours were more common than medullary tumours, accounting for 85.2% of cases. Our study partially matches with the observation of others regarding the tumour type (4, 5, 7, 8, 34), with non-functioning accounting for approximately 48.1% of all adrenal tumours followed by cortisol-secreting adenoma noted in approximately 22.2%, and aldosterone-secreting adenoma in 14.8%. Other types of adrenal tumours, including adrenocortical carcinoma, were not detected in our study population. Although 60-80% of adrenal tumours are asymptomatic with a size less than 4 cm, the main recommendation is to consider all adrenal incidentaloma as a hypersecreting tumour, even without clinical manifestation until otherwise proven by hormonal tests (2, 7, 12). Clinical and biochemical features of tumours seen in our study resulted in the over-production of hormones and metabolites, with over half of all patients developing marked symptoms. Subclinical cortisol-producing adenomas were well recognized and reported to be higher in our study.

The management of adrenal tumours poses a therapeutic dilemma. All patients should undergo hormonal screening to assess functionality and specific radiological imaging and/or scintigraphy in order to recognize lesion-type, assess lesion function and to differentiate between benign and malignant tumours (2, 7, 12, 35).

MDCT is often the first modality utilized in detecting adrenal masses. The sensitivity to differentiating malignant from benign adrenal tumours ranges from 79 to 89% in studies and with specificities of 87-96% (5, 13, 17, 18). Adrenal adenomas may be suggested by CT on the basis of a low attenuation coefficient without enhancement to contrast media images and/or early as well as rapid washout on enhanced scans. A lesion with smooth margins, a size less than 4 cm and density <10 HU without enhancement to contrast media indicates lipid-rich adenomas, although 25–30% of adenomas are lipid poor and have CT attenuation values >10 HU. A density of -10 HU is characteristic of a myelolipoma; if the attenuation is 0-15 HU without enhancement a simple cyst is suspected, while malignant tumours have high pressure impeding the contrast-enhancement and delaying the contrast medium wash-out (5-8, 14. 15, 17, 18).

In our study, most (87%) adenomas were characterized on unenhanced CT using a threshold of ≤10 H. Interestingly, in our series 10% of adenomas were diagnosed in routine contrast enhanced studies using an identical threshold.

Pheochromocytomas can have a varied appearance on non-contrast CT ranging from low-density to soft-tissue attenuation. Although the vast majority have an attenuation value greater than 10 HU, rare low-density pheochromocytoma can have attenuation values similar to adenomas (21, 22, 34, 36, 37), which was the case in our study. The mean arterial and venous phase enhancement of pheochromocytomas in the 2 cases presented in our results was significantly higher than that of adenomas.

MRI is indicated for characterization of adrenal masses that show atypical findings on MDCT. Adrenal adenomas usually present on MRI as isointense or with low signal intensity on T1- and T2-weighted images and rapid contrast and washout after gadolinium administration, although they may contain insufficient lipids, resulting in a loss of signal on the out-of-phase scan (6, 8, 13,16). MRI is superior than MDCT in the characterization of carcinoma infiltration. The presence of calcifications, necrosis and haemorrhage is suspicious but not pathognomonic for malignancy, which show low signal intensity on T1-weighted images and high signal intensity on T2-weighted images with strong enhancement and slow washout after contrast media administration (11, 14, 15). MRI effectiveness was previously reported to correspond to a diagnostic accuracy of 93% to differentiate between benign and malignant adrenal masses. However, on T2-weighted images 30% of lesions present overlapping between benign and malign tumour appearance, such as adrenal carcinoma and metastatic lesions (10, 11, 17, 36, 38). All this may reduce the accuracy of this imaging modality.

The reason for misdiagnosing two adenomas in our study can be explained partially by the fact that for 1 patient a chemical-shift MRI was not available, and the dynamic studies showed a marked enhancement after injection of gadolinium. On the second MRI imaging, 1 adrenal mass that was classified as metastases proved to be a benign adenoma at histology.

Pheochromocytomas show specific MRI features such as a clearly increased signal intensity on T2-weighted images and significant enhancement after gadolinium administration. On T1-weighted sequences, pheochromocytomas are typically isointense or hypointense to muscle (21, 22, 34), which was the case in our study. However, the appearance can be quite variable if there is necrosis or haemorrhage present, which would be hyperintense on both T1- and T2-weighted sequences. Pheochromocytoma can even have low signal intensity on T2-weighted sequences in approximately 35% of cases (15, 36-38).

The main purpose of this study was to evaluate the use of scintigraphic modality in the assessment of AT in order to allow the clinician to make a precise diagnosis and customize the treatment accordingly. Due to the potential overlap in CT and MRI appearances of different tumour types, functional imaging can be helpful for characterizing the nature of AT and to differentiate between cortical and medullar adrenal masses.

MIBG imaging has been well established as a localizing tool and a functional marker of catecholamine secreting tissue, with a high sensitivity range (83%-100%) and a high specificity (95–100%) (24, 25, 30). MIBG demonstrated a high diagnostic accuracy in four patients, who had pheochromocytomas confirmed by histopathology and were positive on the MIBG scan. On the basis of our findings, nuclear imaging modalities using MIBG are able to better characterize pheochromocytomas compared with MRI (1 was misdiagnosed). All other types of AT were MIBG negative, except one case of AT with a faint uptake (grade 1) that was characterized as physiologic adrenal uptake. Comparative studies between MIBG and MRI demonstrated that MIBG uptake in patients with pheochromocytoma is able to differentiate between benign and malignant tumour lesions, while MRI is not useful for this purpose (15).

Somatostatin receptor scintigraphy is currently widely utilized in clinical practice and has been extensively investigated for imaging of sympathomedullary and other neuroendocrine tumours (28-31). This uptake is related to the widespread distribution of cells expressing somatostatin receptors (SSTR), especially type 2 SSTR, in the majority of neuroendocrine tumours, including the adrenal gland (26, 27). The results of various studies revealed that somatostatine radiolabelled analogues (^{99m}Tc-HYNIC-TOC) are a second choice technique for sympathomedullary imaging after MIBG scintigraphy, especially when the MIBG is completely or partly false negative. Conversely, due to its high sensitivity ^{99m}Tc-HYNIC-TOC scintigraphy can be considered as the first choice scintigraphic imaging technique in paragangliomas. (24, 25, 30, 31, 39).

Encouraged by the high sensitivity of SRS in localizing pheochromocytoma and paragangliomas we also performed ^{99m}Tc-HYNIC-TOC scintigraphy in a group of patients with adrenal adenomas. The results of the *in vitro* studies demonstrate that somatostatin receptors are expressed in adrenal tumours in a varied manner, which is specific in each case (26-28).

Successful detection was achieved in majority of the AT with a sensitivity of 77.78%, and a specificity of 89.47%. In contrast, ^{99m}Tc-HYNIC-TOC scintigraphy has a lower diagnostic potential in differentiating the form of these tumours (sensitivity of 57.14%, and specificity of 38.46).

All four of our patients diagnosed with pheochromocytomas were positive on ^{99m}Tc-HYNIC-TOC scintigraphy (three cases with grade 3, one case with grade 2).

Scintigraphy using specific tracers such as ^{131}I-MIBG and ^{99m}Tc-HYNIC-TOC may provide *in vivo* tissue characterization of adrenal tumours. Based upon the present results, somatostatin-receptor analogues can be front-line radiotracers in the imaging of adrenocortical and adrenomedullary tumours, respectively, while performing SRS for hormone secreting forms of adrenocortical adenomas is not advised. ^{99m}Tc-HYNIC-TOC positive scintigraphy accompanied with MIBG negative scintigraphy is likely to belong to the adrenocortical adenoma.

CONCLUSION

On the basis of our findings, nuclear imaging modalities using specific tracers are able to characterize AT with a high sensitivity and specificity compared with MRI and MDCT. Functional scintigraphy using SPECT modality complements anatomy-based imaging and facilitates diagnostic localization. In particular, radionuclide techniques are able to identify the existence of AT and to differentiate between adenoma and pheochromocytoma. Furthermore, whole-body imaging allows the detection of extra-adrenal phaeochromocytomas, multifocal disease, metastatic disease and residual/recurrent tumour. Our data show considerable clinical promise for the future and provide the rationale of different diagnostic and therapeutic possibilities of somatostatin analogues in adrenal tumours.

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ACUTE KIDNEY DAMAGE: DEFINITION, CLASSIFICATION AND OPTIMAL TIME OF HEMODIALYSIS

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AKUTNO OŠTEĆENJE BUBREGA: DEFINICIJA, KLASIFIKACIJA I OPTIMALNO VREME ZA HEMODIJALIZU

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ABSTRACT

Acute damage to the kidney is a serious complication in patients in intensive care units. The causes of acute kidney damage in these patients may be prerenal, renal and postrenal. Sepsis is the most common cause of the development of acute kidney damage in intensive care units. For the definition and classification of acute kidney damage in clinical practice, the RIFLE, AKIN and KDIGO classifications are used. There is a complex link between acute kidney damage and other organs. Acute kidney damage is induced by complex pathophysiological mechanisms that cause acute damage and functional disorders of the heart (acute heart failure, acute coronary syndrome and cardiac arrhythmias), brain (whole body cramps, ischaemic stroke and coma), lung (acute damage to the lung and acute respiratory distress syndrome) and liver (hypoxic hepatitis and acute hepatic insufficiency). New biomarkers, colour Doppler ultrasound diagnosis and kidney biopsy have significant roles in the diagnosis of acute kidney damage. Prevention of the development of acute kidney damage in intensive care units includes maintaining an adequate haemodynamic status in patients and avoiding nephrotoxic drugs and agents (radiocontrast agents). The complications of acute kidney damage (hyperkalaemia, metabolic acidosis, hypervolaemia and azotaemia) are treated with medications, intravenous solutions, and therapies for renal function replacement. Absolute indications for acute haemodialysis include resistant hyperkalaemia, severe metabolic acidosis, resistant hypervolaemia and complications of high azotaemia. In the absence of an absolute indication, dialysis is indicated for patients in intensive care units at stage 3 of the AKIN/KDIGO classification and in some patients with stage 2. Intermittent haemodialysis is applied for haemodynamically stable patients with severe hyperkalaemia and hypervolaemia. In patients who are haemodynamically unstable and have liver insufficiency or brain damage, continuous modalities of treatment for renal replacement are indicated.

Keywords: *acute kidney injury, definition, classification, renal replacement therapy, haemodialysis, continuous dialysis*

SAŽETAK

Akutno oštećenje bubrega ozbiljna je komplikacija kod bolesnika u jedinicama intenzivnog lečenja. Uzroci za nastanak akutnog oštećenja bubrega kod ovih bolesnika mogu biti prerenalni, renalni i postrenalni. Najčešći uzrok razvoja akutnog oštećenja bubrega u jedinicama intenzivnog lečenja je sepsa. Za definiciju i klasifikaciju akutnog oštećenja bubrega u kliničkoj praksi koriste se RIFLE, AKIN i KDIGO klasifikacija. Između akutnog oštećenja bubrega i drugih organa postoji složena ukrštena povezanost. Akutno oštećenje bubrega složenim patofiziološkim mehanizmima uzrokuje akutno oštećenje i poremećaj funkcije srca (akutna srčana slabost, akutni koronarni sindrom, srčane aritmije), mozga (grčevi celog tela, ishemijski moždani udar, koma), pluća (akutno oštećenje pluća, akutni respiratorni distres sindrom) i jetre (hipoksični hepatitis, akutna insuficijencija jetre). Značajnu ulogu u dijagnostikovanju akutnog oštećenja bubrega imaju novi biomarkeri, kolor dopler ultrazvučna dijagnostika i biopsija bubrega. Prevencija razvoja akutnog oštećenja bubrega u jedinicama intenzivnog lečenja uključuje adekvatan hemodinamski status bolesnika i isključivanje nefrotoksičnih lekova i agenasa (radiokontrstna sredstva). Komplikacije akutnog oštećenja bubrega (hiperkalemija, metabolička acidoza, hipervolemija, azotemija) leče se medikamentima, infuzionim rastvorima i terapijom za zamenu funkcije bubrega. U apsolutne indikacije za akutnu hemodijalizu spadaju rezistentna hiperkaliemija, teška metabolička acidoza, rezistentna hipervolemija i komplikacije visoke azotemije. U odsustvu apsolutnih indikacija, hemodijaliza je indikovana kod bolesnika u jedinicama intenzivnog lečenja u stadijumu tri AKIN/KDIGO klasifikacije, a kod pojedinih bolesnika i u stadijumu 2. Intermitentna hemodijaliza se primenjuje kod hemodinamski stabilnih bolesnika sa teškom hiperkalemijom i hipervolemijom. Kod bolesnika koji su hemodinamski nestabilni, kod kojih postoji insuficijencija jetre ili oštećenje mozga indikovani su kontinuirani modaliteti terapije za zamenu funkcije bubrega.

Ključne reči: *akutno oštećenje bubrega, definicija, klasifikacija, terapija za zamenu funkcije bubrega, hemodijaliza, kontinuirana dijaliza*

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INTRODUCTION

Acute kidney injury (AKI) is a common, serious complication in critical patients in intensive care units (incidence of acute kidney damage is 25%) (1, 2). There are numerous causes of acute kidney damage in these patients, including sepsis, abdominal surgery, liver failure and severe weakness of the heart. Organ function disorders, triggered by acute renal damage, play a key role in the survival of critical patients requiring renal replacement therapy. The mortality rate of haemodynamically unstable patients in intensive care units, with shock and insufficiency of multiple organ systems (including acute kidney damage requiring haemodialysis), is high at 60–80% (1, 2).

Definition and classification of acute kidney damage

For the diagnosis and assessment of the severity of acute kidney damage, three classifications are used: Risk, Injury, Failure, Loss of kidney function, and End-stage kidney disease (RIFLE) 2004, Acute Kidney Injury Network (AKIN) 2007 and Kidney Disease Improving Global Outcomes (KDIGO) 2012 (2). According to the recommendations of RIFLE and AKIN, acute renal impairment is defined as an increase of serum creatinine concentration ≥ 26.5 μmol/l (≥ 0.3 mg/dl) over 48 h compared to basal creatinine concentration and/or diuresis of less than 0.5 ml/kg/h for at least 6 h (2). Based on the KDIGO classification, acute kidney damage is defined as an increase of creatinine concentration in serum by ≥ 0.3 mg/dL (≥ 26.5 μmol/l) for 48 h or as an increase of serum creatinine concentration ≥ 1.5 times compared to basal creatinine concentration for the previous seven days and/or diuresis less than 0.5 ml/kg/h for at least 6 h (Table 1) (2). Significant constraints of these three classifications are the definition and assessment of the severity of acute renal damage based on the serum creatinine concentration (loss of muscle mass, increased concentration of substances affecting analytical measurement of serum creatinine concentration, impaired renal function and impaired liver function), defining basal serum creatinine concentration (serum creatinine concentration just before the episode of acute kidney damage) and decision-making on the initiation of treatment with renal replacement therapy (RIFLE-F, AKIN-3 and KDIGO-3). The optimal time for starting a therapy to replace the kidney function in clinical practice is still not clearly and precisely defined (in the absence of absolute indications for haemodialysis) (2). In patients with liver cirrhosis, acute kidney damage is defined based on the ICA-AKI criteria (Table 2) (3-5).

Table 1. Classification of acute kidney damage: RIFLE, AKIN, and KDIGO

RIFLE	Creatinine Criterion	Diuresis Criterion
Risk (1)	≥ 26.4 μmol/l or > 150–200% compared to basal value	> 0.5 ml/kg/h for ≥ 6 h
Injury (2)	> 200–299% compared to basal value	> 0.5 ml/kg/h for ≥ 12 h
Failure (3)	> 300% compared to basal value or > 354 μmol/l with↑ > 44 μmol/l or treatment with dialysis support therapy	> 0.3 ml/kg/h for ≥ 24 h or anuria for 12 h
Loss (4)	Persistent acute kidney damage - a complete loss of kidney function over a period of more than 4 weeks	
ESRD (5)	Final stage of kidney disease over a period of time longer than three months	
AKIN	Creatinine Criterion	Diuresis Criterion
AKIN 1	≥ 26.4 μmol/l or > 150–200% compared to basal value	> 0.5 ml/kg/h for ≥ 6 h
AKIN 2	> 200–299% compared to basal value	> 0.5 ml/kg/h for ≥ 12 h
AKIN 3	> 300% compared to basal value or > 354 μmol/l with↑> 44 μmol/l or treatment with dialysis support therapy	> 0.3 ml/kg/h for ≥ 24 h or anuria for 12 h
KDIGO	Creatinine Criterion	Diuresis Criterion
KDIGO 1	1.5–1.9 times compared to basal value	< 0.5 ml/kg/h for 6–12 h
KDIGO 2	2.0–2.9 times compared to basal value	< 0.5 ml/kg/h for ≥ 12 h
KDIGO 3	3.0 times compared to basal value or serum creatinine concentrations at a value greater than 4.0 mg/dl or starting RRT	< 0.3 ml/kg/h for ≥ 24 h or anuria for ≥ 12 h

RIFLE - Risk, Injury, Failure, Loss of kidney function, and End-stage kidney disease, AKIN - Acute Kidney Injury Network, KDIGO - Kidney Disease Improving Global Outcomes, ESRD - End-stage Renal Disease

Table 2. New diagnostic criteria for acute kidney damage in patients with cirrhosis of the liver (achieved by ICA consensus)

Basal concentration of serum creatinine	Stable concentration of serum creatinine ≤ 3 months. If the previous concentration of serum creatinine is not available, take as a baseline the concentration of serum creatinine at admission
Definition of AKI	↑concentration of creatinine in the serum ≥ 26.5 μmol/l (≥ 0.3 mg/dl) ≤ 48 h or an increase of 50% in the ratio compared to the basal value
Stages of AKI	Stage 1: ↑ SCr ≥ 26.5 μmol/l (≥ 0.3 mg/dl) or↑ SCr ≥ 1.5–2.0 times compared to the basal value
	Stage 2: ↑ SCr > 2.0–3.0 times compared to the basal value
	Stage 2: ↑ SCr > 3.0 times compared to basal value or SCr ≥ 352 μmol/l (4.0 mg/dl) with acute↑ ≥ 26.5 μmol/l (≥ 0.3 mg/dl) or initiation of kidney replacement therapy
Progression of AKI	Progression of AKI to a higher stage or need for treatment with kidney replacement methods
Regression of AKI	Regression of AKI in lower stage
Response to treatment	Absent: no AKI regression
	Partial: regression of AKI: ↓ SCr to a value of ≥ 26.5 μmol/l (≥ 0.3 mg/dl) above the baseline
	Complete: AKI regression:↓ SCr to a value of < 26.5 μmol/l (< 0.3 mg/dl) above the baseline

ICA - International Club of Ascites, AKI - Acute Kidney Injury, SCr - serum creatinine concentration
Modified by reference [2].

Acute kidney damage in patients with liver cirrhosis is defined as an increase in serum creatinine concentration ≥ 26.5 μmol/l (≥ 0.3 mg/dl) compared to basal creatinine concentration over a period of ≤ 48 h or as an increase in serum creatinine concentration ≥ 50% relative to the basal value (3-5). The baseline serum creatinine concentration is precisely defined as the serum creatinine concentration within seven days prior to hospitalisation or serum creatinine concentration over a three month period prior to hospitalisation (if there are more measurements of serum creatinine concentration, the one nearest to hospitalisation is taken) or the serum creatinine concentration at the time of admission to the hospital (if there is no serum creatinine concentration noted within a period of three months prior to hospitalisation) (3-5). Type 1 hepatorenal syndrome, as a specific form of acute kidney damage, is defined on the basis of the hepatorenal syndrome-acute kidney injury (HRS-AKI) criteria (Table 3) (3-5). In patients with liver cirrhosis, serum creatinine levels overestimate the volume of glomerular filtration due to loss of muscle mass (reduced creation of creatinine from creatine in muscles), increased creatinine clearance in the proximal tubules of the kidneys, liver damage and increased serum bilirubin concentration (impact on analytical measurement of serum creatinine concentration). As a result, all of these factors have complicated and delayed the diagnosis of acute kidney damage. Pregnancy-related acute kidney injury (PR-AKI) is defined as a serum creatinine concentration > 71 μmol/l in pregnant women in the absence of clinical data for chronic kidney disease (normal serum creatinine concentration in the third trimester of pregnancy is 62–71 μmol/l) (6, 7). The definition and classification of acute renal disease associated with pregnancy is not entirely clear and precisely defined due to open issues, such as low basal serum creatinine concentration due to increased glomerular filtration in pregnancy by approximately 50% (adaptive changes in pregnancy) and defining an optimal method for measuring glomerular filtration volume (6, 7).

Table 3. Diagnostic criteria for type 1 hepatorenal syndrome (HRS-AKI) in patients with liver cirrhosis

HRS-AKI criteria
• Diagnosis of liver and ascites cirrhosis
• Diagnosis of AKI according to ICA-AKI criteria
• Absence of a response after two consecutive days of discontinuation of the diuretic or plasma volume expansion with albumin 1.0 g/kg/day
• Absence of shock
• Absence of nephrotoxic medicines/agents (NSAIDs, aminoglycosides, and iodine contrast agents)
• Absence of macroscopic signs of kidney structure damage:
• Absence of proteinuria (> 500 mg/24 h)
• Absence of microhaematuria (> 50 RBC/HPF)
• Normal findings on ultrasound examination of the kidney

HRS-AKI - Hepatorenal Syndrome-Acute Kidney Injury, ICA - International Club of Ascites, ICA-AKI - International Club of Ascites-Acute Kidney Injury, NSAIDs - Non-Steroidal Anti-Inflammatory Drugs, RBC - Red Blood Cells, HPF - High Power Field
Modified by reference [2].

The influence of acute kidney damage on the function of other organs

Studies show that there is a complex correlation between acute kidney damage and other organs/systems of organs, including the heart, brain, lung, and liver. Knowledge of the pathophysiological mechanisms of the cross-linked association between acute kidney damage and other organs represents a new potential strategy for the treatment of critical patients in intensive care units (8).

Acute damage to the kidneys and heart

Acute kidney damage can cause acute heart damage and dysfunction (acute reno-cardiac syndrome or cardiorenal syndrome, type 3) (9, 10). Acute heart damage and function disorders include acute heart failure, acute coronary syndrome, cardiogenic shock and cardiac arrhythmias (9, 10). The mechanisms of the effects of acute kidney damage on acute heart damage and function disorders are divided into two groups: direct and indirect. Direct mechanisms are the result of microinflammatory effects on cardiomyocytes (9, 10). After acute kidney damage caused by ischaemia-reperfusion of the kidneys, there is a reinforced response of systemic and local immune systems results in the accumulation of neutrophils in the interstitium of the myocardium (neutrophils amplify the release of free oxygen radicals, proteases and myeloproteases that directly damage the myocardium), increased expression of pro-inflammatory mediators (interleukin (IL)-6 and tumour necrosis factor (TNF)-α) and cardiomyocyte apoptosis (9, 10). Among indirect mechanisms of the effects of acute kidney damage on the development of acute heart damage and heart function disorders there are: oliguria and increase in volume of extracellular fluid (volume overload, hypervolaemia, hypertension, ascites, and intra-abdominal hypertension), electrolyte balance disorders (hyperkalaemia, hyperphosphataemia, and hypocalcaemia) and development of disorders of cardiac rhythm, acid-base balance disorders (metabolic acidosis) and negative inotropic effects (reduced contractility of the left ventricle) (9, 10). For the detection of acute heart damage and function disorders, the measurement of the concentration of serum natriuretic peptides (BNP/NT-pro-BNP) and troponins (cTnI/cTnT) and ultrasound of the heart (echocardiography) are used (9, 10). Excess liquid in patients (fluid overload (FO)) is defined as the difference between the total uptake and loss of liquid divided by the body mass (excess fluid exists if the FO ≥ 10%) (11-13). For evaluation of the early detection of excess fluid, ultrasound of the lungs (estimated fluid in the extravascular lung section–estimation of lung congestion) and the inferior vena cava (indirect assessment of central venous pressure) are used (11-13). With ultrasound examination of the lungs, the vertical hyperechogenic lines are visualised (lung comets), and lung congestion is absent if the sum of lung comets is < 5. A mild degree of congestion of the lungs is present if the sum of the ultrasonic lung comets = 5–15, moderate degree if the sum = 15–30, and with a severe level of lung congestion, the sum of ultrasound comet lungs is greater than 30 (11-13). Through ultrasound examination of the inferior vena cava, the diameter of the inferior vena cava (VCId), the index of the inferior vena cava (VCIi, the ratio of the diameter of the inferior vena cava and the patient's body surface area - VCId/TP mm/m^2) and the index of the collapsibility of the inferior vena cava (VCIci, (VCIci = (VCIexp - VCIinsp)/VCIexp) x 100%) are measured. Euvolaemia exists if VCIci = 50–75%, and VCIci < 50% indicates hypervolaemia (12, 13). Removal of excess liquid is achieved using Henle's loop diuretics and extracorporeal ultrafiltration techniques. In patients with known resistance to the effect of loop of Henle diuretics, their continuous intravenous infusion is applied. If there is no response (increase in diuresis), extracorporeal ultrafiltration is administered: slow continuous ultrafiltration (SCUF) and isolated/sequential ultrafiltration (SUF) (9, 10). Slow continuous ultrafiltration is applied continuously (8 h) with a small blood flow (Qb = 50–100 ml/min) and rate of ultrafiltration (Quf = 100–300 ml/h). With isolated/sequential ultrafiltration, the blood flow is Qb = 200–300 ml/min, and the rate of ultrafiltration is Quf = 500–1000 ml/h (since there is a risk of development of haemodynamic complications) (9, 10).

Acute damage to the kidneys and brain

Acute kidney damage can cause acute brain damage (14-16). Acute brain damage is caused by direct and indirect mechanisms. Direct mechanisms include the accumulation of uremic toxins and microinflammatory conditions, and the most significant indirect mechanisms are disorders of fluid and electrolyte balance (hypervolaemia, hyponatraemia, and hypernatraemia), acid-base balance disorders (metabolic acidosis), lack of thiamine, and the effect of kidney function replacement therapy—dialysis-associated brain injury (DABI) (a rapid decrease in the urea concentration in the serum, intradialysis hypotension) (14-16). Quickly reducing the concentration of urea in the serum may cause dialysis disequilibrium syndrome (DDS), which is caused by brain oedema. to prevent the development of disequilibrium syndrome, the duration of first round of haemodialysis should be limited to 2.0–2.5 h, the blood flow should be limited to 200 ml/min, and the sodium concentration in the solution for haemodialysis should be modelled after the sodium concentration in the serum of the patient (the concentration of sodium in the solution for haemodialysis should not be greater than 10 mmol/l than the sodium concentration in the serum of the patient) using a low-flux membrane of low efficiency (coefficient of mass transfer (CoA) < 300–600), and the target urea reduction ratio (URR) should be 0.40 (short-term low-efficiency haemodialysis) (14-16). In patients with a high risk of developing disequilibrium syndrome (traumatic brain injury and intracerebral haemorrhage), the intravenous administration of mannitol during haemodialysis treatment (1.0 g/kg → increases the serum osmolarity by 8.5–10 mOsm/kg H_2O) and

a continuous dialysis modality should be considered (14-16). Intradialysis hypotension causes ischaemia-reperfusion of the subcortical white matter of the brain, leukoaraiosis (neuronal loss, demyelination, and gliosis), and development of stunning of the brain, which all result in cognitive dysfunction (significant memory loss) (17, 18).

Acute damage to the kidneys and lungs

Respiratory complications are common in patients with acute kidney damage (cardiogenic pulmonary oedema, non-cardiac pulmonary oedema/acute respiratory distress syndrome, and respiratory failure, which requires mechanical ventilation) (19, 20). Cardiogenic lung oedema in patients with acute renal impairment is due to hypervolaemia and metabolic acidosis and is successfully treated with a loop of Henle diuretics and extracorporeal ultrafiltration. Non-cardiac pulmonary oedema/acute respiratory distress syndrome in patients with acute kidney damage is due to the increased systemic and local response of the immune system, neutrophil infiltration of lung parenchyma and apoptosis of endothelial and epithelial cells of the lung (19, 20). A syndrome of increased permeability of capillaries for proteins, capillary leak syndrome (CLS) has an important role in the development of non-arterial oedema of the lungs in patients with acute kidney damage (21). The diagnosis of non-cardiac pulmonary oedema (acute respiratory distress syndrome) is based on the following criteria: rapid onset, bilateral infiltrates on chest radiography, normal function of the heart (the filling pressure of the capillaries of the lung (pulmonary capillary wedge pressure (PCWP) is less than 18 mmHg) and the ratio of the partial pressure of oxygen in the arterial blood and the oxygen fraction in the inhaled air (PaO2/FiO2) is < 200 (Acute Respiratory Distress Syndrome, ARDS), respectively, as well as < 300 in acute lung injury (ALI) (22, 23). Patients with acute kidney and lung damage require mechanical ventilation. Positive pressure ventilation (PPV), with its haemodynamic and non-haemodynamic mechanisms, can exacerbate acute kidney damage (kidney hypoperfusion) (22, 23). In these patients, the lung protective ventilation strategy (LPVS) with a small breathing/respiratory volume (a volume of 6 ml/kg of ideal body weight) is recommended, where the plateau pressure at the end of the inhalation should be less than 30 cm H_2O using the lowest positive pressure at the end of exhalation (PEEP = 5–10 cm H_2O) to achieve satisfactory oxygenation (PaO_2 = 55–80 mmHg or $SaHbO_2$ = 88–90%) (22, 23). In severe forms of acute respiratory distress syndrome (severe hypoxaemia: PaO_2/FiO_2 > 80 mmHg and uncompensated hypercapnia: pH < 7.20), extracorporeal removal of carbon dioxide ($ECCO_2R$) is the indicated therapy (24, 25).

Acute damage to the kidneys and liver

In the prerenal type of acute kidney damage, a reduced effective arterial volume can cause hypoxic hepatitis (HH) (26, 27). Hypoxic hepatitis is characterised by a sudden and substantial transient increase of aminotransferases in the serum, as a result of reduced flow and utilisation of oxygen by the hepatocytes of the liver (26, 27). The most important predisposing clinical conditions for the development of hypoxic hepatitis are heart failure (liver congestion), septic shock, pre-renal acute kidney damage and respiratory insufficiency. The incidence of hypoxic hepatitis in intensive care units is 2.5–10%, and the pathophysiology is multifactorial and includes blood stasis in the hepatic veins, reduced blood flow through the liver, whole body hypoxia, decreased supply of oxygen to the hepatocytes, reduced utilisation of oxygen by hepatocytes, ischaemia-liver reperfusion, increased central venous pressure and increased intra-abdominal pressure (26, 27). The main clinical manifestations of hypoxic hepatitis are pain under the right chest arch, hepatomegaly, and increased aminotransferase concentrations in the serum, and the most significant complications are spontaneous hypoglycaemia, respiratory failure due to hepatopulmonary syndrome and hepatic insufficiency (increased serum ammonia concentration) (26, 27). HH is diagnosed based on the following criteria: a significant increase of aminotransferase concentrations in the serum (≥ 20 times compared to the upper normal limit), the presence of one of the predisposing clinical conditions (acute cardiac, circulatory or respiratory insufficiency), the absence of other possible causes of necrosis of liver cells (toxic effect of medicines, viral hepatitis, acute Budd-Chiari syndrome, HELLP syndrome, acute fatty liver in pregnancy, or autoimmune hepatitis) (26, 27). A liver biopsy is not required for the diagnosis of HH, and the main histopathological feature is centrilobular cell liver necrosis (CLNC) (26, 27). Rapid diagnosis and timely treatment of the underlying disease are of paramount importance. Optimisation of circulation, maintaining adequate mean arterial blood pressure, and preservation of the microcirculation and oxygenation of tissue is achieved by application of inotropes, vasodilators and diuretics. In patients with acute liver failure, consideration should be given to the benefit of the modality of albumin dialysis with the Molecular Adsorbent Recirculating System (MARS) (26, 27).

Diagnosis of acute kidney damage

The diagnosis of acute kidney damage is based on anamnesis, physical examination, review of the urine sediment (erythrocytes altered in shape and erythrocyte cylinders indicate glomerular disease, leukocytes indicate acute bacterial inflammation of the kidneys, and brown granular cylinders indicate acute tubular necrosis of the kidneys). Fractional sodium excretion (FE_{Na+}) < 1.0% and FE_{urea} < 35% with normal urine sediment suggest a prerenal (functional) type of acute kidney damage (28). Some patients require tests for the evaluation of the immune system: antibodies to the antigens of the cytoplasm of neutrophils (renal vasculitis), antibodies directed against the basement membrane of the glomeruli (fast progressing glomerulonephritis), and antinuclear antibod-

ies (systemic lupus erythaematosus) (28). For diagnosis of aHUS/TTP, the number of platelets, concentration of haptoglobin and lactate dehydrogenase in the serum, Coombs test, activity of metalloproteinase enzyme ADAMTS13 and titre of antibodies for factor H of the complement system should be determined (29-30). In the last decade, for the diagnosis of acute kidney damage, the following new biomarkers have been used: cystatin C (marker of glomerular filtration), microalbuminuria (marker of integrity of the glomerulus), neutrophil gelatinase-associated lipocalin (NGAL) (marker of tubular damage), kidney injury molecule-1 (KIM-1) (marker of tubular damage), Liver fatty acid-binding protein (L-FABP) (marker of tubular damage), interleukin 18 (a kidney inflammatory marker), and insulin-like growth factor-binding protein-7 (IGFBP-7) (marker of the stress of tubules) (31-33). The concentration of new biomarkers in the urine increases 24–48 h before the increase in serum creatinine concentration. In critical patients in intensive care units, a concentration of NGAL in urine greater than 150 ng/ml may indicate the development of acute kidney damage in the phase before the increase of the creatinine concentration in the serum (non-creatinine increase-acute kidney injury (NCI-AKI)) (32). For the diagnosis of acute kidney damage and the assessment of renal perfusion, colour Doppler ultrasonography of the kidney is used (34). Colour Doppler ultrasonography and measurement of the resistance index from the blood flow curve through the segmental and interlobular arteries allows the evaluation of kidney perfusion and the distinction of the prerenal type of acute kidney damage from acute tubular necrosis. In patients with the prerenal type of acute kidney damage, the resistance index (RI) is < 0.75, whereas a RI ≥ 0.75 indicates a transition from the prerenal to renal type of acute kidney damage (extending of renal hypoperfusion results in the development of acute tubular necrosis) (34). Patients with acute damage to the kidney who have clinical suspicion of acute glomerulonephritis or renal vasculitis require a kidney biopsy (35).

Treatment of acute kidney damage

Prevention of acute kidney damage in intensive care units includes adequate haemodynamic status of the patient (central vein pressure (CVP) = 8–12 mmHg, diuresis ≥ 0.5 ml/kg/h, mean arterial blood pressure (SAP) ≥ 65 mmHg) and exclusion of nephrotoxic drugs and agents (radiocontrast agents) (36). Complications of acute kidney damage (hyperkalaemia, metabolic acidosis, hypervolaemia, and azotaemia) are treated with medications (Resonium A, 10% calcium chloride/calcium gluconate, and Henle's loop diuretics), infusion solutions (8.4% $NaHCO_3$ and 10% or 50% glucose + quick acting insulin) and kidney replacement therapy (36).

Optimal starting time for kidney replacement therapy

Patients with acute renal impairment in intensive care units require enhanced cooperation between doctors and medical technicians of the following specialties: anaesthesiologist intensivists, nephrologists and medical technicians of the general and nephrological intensive care units (37). For treatment of acute kidney damage in critical patients in intensive care units, the following modalities of kidney replacement therapy (renal replacement therapy (RRT)) are used: acute peritoneal dialysis, acute intermittent haemodialysis, and continuous dialysis modalities (continuous venous vein haemodiafiltration (CVVHDF)) (38-48). Intermittent haemodialysis provides rapid clearance of the substance/electrolyte and a high degree of ultrafiltration. Additionally, intermittent haemodialysis is a first-line modality of therapy for kidney replacement function in haemodynamically stable patients for the treatment of life-threatening hyperkalaemia and hypervolaemia (malignant chamber disorder rhythms of the heart and acute oedema of the lungs in acute renal damage) and poisoning caused by overdosage of medicines (41-48). Continued dialysis modalities are indicated in haemodynamically unstable patients with acute kidney damage caused by cardiogenic or septic shock, as well as in patients with acute kidney damage associated with brain or liver damage (increased intracranial pressure) (41-48). The time to initiate kidney replacement therapy is completely clear when there are complications associated with acute kidney damage that are life-threatening for patients, such as resistant hyperkalaemia, severe metabolic acidosis, oedema of the lungs, and complications of high azotaemia (uremic encephalopathy and uremic pericarditis) (49-52). However, in the absence of absolute criteria, the optimal time to initiate dialysis is not clearly defined (there is no consensus) (49-52). Patients with acute renal impairment of stage 3 of the AKIN/KDIGO classification require treatment with kidney replacement methods. For stage 2 of the AKIN/KDIGO classification, randomised clinical studies are required to precisely define the criteria for initiating treatment with renal replacement methods (49-52). The arguments for early initiation of RRT are better volaemia control (avoiding the accumulation of water, especially in patients where there is resistance to the use of diuretics), better control of the electrolyte and acid-base status, the clearance of toxins of small- and medium-molecular weight (modulation of the immune system and clearance of mediators of inflammation), and avoiding severe complications associated with acute kidney damage (heart rhythm disorders due to hyperkalaemia) (49-52).The arguments for the late initiation of RRT are exposure of patients to complications associated with the placement of central venous catheters, exposure of patients to complications associated with *RRT* (intradialysis hypotension (an iatrogenic episode of haemodynamic instability in patients may aggravate acute kidney damage and slow down/delay kidney function recovery), cardiac rhythm disorders, and antibiotic clearance), complications associated with anticoagulant therapy (haemorrhage due to systemic anticoagulation caused by the use of unfractionated heparin), risk of increased clearance of medications (suboptimal

therapeutic concentrations in serum), and high costs of treatment (especially in patients with slow kidney function recovery) (49-52). Based on the results of the clinical trials done so far, three sets of indications for the initiation of treatment with kidney replacement methods have been identified. The first group consists of the traditional indication (hyperkalaemia ($K^+ \geq 6.5$ mmol/l), serum urea concentration ≥ 84 mg/dl, pH of arterial blood < 7.15, serum bicarbonate concentration < 10 mmol/l, acute pulmonary oedema, acute uremic encephalopathy or acute uremic pericarditis) (49-52). The second group of indications constitute a severe form of acute kidney damage (stage 3 AKIN/KDIGO) in the absence of traditional indications, and the third group indicates acute kidney damage (stage 2 AKIN/KDIGO) in extreme situations (severe sepsis and rapid deterioration of acute kidney damage) (49-52). Two randomised clinical studies, Early vs. Late Initiation of Renal Replacement Therapy in Critically Ill Patients with Acute Kidney Injury (ELAIN) and Acute Kidney Initiation in Kidney Injury (AKIKI) showed a different impact of the early onset of dialysis on the survival rate of patients with acute kidney damage in intensive care units (49-52). The ELAIN Study demonstrated improved survival in patients with the early initiation of dialysis (stage 2 AKIN/KDIGO), while the results of the AKIKO Study have not confirmed this (there was not shown the improved survival of patients who started early treatment with dialysis supportive therapy was not shown, while the improved survival of in patients who started late dialysis supportive therapy (stage 3 AKIN/KDIGO) was observed (49-52). Randomised clinical studies of the Veterans Affairs/National Institutes of Health Acute Renal Failure Trial Network (VA/NIH ATN) and the Randomised Evaluation of Normal versus Augmented Level (RENAL) have shown that patients treated with continuous dialysis modalities have no statistically significantly higher survival rates in comparison to patients with acute kidney damage who are treated with intermittent haemodialysis. With increasing dose of renal replacement therapy (RRT), the survival rates of patients with acute kidney damage are not significantly increased (49-52). According to the recommendations of KDIGO, the dose of individual treatment of intermittent haemodialysis, expressed through the kinetic model of urea, should be $Kt/V \geq 1.20$-1.4, and the dose of the continuous dialysis modality, expressed over the effluent rate, should be 20–25 ml/kg/h. In patients with severe sepsis and acute kidney damage, the dose of continuous modality of CVVHDF, expressed over the effluent rate, should be 35 ml/kg/h (49-52).

CONCLUSION

Acute kidney damage is an independent risk factor for an adverse outcome for patients in intensive care units. Due to the complexity and severity of acute kidney damage syndrome (multiple organ systems insufficiency), patients in intensive care units require a team approach, technical knowledge, well-trained staff, enhanced collaboration between anaesthesiologist intensivists and nephrologists as well as precisely defined treatment protocols that should include therapeutic support for more organ systems. Early detection of patients who have a high risk of developing acute kidney damage, timely application of appropriate prevention and treatment, and adequate monitoring of patients can significantly prevent the development of acute kidney damage and reduce the mortality rate of these patients.

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POSSIBILITY OF OPERATIVE TREATMENT OF UTERINE SARCOMA: CASE REPORT

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MOGUĆNOST OPERATIVNOG LEČENJA SARKOMA UTERUSA: PRIKAZ SLUČAJA

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ABSTRACT

Uterine sarcomas make up only 5% of all malignancies in gynecology. Their classification is complicated due to low incidence and large histological differences. Uterine sarcoma is usually diagnosed in postmenopausal women, and this is most often done accidentally at the postoperative stage. The existence of uterine sarcoma should be suspected in cases of rapid uterine growth in postmenopausal state. Postmenopausal abnormal bleeding is the most common reason for a medical examination.

In this paper, a 48-year-old patient is presented in whose case during a regular gynecological examination; the existence of tumour change in the uterine part of uterus has been noticed. The patient did not have gynecological problems until then. The patient was then subjected to a diagnostic exploratory curettage. The pathohistological finding was negative. Given that the onset change is present after the control check, it is decided to proceed with an operative procedure. A pathohistological finding (uterus and adnexa) indicates that it is a uterine sarcoma. After that, the patient was re-treated with two more operations and then had chemotherapy and radiation therapy. After completing the whole treatment, for the period of six years, the patient now feels well and performs her usual work tasks.

Keywords: *uterine sarcoma, operative treatment, chemotherapy*

SAŽETAK

Sarkomi uterusa obuhvataju samo 5% od svih malignitета u ginekologiji. Njihova klasifikacija je komplikovana zbog male učestalosti i velikih histoloških različitosti. Sarkoma uterusa se obično dijagnostikuje kod postmenopauzalnih žena i to najčešće slučajno postoperativno. Na postojanje sarcoma uterusa treba posumljati u slučajevima rapidnog rasta uterusa u postmenopauzi . Postmenopauzalno abnormalno krvarenje je najčešći razlog za medicinski pregled.

U ovom radu prikazan je slučaj bolesnice, starosti 48 godina kod koje se na redovnom ginekološkom pregledu uoči postojanje tumorske promjene u istmičnom dijelu uterusa. Pacijentkinja nije imala ginekološke tegobe do tada. Pacijentkinja je potom bila podvrgnuta dijagnostičkoj eksploratívnoj kiretaži . Patohistološki nalaz je bio uredan. S obzirom da se uočna promjena I na kontrolnom pregledu održava odluči se uraditi operativni zahvat. Patohistološki nalaz (uterus I adnexa) pokazuje da se radi o sarkomu uterusa. Nakon toga pacijentkinja u dva navrata bila podvrgnuta ponovnim operativnim zahvatima a potom hemio I zračnoj terapiji. Nakon sporovedenog kompletnog liječenja pacijentkinja se sada, sest godina , nakon okončanog liječenja osjeća dobro I obavlja uobičajne radne zadatke.

Ključne riječi: *sarkom uterusa, operativno liječenje, hemioterapija*

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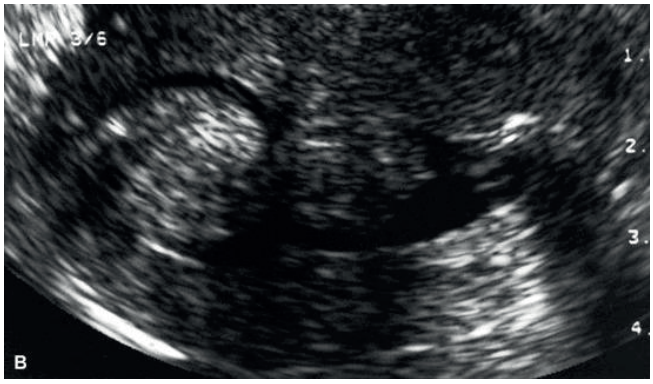
INTRODUCTION

Adenosarcomas of the uterus are generally neoplasms of low grade, and are capable of recidivism after polypectomy or hysterectomy and are only very rarely metastatic. The two most important negative prognostic factors, which are occasionally present, are deep myometrical invasion with a predominant sarcomatous component and high morphological grade, which is followed by the loss of hormone receptors and CD10. Adenosarcoma can be mistaken for various lesions, and the main differential diagnosis is adenofibroma, which by definition has a morphologically benign component (3). The main symptom of these lesions is abnormal vaginal bleeding, pain in the lower abdomen, a palpable tumour mass in the lower abdomen, unusual urinary symptoms, increased and prolonged vaginal discharge, and sometimes the presence of polypoid tissue that can be seen in a dilated cervical canal (4).

These tumours have a tendency to appear in postmenopausal patients with an average age of 66 years. The average time interval from the first symptoms to the final diagnosis is approximately 13 months. The risk factors are similar to those of uterine adenosarcoma and include obesity, oestrogen therapy, radiation exposure, nulliparity, and potentially tamoxifen exposure (5). The prognosis is much better in the early phase and in younger patients, although these are often incorrectly treated due to an incorrect diagnosis (6).

CASE REPORT

Patient S.R. age 48, attends a regular gynecological examination in 2008 (until then regular gynecological examinations were negative). An ultrasound and gynecological examination in the area of the isthmus (lower part of the uterus) detect a tumour change (myoma). Doppler index in the region of tumour changes shows reduced resistance to flow through blood vessels (Ri <0.42). On 1/8/2008 in the Gynecology Department of Hospital in Foca, exploratory curettage was performed. Pathohistological diagnosis: endometrium pieces, smooth muscle tissue pieces without atypical elements. On the control examination, in 3 months, a further increase in the described tumour changes was observed. During gynecological examination in the area of tumour changes, softening of the uterine wall in relation to the remainder of the uterus was noticed. On 25/11/2008, an operation was performed (Hysterectomia totalis abdominal cum adnexectomia bill) PHD (1674/08): Leiomyosarcoma uteri. Control checks 3, 6 and 9 months after surgery indicate a negative post-operative report. Control examination 15/12/2009 (EHO and NMR): Urinary bladder without pathological contents. Along the left wall of the bladder, a tumour with dimensions of 48x46 mm, in structure similar to myometrium with signs of central tumour necrosis with very rich vascularization. By decision of the Consilium for skin and soft tissue at the Institute of Oncology



Picture 1. Tumor changes in the lower part (isthmus) of the uterus

and Radiology of Serbia (17.12.2009), on April 4, 2010, an operation was performed at the Clinic for Gynecology and Obstetrics of the Clinical Centre of Serbia (KGA KCS) Extirpatio tumoris in toto. Omentectomia partialis. Pathohistological diagnosis 222/10: Leiomyosarcoma (high degree of malignancy).

Examinations 3 and 6 months after surgery indicate a negative finding (PET / CT and MRI abdomen and pelvis) Control examination on 25.11.2010 (PET / CT Institute of Oncology of Vojvodina: Two focuses of intense gathering FDG localised at right side in the obturator foramen (above the urinary bladder) and to the left next to the anterior abdominal wall in the projection of the external iliac lymph nodes, differential diagnosis. They correspond to metabolically active secondary deposits.

On 27.12.2016, the patient was operated at the East Sarajevo Clinical Center, Foca hospital (Extirpatio tumoris in toto. Adhesiolysiss) Histopathology diagnosis: 2021-2022 / 10: metastaticum leiomyosarcoma, grade 2, total score 4 metastaticum et leiomyosarcoma, grade 2, the total score 4.

Council For the pelvic surgery of the Clinical Center of Serbia and the Oncology Council of the Clinical Center Eastern Sarajevo made a decision on 09.02.2011 that the patient is to be administered by systemic chemotherapy protocol MAI 3 cycles, followed by radiation treatment and by an additional 3 cycles of chemotherapy after MAI protocol (ADM-CDDP).

After therapy completion, according to the decision by the oncology console, the patient is to attend regular examinations. A local finding in a small pectoris was negative. NMR and PET/CT examination came negative. MRI of the abdomen and pelvis on 07/10/2015 indicate: "...In addition to the findings of hemangioma of the liver and kidney cysts other findings in the abdomen and pelvic are negative. Finding PET / CT 10/10/2016..." On the obtained PET sections, zone of pathological accumulation of FDG, which would indicate the presence of a malignant tumour is not visible."

DISCUSSION

The classification used for the staging of uterine sarcoma is the revised International Federation of Gy-

necology and Obstetrics (FIGO) 2009 classification for uterine sarcoma. Stage I includes disease limited to the uterus. Stage II includes disease extending beyond the uterus, within the pelvis. Stage III is defined by an abdominal extension of the disease, and stage IV involves direct invasion of the bladder or the rectum, or the presence of distant metastasis (7). Cervical cancer cases in young girls are described in the form of cluster formations that can fill the entire vagina. This is the so-called Botrioid Sarcoma (sarcoma botryoides) (10). Early and complete resection is the best-evidenced treatment for uterine leiomyosarcoma. Oophorectomy and lymphadenectomy may be safely omitted for clinically uterus-confined leiomyosarcoma. Chemotherapy increases survival of women with metastatic leiomyosarcoma (8). Patients suffering from breast cancer, estrogen-dependent, use Tamoxifen in therapy. Tamoxifen is an antiestrogenic drug that is used to prevent or slow down the recurrence of breast cancer after primary therapy, including breast surgery, radiation therapy and chemotherapy. The question is whether the use of Tamoxifen (TAM) increases the risk of developing uterine cancer. Conclusion For most women, the benefits of TAM in preventing a recurrence of BC outweigh by far the potential risk of uterine cancer. Furthermore, benefit from the TAM has evident survival. In the adjuvant setting, TAM is recommended for a maximum of 5 years (9).

CONCLUSION

Many questions about sarcomas do not yet have a clear answer, nor do they have clear guidelines for their early diagnosis. It is believed that with uterine sarcomas, due to the conservative possibilities of treatment of uterine myoma with GnRH-analogs and more numerous hysteroscopic procedures, it will be diagnosed more often in the upcoming years.

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WELLS' SCORE IN DIAGNOSIS OF PULMONARY EMBOLISM IN PATIENT WITH THROMBOCYTOPENIA: A CASE REPORT

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VELSOV SKOR U DIJAGNOSTICI PLUĆNE EMBOLIJE KOD PACIJENTA SA TROMBOCITOPENIJOM: PRIKAZ SLUČAJA

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ABSTRACT

Current diagnostic workup of patients with suspected acute pulmonary embolism (PE) usually starts with the assessment of clinical pretest probability, using clinical prediction rules and plasma D-dimer measurement. Although an accurate diagnosis of acute pulmonary embolism (PE) in patients is thus of crucial importance, the diagnostic management of suspected PE is still challenging.

A 60-year-old man with chest pain and expectoration of blood was admitted to the Department of Cardiology, General Hospital in Cuprija, Serbia. After physical examination and laboratory analyses, the diagnosis of Right side pleuropne monia and acute pulmonary embolism was established. Clinically, patient was hemodynamically stable, auscultative slightly weaker respiratory sound right basal, without pretibial edema. Laboratory: C-reactive protein (CRP) 132.9 mg/L, Leukocytes (Le) 18.9x109/L, Erythrocytes (Er) 3.23x1012/L, Haemoglobin (Hgb) 113 g/L, Platelets (Plt) 79x109/L, D-dimer 35.2. On the third day after admission, D-dimer was increased and platelet count was decreased (Plt up to 62x109/L). According to Wells' rules, score was 2.5 (without symptoms on admission), a normal clinical finding with clinical manifestation of hemoptysis and chest pain, which represents the intermediate level of clinical probability of PE. After the recidive of PE, Wells' score was 6.5. In summary, this study suggests that Wells' score, based on a patient's risk for pulmonary embolism, is a valuable guidance for decision-making in combination with knowledge and experience of clinicians. Clinicians should use validated clinical prediction rules to estimate pretest probability in patients in whom acute PE is being consiered.

Keywords: *pulmonary embolism, thrombocytopenia, Wells` score, antiphospholipid syndrome*

SAŽETAK

Aktuelni dijagnostički pristup kod pacijenata sa sumnjom na akutnu plućnu emboliju (PE) obično počinje sa procenom kliničke pretest verovatnoće korišćenjem kliničkih prediktivnih skorova i određivanjem D-dimera u plazmi. Iako je precizna dijagnoza akutne plućne embolije kod pacijenata od velikog značaja, dijagnoza suspektne PE je i dalje veliki izazov.

Pacijent muškog pola, 60 godina star, je primljen na Odeljenje kardiologije Opšte bolnice u Ćupriji, Srbija, zbog bola u grudima i iskašljavanja krvi. Nakon fizikalnog pregleda i urađenih laboratorijskih analiza, postavljena je dijagnoza pleuropneumonije sa desne strane i sumnja na plućna emboliju. Klinički, pacijent je bio hemodinamski stabilan, auskultatorno neznatno slabiji respiratorni šum desno, bazalno, bez pretibijalnih edema. Laboratorija: C-reaktivni protein (CRP) 132.9 mg/L, leukociti (Le) 18.9x109 /L, eritrociti (Er) 3.23x1012/L, hemoglobin (Hgb) 113 g/L, trombociti (Tro) 79x109/L, D-dimer 35.2. Trećeg dana nakon prijema, dolazi do porasta D-dimera i pada broja trombocita na 62x109/L. Korišćenjem Velsovog skora kliničke verovatnoće dobijen je rezultat 2,5 (bez simptoma na prijemu) uz normalan klinički nalaz i jedine kliničke manifestacije (hemoptizije i bol u grudima), što predstavlja srednji stepen kliničke verovatnoće za PE. Posle recidiva PE, Velsov skor je porastao na 6,5. Ukratko, ova studija ukazuje na to da je korišćenje Velsovog skora za procenu rizika od plućne embolije predstavlja dragocen dijagnostički vodič, u kombinaciji sa znanjem i iskustvom kliničara. Kliničari treba da koriste proverene prediktivne kliničke skorove za procenu kliničke pretest verovatnoće, kod pacijenata kod kojih se razmatra akutna PE.

Ključne reči: *plućna embolija, trombocitopenija, Velsov skor, antifosfolipidni sindrom.*

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INTRODUCTION

European Guidelines for the diagnosis and treatment of pulmonary embolism (PE) estimated annual incidence rate of venous thrombosis and pulmonary embolism at about 0.5 to 1.0 per 1000 inhabitants. [1,2] Diagnosis of PE is not easy and autopsy studies have already confirmed that fact. Percentage of correct diagnosis during the lifetime is approximately 30% (10-65%). [3] Untreated PE is associated with a high mortality rate ranging up to 30%, while the mortality in diagnosed and treated PE is 8%. About 10% of patients with PE die of sudden death. [2] Pulmonary embolism is a potentially lethal complication in surgery patients and the proximal deep venous thrombosis was complicated by pulmonary embolism in approximately one-third of cases, often without clinical manifestations.[4,5]

Current diagnostic workup of patients with suspected acute pulmonary embolism (PE) usually starts with the assessment of clinical pretest probability, using clinical prediction rules and plasma D-dimer measurement. Indeed, recent studies have demonstrated the safety of rejecting the diagnosis of PE by the combination of a low clinical probability and a normal quantitative D-dimer test result, thereby decreasing the need for further radiologic diagnostic imaging in up to 30% of patients. On the third day of hospitalization the patient we are talking about had symptoms of sudden anxiety and heart palpitations.

Although an accurate diagnosis of acute pulmonary embolism (PE) in patients is thus of crucial importance, the diagnostic management of suspected PE is still challenging.

CASE PRESENTATION

A 60-year-old man with chest pain and expectoration of blood was admitted to the Department of Cardiology, General Hospital in Cuprija, Serbia. Problems had begun the day before the admission when he started coughing blood content and had a long-lasting pain in the right shoulder blade. His medical history reveals that 10 days before that he had Aorto-Bi-Iliac bypass as a surgical intervention for abdominal aortic aneurysm. After that, patient was discharged from vascular surgery with symptoms and signs of urinary infection and he was prescribed a macrolide antibiotic therapy.

After physical examination and laboratory analyses, the diagnosis of *Right side pleuropneumonia* and *pulmonary embolism* was established. Clinically, patient was hemodynamically stable, auscultative slightly weaker respiratory sound right basal, without pretibial edema. Laboratory findings were the following: C-reactive protein (CRP) 132.9 mg/L (normal (N) 0-3) , Leukocytes (Le) 18.9x10^9/L (N 4,1-10,9 G/L), Erythrocytes (Er) 3.23x10^{12}/L (N 4.2-6.3 T/L) Haemoglobin (Hgb) 113 g/L (N 120-180 g/L), Platelets (Plt) 79x10^9/L (N 140-440 G/L), D-dimer 35.2 (N < 0,5 mg/L FEU), 2 plus protein in urine, 30-35 Le in urine, bacteriuria (the following devices were used to obtain laboratory parameters: the hematology analyzer *CELLDYN 1800 Abbot;* for biochemical analysis *DIMENSION RXL MAX Siemens* and for obtaining D-dimer *SISMEX 1500 Siemens*). Electrocardiography record: sinus rhythm: frequency 70 beats per minute, present left deviation of heart (Figure 1A). Radiographic findings: the right costophrenic angle bronchopneumonic shading in lungs. Echocardiographic findings: dimensions of cardiac chambers in reference values (Figure 2). Bacteriological examination of sputum: Streptococcus pneumoniae. Therapy: the third generation cephalosporin, half a dose of low molecular weight heparin (LMWH)-enoxaparin.

On the third day of hospitalization the patient had symptoms of sudden anxiety and heart palpitations (Figure 1B) with normal blood pressure and a heart rate of 110/min, respiration rate of 20/ min and oxygen saturation (Sa O2) of 92%. Urgent echocardiography showed signs of acute increased load of the right ventricle (RV dilated with TR 2-3 + SPDK to 57 mm Hg). MSCT pulmonary angiography confirmed thrombosis of distal part of the right pulmonary artery and the segmental branch of the right pulmonary artery with subsequent infarct Right side Pleuropneumonia. D-dimer was increased and platelet count was decreased (Plt up to 62x10^9/L). Oral and parenteral anticoagulants were included and in the next few days patient's status was slowly stabilizing with the normalization of platelet counts (170 and 300 x10^9/L).

On the sixth day of first admission, only discreetly larger circumference of the left lower leg, without painful sensitivity, was registered. A Color Doppler Ultrasound imaging device was used to evaluate partially passable femoralis vein filled with thrombus masses. Additional microbiological analy-

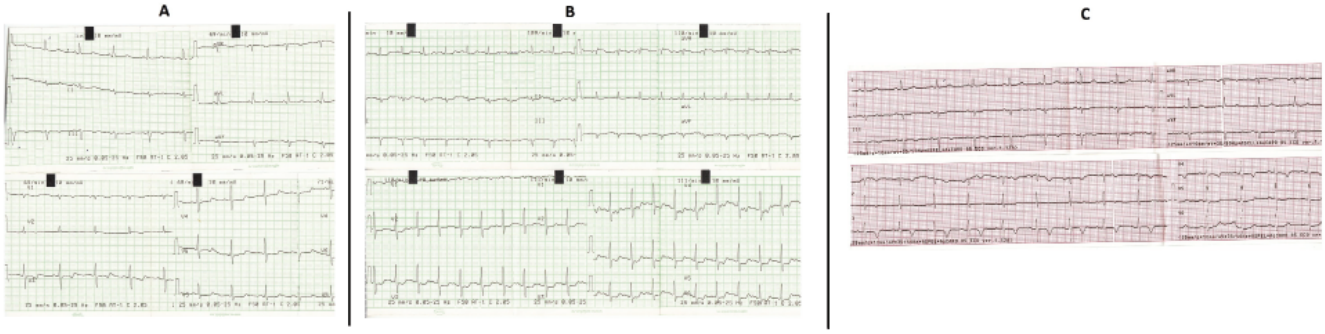

Figure 1. An electrocardiogram (ECG) records the electrical activity of the heart at admission (**A**), during the worsening of disease and hospitalization (**B**) and at discharge (**C**).

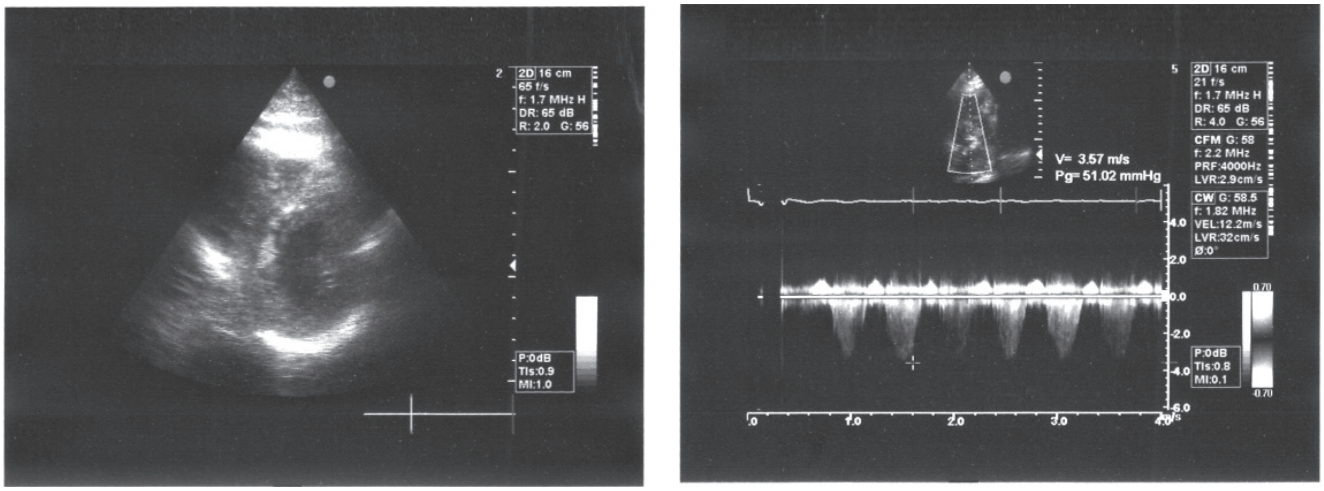

Figure 2. Echocardiographic findings in PE: normal left ventricle and enlarged right ventricle, elevated RVSP (right ventricular systolic pressure).

ses were done: sputum-culture - *Streptococcus pneumoniae*, urine culture - negative finding, hemoculture - negative finding, stool culture - *Clostridium difficile toxin A/B*-positive finding. At the same time, this patient was diagnosed with *acute Enterocolitis* with diarrhea, the existing anemia (Hgb 92 g/l) and hypoproteinemia (albumin 20.5 g/L, N 34-50 g/l). The infectologist who set that diagnosis suggested intensive therapy: rehydration, albumin solution, metronidazole (parenteral) and vancomycin (*per os*) administration. After the laboratory tests, Antiphospholipid syndrome (APS) was established as a definitive diagnosis. The patient recovered and on the seventeenth day of hospitalization (Figure 1C), he was dismissed from the department with a recommendation to do consultative hematologist examination.

DISCUSSION

Diagnosis of PE is a major challenge for clinicians because the spectrum of clinical symptoms can range from dramatic clinical picture followed by cardiogenic shock and sudden cardiac death to absent, minor and clinically nonspecific symptoms that can mimic many other diseases. Concomitant diseases and dominant clinical presentation of the underlying disease may mask symptoms of PE. The clinical form of the disease is conditioned by numerous factors such as the size of affected blood vessels in the lungs, the presence of arterial spasm, the size and distribution of thrombus, the nature of occlusion, the previous cardiopulmonary status, the application of therapy, and others. For the rapid orientation and triage of patients with suspected PE, there are several scores of clinical probability of PE. Most often used are Wells' and Geneva score, but Wells' score has the priority in suspected PE cases as well as in elderly high risk patients. [6,7,8,9] These score systems are based on scoring the presence of certain clinical parameters and, on the basis of the total score, assign the patient to the category with the corresponding degree of probability for PE. Since the PE disease is a highly variable clinical course, the emergence of new clinical symptoms and the signs that these score systems show are necessary because the overall score can be significantly changed over a short period of time. At first glance our case seems simple, but it hides diagnostic and therapeutic dilemmas. No symptoms on admission, a normal clinical finding with clinical manifestation of hemoptysis and chest pain were present the day before admission. If these symptoms apply to Wells' original score of the clinical probability of PE (hemoptysis, 1 point + recent surgery, 1.5 points), we get 2.5 points. This means that diagnosis of pulmonary embolism is unlikely (grading on two levels) or there is an intermediate level of clinical probability of PE (grading in three levels) (Table 1). The same level of clinical probability we get if we apply the original revised Geneva score (hemoptysis, 2 points + major surgery in the past month, 2 points). Thirty-day mortality rate estimated over PESI score was 1.7-3.5% (Class II). [6,7] The normal clinical findings on admission and good general condition of the patient did not speak in favor of a pulmonary embolism, or the emergence of new clinical symptoms. But signs of the situation had changed drastically in less than 48 hours. Wells' score rose by 6.5 points (heart rate > 100, 1.5 points, an alternative diagnosis is less likely, 3 points) and the revised Geneva score increased by 5 points (heart rate > 95), which made the diagnosis of pulmonary embolism more likely in both cases. At the same time the thirty-day mortality by PESI soon grew to double the value (Table 1).

Those normal clinical findings on admission must not be misleading for doctors because pulmonary embolism is often an asymptomatic disease. This fact was indicated in a review of 28 trials with 5233 patients with deep venous thrombosis where asymptomatic pulmonary embolism occurred in 1665 patients (32%).[10] The majority of patients with PE is normotensive on admission and almost a third of them are without clinical symptoms. However, it is in this group of patients that most of the early deaths occure due to the sudden deterioration during hospital treatment.[11] In our case, the patient was hemodynamically stable on admission, which is in accordance with the fact that microembolites obstructing

Table 1. Well`s score on admission and after repeated PE episode

Variable	Points	Points
Clinical signs and symptoms of DVT (minimum of leg swelling and pain with palpation of the deep veins)	0	0
An alternative diagnosis is less likely than PE	0	0
Heart rate greater than 100	1.5	1.5
Immobilisation or surgery in the previous four weeks	0	1.5
Previous DVT/PE	0	3
Hemoptysis	1	1
Malignancy (on treatment, treated in the last 6 months or palliative)	0	0
Clinical probability for PE *Low* *Intermediate* *High*	(Score is 2.5) <2 total **2–6 total** >6 total	(Score is 6.5) <2 total 2–6 total **>6 total**

DVT, deep vein thrombosis; *PE*, pulmonary embolism.

pulmonary circulation are most often clinically silent or give unspecified symptomatology. However, massive thrombus, suddenly released, usually from the venous system of the lower extremities, can obstruct the large pulmonary artery branch and lead to pulmonary hypertension with acute right ventricle load, followed by dramatic clinical symptomatology. Due to ventricular blindness, there is hypoxia with subsequent tachycardia, and reflex hyperventilation initially causes formation of hypocapnia. Associated bronchopulmonary constriction leads to the occurrence of pulmonary hypertension, resulting in desiccation of the right ventricle with compressed left ventricular filling and manifest clinical symptomatology. Nonspecific symptoms and signs should be carefully considered: confusion, unexplained fever, wheezing, transient shortness of breath, pleural effusions, infiltrates in the lungs, coughing, resistant cardiac arrhythmias, cardiovascular collapse, syncope, and hemoptysis.

Chest pain in PE is present in almost every second patient (52%), while incidence of hemoptysis is considerably lower, ranging 7%-11% (mostly related to pulmonary infarction).[1,2,7] The guide of the European Association of Cardiologists diagnostic algorithm for patients without shock and hypotension with low and medium level of clinical probability for PE, implies that after positive D-dimer, CT angiography is necessary, and in case of positive finding the PE should be treated.[7] In our case due to highly positive inflammatory syndrome at the reception, the absence of clinical signs of DVT, left heart deviation on the ECG and normal echocardiographic findings, we did not do CT angiography at the reception. Particularly disturbing were the clinical facts such as a major surgical intervention two weeks earlier and the findings of thrombocytopenia on admission ($79x10^9$/l), which in the case of massive thromboembolism further complicated the narrowed range of therapeutic options. [12] Thrombolytic therapy would not be taken into account in this case since, due to the recent surgery, its application was absolutely contraindicated. According to ESC, recommendations for the treatment of pulmonary embolism in case of a contraindication for thrombolytic therapy or the failure of thrombolytic therapy are surgical embolectomy (Class I, level of evidence C) as well as percutaneous treatment with a guidance catheter (class IIa, level of evidence C) that are limited in our conditions. [7]

Thrombocytopenia is not the state that favors thrombosis and its findings required careful analysis. Numerous cases of pulmonary embolism and thrombocytopenia were recorded in literature. In the study of 225 patients with PE, in the group of patients with thrombocytopenia on admission, thirty-day mortality was 38.7% and in the group without thrombocytopenia was 15.95%. The predictive value of thrombocytopenia for the thirty day mortality had a sensitivity of 78.4%, a specificity of 49%. [13]

Mild thrombocytopenia is a common cause of venous thromboembolism, but pronounced thrombocytopenia may be the cause of diagnostic confusion especially when it occurs during the treatment with heparin. In our case thrombocytopenia on admission was new symptom since it was not present during the previous surgery. It could be a reflection of septic conditions, iatrogenic in connection with the previous hospitalization, but also due to consumption caused by extensive thrombosis, as well as of many other, less likely, complex reasons. The possibility of pseudothrombocytopenia was discarded since no platelet aggregates were detected under the microscope and a low platelet count was also obtained by determining the number of platelets using citrate as an anticoagulant. Because of thrombocytopenia, as well as the fact that the clinical finding on admission was unlikely to PE, we decided to use a half of the therapeutic dose of low molecular weight heparin. But on the third day, when we had clear signs of PE and the lowest level of platelets ($62x10^9$/L), we used full therapeutic dose of enoxaparin that led to the stabilization and normalization of the patient's platelet count (up to $300x10^9$/L). According to ESC recommendations for the treatment of pulmonary embolism in patients with no shock and hypotension (no high risk patients), as our patient was, the therapeutic approach during the acute phase involves the use of parenteral anticoagulant therapy-LMWH or fondaparinux (class I, level of evidence A) with parallel

administration of oral anticoagulant therapy (class I, level of evidence B). However, thrombolytic therapy should also be considered in patients with moderately high risk with clinical signs of haemodynamic instability (class IIa, level of evidence B). [7] Since LMWH and fondaparinux significantly less induce large haemorrhage, most patients do not require laboratory monitoring in relation to unfractionated heparin and carry significantly less potential to induce heparin-induced thrombocytopenia, in addition to non-heparin anticoagulants (lepirudin, danaparoid, bivalirudin, argatroban), where fondaparinux is preferred because there is only a small number of reported cases of heparin-induced thrombocytopenia. [14,15] Since the European Guidelines for the diagnosis and treatment of pulmonary embolism do not provide guidelines for therapeutic approach for patients who have, in addition to pulmonary embolism, initial thrombocytopenia, individual approach and careful evaluation of each patient is needed. Decrease in platelet count during treatment with heparin was not a result of heparin-induced thrombocytopenia as has already been described in the literature. In earlier literature, four patients with PE were described, where in average in the first 18 hours after initiation of therapy with heparin, a decrease in the number of platelets was shown and continuation of therapy with heparin that had already started had an excellent final hemodynamic result and normalization of platelet counts due to inhibition of platelet consumption. [16]

Our case is complicated by severe diarrhea accompanied by dehydration, hypoalbuminemia and anemia that required prolonged hospitalization and patient immobilization because there was the increased risk of new thromboembolic events and not rarely relapsed diarrhea induced by Clostridium difficile. The risk of re-infection within eight weeks is 10-20%. [17] The final closure is certainly a definitive diagnosis of antiphospholipid syndrome where thrombocytopenia is the most common hematologic event.

CONCLUSION

In summary, this study suggests that the Wells' score based on a patient's risk for pulmonary embolism is valuable guidance for decision-making in combination with knowledge and experience of clinicians. Clinicians should use validated clinical prediction rules to estimate pretest probability in patients in whom acute PE is being considered.

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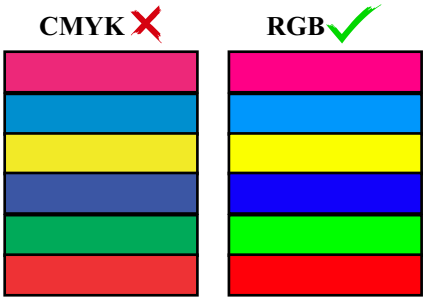

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