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NAJZNAČAJNIJE OSNOVE PATOGENEZE COVID-19

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ABSTRACT

At the end of 2019, a new coronavirus infection occurred in the People's Republic of China with an epicentre in the city of Wuhan. On February 11th, 2020, the World Health Organization assigned the official name of the infection caused by the new coronavirus – COVID-19. COVID-19 has affected people from all over the world given that the infection was noted in 200 countries resulting in annunciation of the pandemic situation. Human corona viruses cause mild to moderate respiratory infections. At the end of 2002, a new coronavirus appeared (SARS-CoV), the causal agent of atypical pneumonia, which caused acute respiratory distress syndrome (ARDS). The initial stage of COVID-19 infection is the penetration of SARS-CoV-2 into target cells that have angiotensin converting enzyme type II receptors. The virus enters the body through the respiratory tract and interacts primarily with toll-like receptors (TLRs). The events in SARS-Cov-2 induced infection follow the next scenario: epithelial cells via TLRs recognize and identify SARS-Cov-2, and after that the information is transmitted to the transcriptional NF-κB, which causes expression of the corresponding genes. Activated in this way, the epithelial cells begin to synthesize various biologically active molecules. The results obtained on preclinical material indicate that ROS generation increases and the antioxidant protection decreases, which plays a major role in the pathogenesis of SARS-CoV, as well as in the progression and severity of this respiratory disease.

***Keywords**: World Health Organization, coronavirus – COVID-19, respiratory infection, respiratory distress syndrome*

SAŽETAK

Krajem 2019. godine, infekcija novim korona virusom se dogodila u Narodnoj Republici Kini sa središtem u gradu Vuanu. Jedanaestog februara, 2020. godine, Svetska Zdravstvena Organizacija je dala zvanično ime infekciji prourokovanoj novim korona virusom – COVID-19. Od COVIDA-19 su oboleli ljudi širom sveta uzimajući u obzir činjenicu da je infekcija zabeležena u 200 država što je imalo za posledicu objavu pandemije. Korona virusi od kojih oboljevaju ljudi prouzrokuju blage do umerene respiratorne infekcije. Krajem 2002. godine, pojavio se novi korona virus (SARS-CoV), uzročnik atipične pneumonije koja je prouzrokovala akutni respiratorni distres sindrom (ARDS). COVID-19 infekcija počinje prodiranjem SARS-CoV-2 u ciljne ćelije koje imaju receptore angiotenzin konvertujućeg enzima 2. Virus ulazi u telo kroz respiratorni trakt i intereaguje prvenstveno sa Toll sličnim receptorima. Redosled dešavanja kod infekcije izazvane virusom SARS-Cov-2 je sledeći: epitelne ćelije preko TLR (eng. Toll Like Receptors) sličnih receptora prepoznaju i identifikuju SARS-Cov-2, i posle toga se informacija prenosi do transkripcionog NF-kB koji prouzrokuje ekspresiju odgovarajućih gena. Aktivirane na ovaj način, epitelne ćelije počinju da sintetišu različite biološki aktivne molekule. Rezultati dobijeni na prekliničkom materijalu nagoveštavaju da se stvaranje ROS povećava a antioksidativna zaštita smanjuje što igra glavnu ulogu u patogenezi SARS-CoV, kao i u progresiji i težini ove respiratorne bolesti.

***Ključne reči:** Svetska zdravstvena organizacija, koronavirus – KOVID-19, respiratorna infekcija, respiratorni distres sindrom*

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CLASSIFICATION AND EPIDEMIOLOGICAL CHARACTERISTICS OF CORONAVIRUSES

At the end of 2019, an outbreak of a new coronavirus infection occurred in the People's Republic of China (PRC) with an epicentre in the city of Wuhan (Hubei province), the causative agent of which was given the temporary name 2019-nCoV. On February 11th , 2020, the World Health Organization (WHO) assigned the official name of the infection caused by the new coronavirus – COVID-19 ("Coronavirus disease 2019"). The International Committee on virus taxonomy on February 11th , 2020, assigned the official name of the infectious agent-SARS-CoV-2 due to its high homology (approximately 80%) with SARS-CoV, which caused acute respiratory distress syndrome (ARDS) and high mortality during 2002-2003 (1).

COVID-19 has affected people from all over the world given that the infection was noted in 200 countries resulting in annunciation of the pandemic situation by WHO (2,3).

It was believed that the first outbreak of SARS-CoV-2 occurred through the zoonosis transmission in the market of seafood in Wuhan, China. After some time, it was admitted that the transmission of virus among people played the main role (4).

Corona virus belongs to *coronaviridae* family – it is a large family of RNA-containing viruses, 30 kb in size, which are capable of infecting humans and some animals (5).

Currently it is known that there are four types of coronaviruses (HCoV-229E, -OC43, -NL63 и -HKU1) circulating in human population. Human corona viruses, such as 229E and NL63, cause mild to moderate respiratory infections. According to serological and phylogenetic analysis, coronaviruses are divided into four genera: Alphacoronavirus (Alpha-CoV), Betacoronavirus (Beta-CoV), Gammacoronavirus (Gamma-CoV) and Deltacoronavirus (Delta-CoV). Alpha-CoV and Beta-CoV coronaviruses affect only mammals (6).

Before 2002, coronavirus was recognized as a cause of mild respiratory infections that have been fatal very rarely. At the end of 2002, a new coronavirus appeared (SARS-CoV), the causal agent of atypical pneumonia, which caused acute respiratory distress syndrome (ARDS). SARS-CoV belongs to the genus Beta-CoV. The natural reservoir of SARS-CoV are bats, intermediate hosts are camels and Himalayan civets. In total, more than 8000 cases were registered in 37 countries around the world during the epidemic period, of which 774 were fatal. No new cases of SARS-CoV-induced ARDS have been reported since 2004. In 2012, the world encountered a new MERS coronavirus (MERS-CoV), a pathogen of the Middle East respiratory syndrome, which also belongs to the genus Beta-CoV. The main natural reservoir of MERS-CoV coronaviruses are single-humped camels. From 2012 to January 31st , 2020, 2519 cases of coronavirus infection caused by the MERS-CoV virus were registered, of which 866 were fatal. All cases were geographically associated with the Arabian Peninsula (82% of the cases are reported in Saudi Arabia). At the moment, MERS-CoV continues to circulate and causes new cases of the disease. The new SARS-CoV-2 coronavirus is classified as group II pathogenicity, as are some other members of this family (SARS-CoV virus, MERS-CoV). SARS-CoV-2 coronavirus is believed to be a recombinant virus between a bat coronavirus and coronavirus of an unknown origin

ROLES OF ACE2 AND TLR RECEPTORS IN SARS-COV-2 INFECTION

The entrance gate of the pathogen is the epithelium of the upper respiratory tract and epithelial cells of the stomach and intestines. The initial stage of infection is the penetration of SARS-CoV-2 into target cells that have angiotensin converting enzyme type II (ACE2) receptors (7,8).

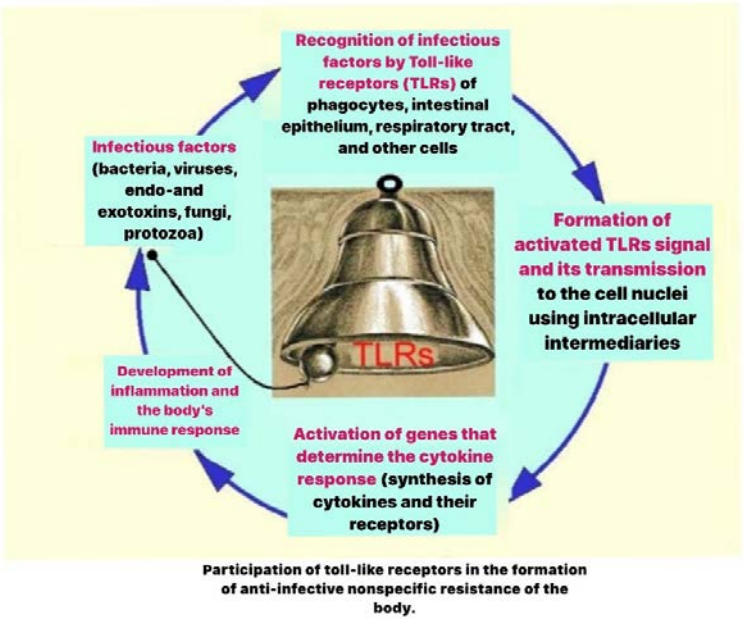
ACE2 receptors exist in the population of endothelial cells in the respiratory tract, kidneys, oesophagus, bladder, ileum and central nervous system (CNS). ACE2 can be disposed by two proteases, ADAM17 and TMPRSS2. It was shown that the cleavage of ACE2 by TMPRSS2 can facilitate the penetration of SARS-CoV-2 into cells, while the action of ADAM17 on ACE2 may have a protective effect (9).

Virus (SARS-Cov2) → gets into the respiratory tract → interacts with Toll-like receptors of the bronchial epithelium → activates NF-κB (core factor of kappa B) → synthesis of interferons→ activation of ACE2 (receptor of angiotensin converting enzyme) → penetration of SARS-Cov2 into the cell (bronchial epithelial cell)→ replication of virus → damage of epithelial cell and its death

The virus enters the body through the respiratory tract and interacts primarily with Toll-like receptors (TLRs). TLRs are the main specialized cellular structures that are able to recognize various infectious agents, such as microbes, viruses, some protozoa (primarily their exotoxins and endotoxins) and initiate the expression of cytokines - biologically active substances that determine the formation and launch of mechanisms of non-specific resistance of the body. By recognizing the infectious aggression, TLRs immediately "sound the alarm", initiating the activation of anti-infectious protective and adaptive mechanisms of the body at the cellular level (Figure 1).

It is noteworthy that Toll-like receptors are expressed not only on phagocytes (monocytes, tissue macrophages and neutrophils) and immune-competent cells (T and B lymphocytes), but also on many other types of cells - epithelial cells of the respiratory and urinary tracts, intestines, endothelial cells, keratocytes, microglial cells, that participate in the formation of anti-infective resistance of the body.

Figure 1. Participation of Toll-like receptors in the formation of anti-infective non-specific resistance of the body



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13 types of TLRs have been identified in humans so far: from TLR-1 to TLR-13, but it is speculated that there are probably more. The majority of TLRs are transmembrane receptors: TLR-1,-2,-4,-5,-6,-10,-11 (Figure 2).

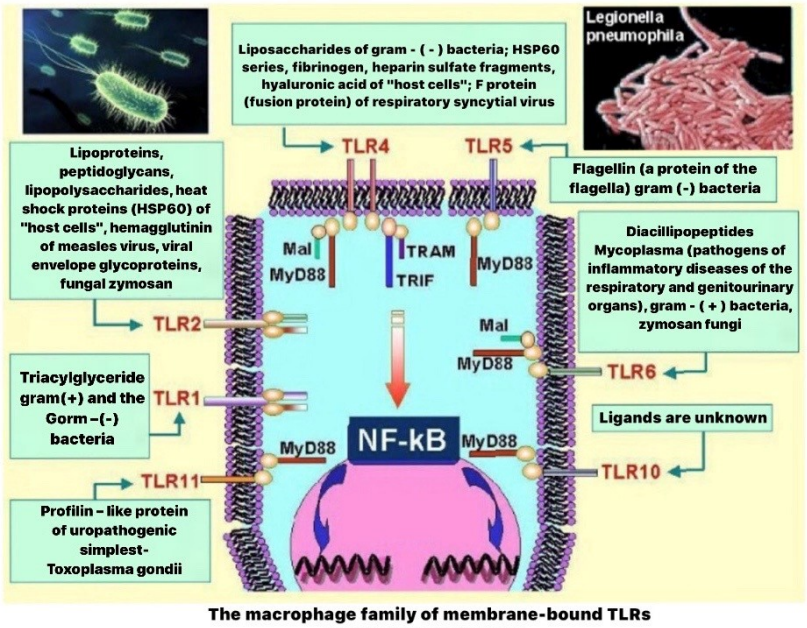

Figure 2. The macrophage membrane-bound TLRs family and their main ligands

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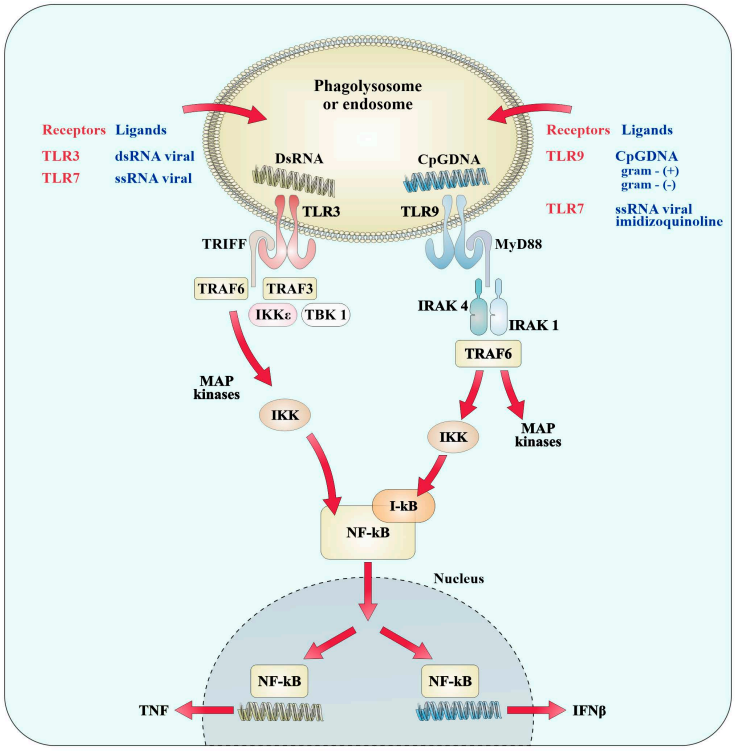
[Comment: Mal (or TIRAP), TRAM (or TICAM), TRIF (or TICAM-1), MyD88 – these are intracellular adapter proteins that belong to the TIR domain group-containing proteins and participate in the transmission of signals from Toll-like receptors. Among them, MyD88-adapter-like (myeloid differentiated factor 88) is a universal adapter protein (almost all TLRs use MyD88 for their signal transduction) and, at the same time, - a specific protein for transmitting signals from TLR4 and TLR5. It is established that TLR 5 mutations cause a predisposition to Legionnaire's disease (legionnaires), the cause of which is "Gram-negative flagellated bacteria" (pictured is an electronogram of Legionella pneumophila). Regarding the fact that flagellin is astrong adjuvant, it was found out that the mechanisms of its action at the cellular level can contribute to the creation of vaccinepreparations for the prevention of Legionnaire's disease.]

Transmembrane receptors usually consist of 2 domains – the extracellular, which provides direct interaction with ligands of microorganisms or their products, and the intracellular (cytoplasmic), which initiates translation of signals of activated TLR. After interacting with ligands, TLRs acquire the ability to bind intracellular adapter proteins that provide subsequent signal transmission.

Detection of viruses (SARS-Cov-2) and other intracellular microorganisms is the main goal of functioning of another family of TLRs, which are localized in the cytoplasm and on the internal structures of cells (Golgi apparatus). This small intracellular family combines TLR-3, TLR-7 and TLR-9 (Figure 3).

Figure 3. The family of intracellular TLRs and their main ligands



[**Abbreviations**: CpGDNA-cytosine phosphate guanosine containing the " motive " of DNA gram - (+) and gram - (-) bacteria; ssRNA viral-single-stranded RNA, dsRNA viral double - double-stranded RNA of viruses; IRAK1, IRAK4-cytosolic enzymes of the group signal kinases; imidizoquinoline (imidizoquinoline) - synthetic antiviral drug; MyD88-myeloid differentiated factor 88; universal adapter protein; TRAF6-cytosolic adapter protein associated with the tumor necrosis factor receptor 6; TRAF3-cytosolic adaptive protein associated with the tumor necrosis factor receptor 3; MAP kinases-a cascade of enzymes that performs regulatory phosphorylation nuclear transcription factors; IKK is an inhibitor of the I-kB kinase complex (NF-κB inhibitor); TRIF or TICAM-1-intracellular adapter protein, which belongs to the group of TIR domain-containing proteins and participates in signal transmission from toll-like receptors; TBK1-serotonin/threonine – kinase involved in the cascade of signals that lead to the activation transcription of NF-κB; TNF-tumor necrosis factor, IFN - interferon]

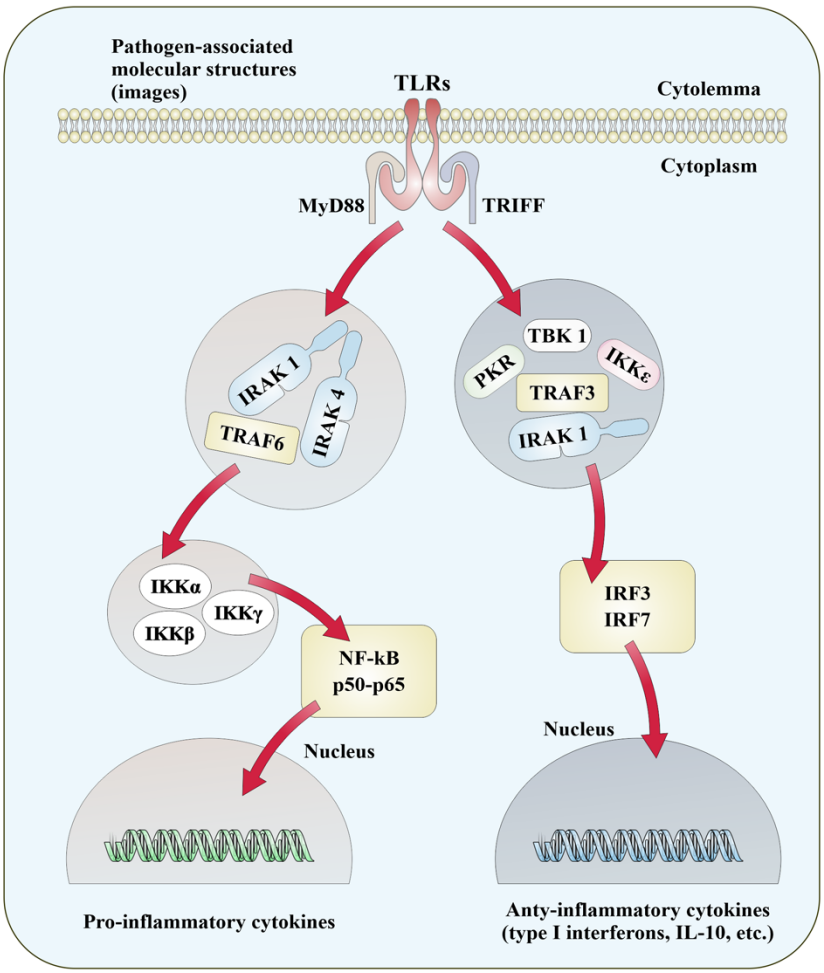
Currently there are two variants of the molecular mechanisms for transmitting signals from activated TLRs: 1) MyD88-dependent and 2) MyD88-independent. The second pathway involves other (non-MyD88) adapter molecules, their combinations with each other or with MyD88. In particular, MyD88-dependent signal translation leads, for example, to the activation of NF-κB and MARK kinase (Figure 4).

A landmark event in the studying of innate immunity was a detection of TLRs on endothelial cells and epithelial cells of the skin and mucous membranes. The traditional idea of the epithelial cover of the body as a passive mechanical barrier to infection has been enriched by a fundamentally new position on the active participation of the epithelium in the formation of anti-infective resistance of the body. The epithelium, recognizing an infectious factor with the help of TLRs, initiates immediate mobilization of mechanisms for its elimination by its own "forces" or by attracting the adaptive immune response elements.

The events in SARS-Cov-2 induced infection follow the next scenario: epithelial cells via TLRs recognize and identify SARS-Cov-2, and after that the information is transmitted to the transcriptional NF-κB, which causes expression of the corresponding genes. Activated in this way, the epithelial cells begin to synthesize various biologically active molecules, including chemokines (cytokines with a chemoattracting effect), resulting in the attraction of macrophages and polymorphic-nuclear leukocytes (neutrophils, basophils, eosinophils) to the site of infection. The activated macrophages and polymorphic-nuclear leukocytes result in phagocytosis of the infectious factor and allocation of their own set of pro-inflammatory mediators.

Thus, the intracellular infectious factors (viruses, such as SARS-Cov-2), and a number of other infectious agents (pathogens of syphilis, tuberculosis, leprosy, etc.) are recognized by TLRs localized on the internal structures of cells. Due to this, it can be assumed that BCG vaccination protects the body from the pathogenic effects of COVID-19. Apparently, due to this, children are in the low-risk zone of COVID-19 (10).

Figure 4. Intracellular mechanisms of the activation of cytokine synthesis during stimulation of the TLRs receptor families



[**Comment:** I. Pathogenic ligand recognized by the TLR receptor family includes MyD88 or TRIF adapter proteins. MyD88 interacts with members of the IRAK family that mobilizes TRAF6, which transmits the signal to the IKK - complex. Then follows the activation of NF-κB(RelA-p50), its penetration into the nucleus and subsequent activation of proinflammatory cytokine genes. There is possible MyD88 independent signal transmission path via TRIF adapter protein, which transmits a signal to the TBK1 and IKK kinases that are in communication with TRAF3. The subsequent activation of IRF3 and IRF7 leads to their penetration into the nucleus and stimulation of the synthesis of anti-inflammatory cytokines: type I interferons -IFN and IFNβ; interleukins: IL-10, etc.. Specifically launched response to the activation of TLR3 synthesis of type I interferons strengthens the antivirus protection. In addition, TLR3 activation causes differentiation and maturation of dendritic cells and thus initiates the adaptive immune responses. II. MyD88-myeloid differentiated factor 88; universal adapter protein; TBK1-serotonin / threonine-kinase is involved in the cascade transmission of signals that lead to the activation of NF-κB transcription. It was found out, that the TBK-1 protein is, on the one hand, an antiviral factor, and with the other is involved in maintaining the viability of tumor cells. Exposure to this protein by substances which block TBK-1 activity may be a fairly promising component of the antiblastomic therapy, however, it has to be taken into account that the inactivation of TBK-1 reduces the body's ability to resist viral infections infections; TRAF6 is a cytosolic adapter protein associated with the receptor of tumor necrosis factor 6; IRAK1, IRAK4 - cytosolic enzymes of the group of signal kinases [IRAK4 is the most important kinase in its family; its insufficiency leads to a violation of the immune response to bacterial infection due to the blockage of the TLR4 receptor signaling pathway; mutations in IRAK4 were found in children suffering from persistent pyogenic bacterial infections. The main threat to these patients is Gram-positive bacteria (Staphylococcus aureus, Streptococcus pneumoniae)]; IKKa, IKKβ, IKKy - are inhibitors of the I-kB kinase complex; phosphorylating I-kB destroys it and thus releases NF-κB and allows it to translocate to the nucleus to activate transcription; TBK1-TANK-is a binding kinase; this is an IKK-related kinase that forms complexes with TRAF2...and activating transcription factor...; TRIF is an adapter protein, which belongs to the group of TIR domain-containing proteins and participates in signal transmission from Toll-like receptors; IRF-transcription factor, which is activated in cells when they are affected by type I interferons: IFN and IFNß; IRF "turns on" the transcription of genes that encode synthesis antiviral proteins; PKR-dcRNA (double-stranded RNA) is a dependent protein kinase, induced by interferon, performs at least three functions in the interferon system: participates in providing the antiviral interferon effect; is a mediator of the NF-κB dependent signaling transduction when activating the transcription of the IFN-beta gene; is one of the factors of IFN-dependent inhibition of cell proliferation. A new method of treatment of tumor neoplasms is being developed on the basis of the use of medications that activate protein kinase PKR.]

As previously mentioned, the activated TLRs activate the transcription nuclear factor NF-κB. NF-κB in an inactive state is linked with a specific inhibitor in the cytoplasm of many cell types, but its expression can be increased due to the response of various stimuli: cytokines, acute phase proteins, regulators of apoptosis and cell proliferation, bacterial toxins, viruses, DNA damage, oxidative stress, and many other factors. The stimulated NF-κB acquires the ability to move to the nucleus and cause changes in the activity of a large number (more than 150) of a wide variety of target

genes, which can be involved in the formation of numerous physiological and pathological processes - immune, inflammatory, proliferative, programmed cell death (apoptosis), etc. The activated NF-κB causes the increase of expression of genes that determine the synthesis of pro-inflammatory cytokines (interleukin(IL)-1β, IL-2, IL-6, IL-12, IL-18, tumor necrosis factor (TNF)-α, TNF-β, lymphotoxin alpha (LT-α), lymphotoxin beta (LT-β), granulocyte colony-stimulating factor (G-CSF)), as well as interferons (β and γ). SARS-CoV-2 penetrates the cell using the ACE2 receptor (11,12) and enzyme TMPRSS2. But the most unusual thing was that the ACE2 gene, which encodes the receptor used by SARS-CoV-2 to enter human cells, is stimulated by interferon — one of the body's main defence forces when detecting the virus. Interferon actually turns on the ACE2 receptor at higher levels, giving the virus new "portals" to penetrate. Thus, the use of interferon at the stage when the virus actively penetrates human cells can further worsen its condition (13).

The life cycle of a virus with a host consists of the following 5 stages: attachment, penetration, biosynthesis, maturation and release. Once the virus binds to host receptors (attachment), they enter host cells through endocytosis or membrane fusion (penetration). As soon as the viral content is released inside the host cells, the viral RNA enters the nucleus for replication. The viral mRNA is used to create the viral proteins (biosynthesis). Then new viral particles are created (maturation) and released (14).

Coronavirus consists of four structural proteins: spike, membrane, envelop and nucleocapsid (15).

The spike consists of two functional subunits, the S1 subunit is responsible for binding to the host cell's receptor, and the S2 subunit is responsible for merging viral and cell membranes. ACE2 has been identified as a functional receptor for SARS-CoV (16). The structural and functional research has shown that the outbreak of SARS-CoV-2 is also connected with ACE2 (5,11-13).

The expression of ACE2 is high in lungs, heart, ileum, kidneys, endotheliacytes and bladder (17). In the lungs, ACE2 is highly expressed in the epithelial cells of bronchi. It was shown, that upon the binding of the SARS spike protein to the ACE2 receptor, the whole unit is undergoing proteolysis by TMPRSS2, which leads to the ACE2 cleavage and activation of spike protein (18,19) resulting in penetration of the virus into the target cell. Thus, the cells which express both ACE2 and TMPRSS2 are mostly affected by SARS-CoV (20). It was shown that SARS-CoV-2 also needs ACE2 and TMPRSS2 to entry a cell (21). The unique characteristics of SARS-CoV-2 among coronaviruses are the presence of a furin cleavage site at the S1/S2 site. The S1/S2 site was completely cleaved during biosynthesis in sharp contrast to the SARS-CoV spike that was incorporated into the assembly without cleavage, making this virus very pathogenic (11,18,19,22).

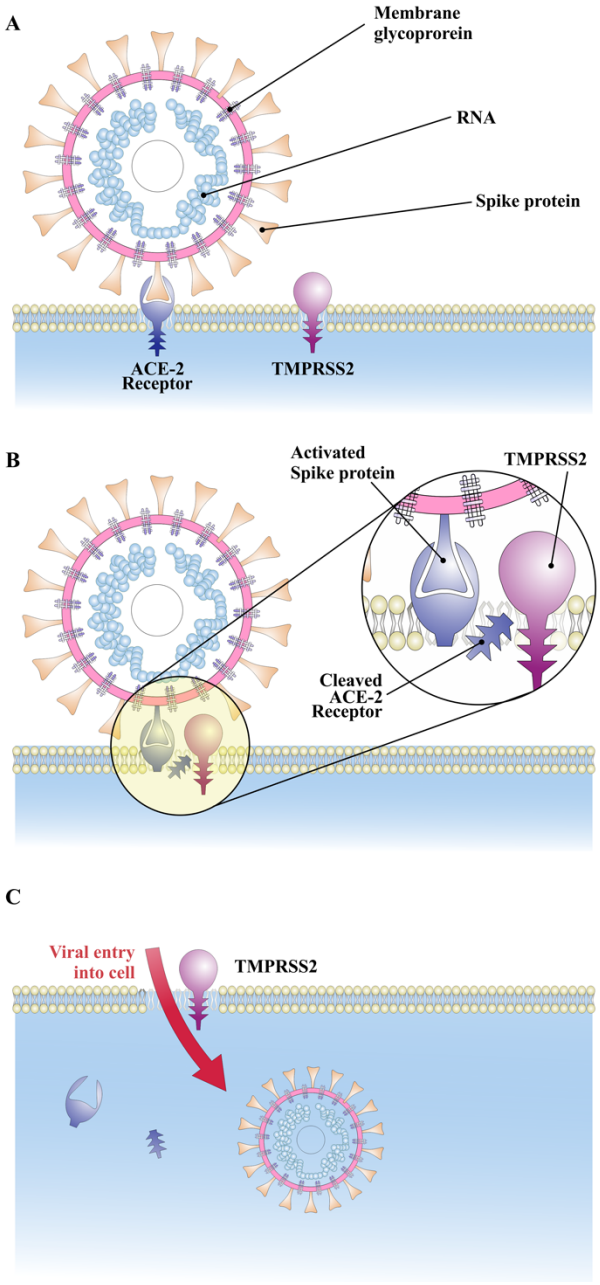
Because ACE2 is highly expressed at the apical side of epithelial cells of the lungs in the alveolar space (23), this virus can penetrate inside and destroy them (Figure 5).

Virus (SARS-Cov2) → enters the pulmonary tract → interacts with Toll-like receptors in aleovocytes → activation of NF-κB (nuclear factor kappa B) → interferon synthesis → activation of ACE2 (receptor of angiotensin converting enzyme) → penetration of SARS-Cov2 into a cell (pneumocyte II type)→replication of the virus → damage of pneumocyte type II and its death → disruption of surfactant's synthesis → scarring of the alveoli → acute respiratory distress syndrome → acute respiratory failure

LUNG TISSUE DAMAGE CAUSED BY COVID-19

SARS-CoV-2 spreads very fast and migrates down the respiratory tract. Unfortunately, around 20% of the infected patients develop pulmonary infiltrates, and some of them develop a very serious condition (24). Not only SARS-CoV and flu virus, but also SARS-Cov-2 mainly infect pneumocytes type 2 (25,26). The infected pneumocytes typemainlyhave peripheral and subpleural localization (27). SARS-CoV multiplies in pneumocytes type 2, releases a large number of viral particles, and the cells undergo apoptosis and die (28), resulting in reduction of synthesis of surfactant. With a deficiency of surfactant, the declination (atelectasis) of some alveoli and overtension of other alveoli with their subsequent rupture occur. This situation is determined by the heterogeneity of the lungs and, consequently, by the different degree of severity of the deficiency of cells-producers of the lungs surfactant (surface-active agent) that occurs in the multiple organ dysfunction syndrome (MODS). The surfactant deficiency also leads to the development of obstructive alveolar hypoventilation due to a decrease in the patency of the lower respiratory tract: their decline, and then - compression (the result of increased transpulmonal pressure due to the need to perform active exhalations to overcome the increased resistance to air flow in the sleeping lower respiratory tract). When surfactant is deficient in the lungs, Laplace's law shows its effect: the smaller the diameter of the vesicle (i.e., the larger the diameter of the alveoli, etc.), the greater the surface tension of the liquid and, consequently, the air pressure in this bubble. If there is a capacity between the bubbles, the air will move along the pressure gradient to the large-diameter bubbles from the small-diameter bubbles until they disappear. The products of lipooxygenase collapse of the phospholipid components of the surface-active agent– leukotrienes are powerful constrictors of the bronchial tubes. Their excessive formation causes bronchiolospasm, which exacerbates the development of obstructive alveolar hypoventilation. In addition, when surfactant is deficient, recovery of the alveoli and lower respiratory tract is disrupted (for instance, the movement of mucus to the zone of mucociliary transport due to a decrease in the longitudinal gradient of surface pressure).

Figure 5. The mechanism of penetration of the virus (SARS CoV2) into the cell



[**Comment:** (A) Spike proteins on the surface of the coronavirus bind to angiotensin-converting enzyme 2 (ACE-2) receptors on the surface of the target cell; (B) Transmembrane serine protease type II (TMPRSS2) binds and cleaves the ACE-2 receptor. In the process, the spike protein is activated; (C) Splited ACE-2 and activated spike protein help the virus to entry. TMPRSS2 expression increases the cellular uptake of the coronavirus]

Also, under the influence of SARS-CoV-2, the forces of surface tension can cause not only the decline of the alveoli diameter, but also the suction of liquid from the capillaries into the alveoli. The forces of molecular interaction at the liquid/air phase section (water molecules more easily overcome the interphase section) can contribute to fluid retention in the alveoli, resulting in the development of alveolar edema. Thus, the surfactant deficiency is a sufficient factor that leads to the inclusion of all known pathogenetic mechanisms of ARDS.

Virus (SARS-Cov2) → enters the respiratory tract → damages the type II pneumocytes → activation of the type I pneumocytes → formation of fibrosis between the alveoli and blood vess els → acute respiratory failure

Pneumocytes type 2 are the progenitor cells for pneumocytes type 1 (29). The accumulation of fibrin in the lung blood vessels is facilitated by a decrease in the content of fibrinolysis activators in the pulmonary endothelium. Fibrin can enter the interstitium and lead to the formation of sclerosing alveolitis. The output of fibrin in the lumen of the alveoli is favoured by the increasing defeat of pneumocytes type 2. Extravasates of fibrin in the alveoli cause the formation of hyaline membranes, which are one of the main signs of ARDS.

Thus, the pathologic result of SARS and COVID-19 is a diffused damage of the alveoli with hyaline membranes enriched with fibrin and few multinuclear gigantic cells (30,31). The aberrant wound healing can lead to more severe scarring and fibrosis than the other forms of ARDS.

Virus (SARS-Cov2) → enters the respiratory tract → interacts with Toll receptors of epithelial cells of the bronchi → activation of NF-κB → synthesis of chemokines → attraction of macrophages and neutrophils to the submucosa of the bronchi

INFLAMMATORY RESPONSE TO COVID-19 INFECTION

Epithelial cells firstly use TLRs to recognize and identify SARS-CoV-2. The resulting information is transmitted to the transcriptional nuclear factor NF-κB, which causes the expression of the corresponding genes. Activated in this way, epithelial cells begin to synthesize various biologically active molecules, including chemokines: the growth regulatory oncogenes - GROα, GROβ, GROγ, IL-8 (CXCL8), interferon-γ (IFN-γ), MIP-1α (macrophage inflammatory protein-1α), MIP-1β (macrophage inflammatory protein-1β), a regulator of activation of normal T-cell expression and secretion (RANTES, CCL5). These cytokines, having a chemoattracting effect, attract macrophages and polymorphonuclear leukocytes to the site of infection.

In addition, epithelial cells produce G-CSF and granulocyte-macrophage colony-stimulating factor (GM-CSF). These colony-stimulating factors induce differentiation of cells of myeloid origin: G-CSF enhances differentiation and proliferation of neutrophils, while GM-CSF stimulates

proliferation and differentiation of various types of immune progenitor cells. In the lung tissue, GM-CSF causes proliferation and activation of the pulmonary dendritic cells and macrophages. Mice with GM-CSF deficiency are highly sensitive to respiratory viruses. The CXCL8 chemokine purposefully recruits neutrophils to the lung lesion (32,33).

Hyperproduction of cytokines of CXCL8 Chemokines causes recruitment of neutrophils in the affected tissues. Many mediators released by neutrophils themselves are neutrophil chemoattractants, so neutrophils can recruit other neutrophils. In turn, neutrophils produce proinflammatory cytokines and chemokines, and recruit monocytes. High levels of neutrophils in the peripheral blood are associated with an unfavorable prognosis of COVID-19 infection (34). Neutrophils are characterized by a rapid rate of phagocytosis and higher intensity of generation of reactive oxygen species (ROS). Granules of neutrophils contain a fairly wide range of enzymes that are secreted into the extracellular space and can cause tissue destruction (35). Despite the presence of neutrophils in tissues infected with SARS-Cov-2, their role in the clearance of coronaviruses remains unknown.

Epithelial cells, alveolar macrophages, and dendritic cells (DC) are three main components of the innate respiratory immunity (36). DC are located under the epithelium. Macrophages are located on the apical side of the epithelium. DC and macrophages are innate immune cells for fighting viruses, as long as the adaptive immunity is not involved.

Responses mediated by T cells against coronaviruses were previously considered (5).

DC and macrophages can phagocyte apoptotic cells infected with the virus (37). For example, apoptotic epithelial cells infected with the virus can be phagocytised by DC and macrophages, which leads to presentation of the antigen to T cells and their infection. Or DC and macrophages may be infected with the virus in the first place. This requires future research.

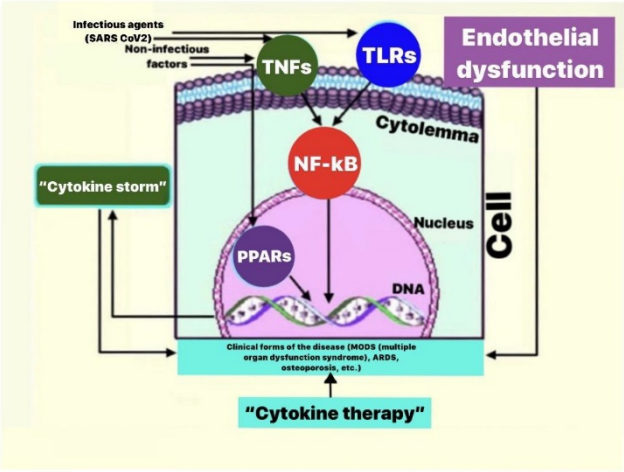
Virus (SARS-Cov2) →interacts with Toll-receptors of epithelial cells of bronchi, macrophages, neutrophils →activation of NF-κB (nuclear factor Kappa B)→violation of inhibition of cytokine synthesis by the feedback principle →synthesis of proinflammatory cytokines in large quantities →cytokine "storm" ("storm»)

The processes of biosynthesis of different types of cytokines are carried out according to a single scenario, despite some differences in its implementation. The receptor-mediated effect on cytokine-producing cells of various signals: exo and endogenous factors of infectious and non-infectious nature (including cytokines) initiates the activation of intracellular (cytosolic) mechanisms of their transmission, leading to the activation of transcription factors and, ultimately, changes in the genetic information that cause the expression of biologically active molecules.

The typical process of cytokine biosynthesis and development of the subsequent "cytokine storm" caused by them includes the following consistently implemented mechanisms (Figure 6):

1. Activation of TLRs by SARS-CoV-2.
2. Stimulation of a nuclear transcription factor located in the cytoplasm in combination with its inhibitor. The signal from the activated TLR activates the mechanism of degradation of such an inhibitor, which leads to the release of a transcription factor from its blocking complex.
3. Transcription (this is the first stage of the process of implementing the genetic information). The stimulated transcription factor penetrates the nucleus and binds to specific parts of DNA, which determines the transfer of information from the DNA molecule to the structure of the matrix RNA.
4. Translation is the next stage in the process of implementing the genetic information, during which the synthesis of proteins occurs: cytokines and other biologically active molecules.
5. Release (exocytosis) of formed cytokines from producing cells into the intercellular space and blood vessels.
6. Receptor-mediated effects of newly formed cytokines and other molecules on target cells.
7. Synthesis and isolation of cytokines and other expressed biologically active substances by the activated target cells, which then cause the "phenotypic (target) effects".

Figure 6. Mechanism of cytokine storm development



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[**Comment**: TNFs (tumor necrosis factor superfamily); possible without an exaggeration to say that any pathological process and any disease do not develop without the participation of members of this superfamily, which consists of two dozen of cytokines and three dozen of receptors to them. A representative of this TNF family, with a wide range of actions, affects the functional activity of immunocompetent

cells; is the most important mediator of inflammatory and tumor processes, etc. The introduction of inhibitors of this cytokine into clinical practice is considered as one of the the most important achievements of modern medical science and practice. PPARs (peroxisome family of proliferator-activating receptors); these recently discovered receptors play an important role in lipid metabolism and carbohydrates, present a new explanation of the pathogenesis of obesity, insulin- resistance, as well as-many clinical effects of medications, used for the treatment of dyslipidemia, reducing the risk of cardiovascular diseases and their complications in the development of metabolic syndrome. NF-κB (Kappa B nuclear transcription factor) is a key transcription factor for various numerous cytokine and immuno-regulatory genes that determine the development of the broadest spectrum of immune and inflammatory, gerontological diseases, tumors, viral infections, etc. In combination with its inhibitor, NF-κB is at the intersection of a number of important cascades of biochemical events that lead to the cell activation. TLRs (family of toll-like receptors); the discovery of these receptors has led to the fundamental changes in the perception of the role and mechanisms of formation of the innate immunity, its connection with the adaptive (acquired) immunity, microbiocenosis of the body; has designated prospects for development of more effective prevention and treatment methods of chronic inflammatory and allergic diseases. Endothelial dysfunction is one of the independent risk factors of almost all cardiovascular diseases, including ischemia, atherosclerosis, primary arterial hypertension, as well as diabetes, inflammatory, autoimmune and tumor diseases].

Among many known transcription factors, NF-κB has received most attention in the medical world. To date, it has been established that NF-κB is a key transcription factor for genes that determine the development of a wide range of diseases – immune and inflammatory diseases, gerontological diseases, tumors, viral infections, etc.

Up to date, it has been established that NF-κB is not a single factor, but the whole family of transcription factors that differ in specificity of the DNA-binding and transcriptional activity. This family of NF-κB includes 5 Rel proteins: RelA (or p65), c-Rel, RelB, Nfkb1 (or p50), and Nfkb2 (or p52). All proteins in the NF-κB family are homologous to the retroviral cancer protein v-Rel and are therefore classified as NF-κB/Rel proteins. Rel proteins are responsible for the interaction of each representative of NF-κBs with its inhibitor IκB and binding to DNA. The heterogeneity of the NF-κBs family allows the cell to subtly regulate the expression of target genes. In the cytoplasm, NF-κBs are in an inactive state, which is due to the binding of Rel proteins with inhibitory proteins belonging to the IκBs family (NF-κBs inhibitors). Usually, inactive NF-κB is associated with one of seven inhibitors: IκBα, IκBβ, IκBε, IκBγ, Bcl3, p105, and p100. Each member of the IκBs family has its own specificity for a particular representative of the NF-κB family. The combination of NF-κB with IκB forms an IKK (inhibitor kinase complex NFκB). The activation of the IKK kinase family is necessary for the release (activation) of NF-κB: IKK phosphorylates IκB. NF-κB released due to degradation of IκB is translocated to the nucleus. By entering the nucleus, NF-κB stimulates the expression of its IκB. Then, this newly synthesized (expressed) IκB molecule translocates into the nucleus and prevents further interaction of NF-κB with regulatory DNA sites. In other words, there is a negative feedback loop that restricts the activity of NF-κB by the mechanism of autoregulation.

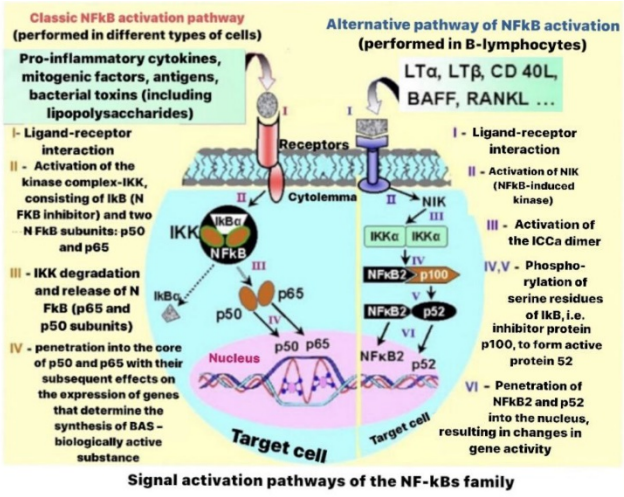
It can be assumed that SARS-Cov-2 violates the interaction of NF-κB with IκB. It inhibits the expression of IκB and thus does not prevent further interaction of NF-κB with regulatory DNA sites. This leads to the uncontrolled formation of pro-inflammatory cytokines.

As it turned out, the functional state of NF-κB can be controlled not only by IκBs, but also by other factors. Various ways of activation and suppression of excessive stimulation of NF-κBs have been established. There are two main variants of sequential events that lead to NF-κB activation: the classic ("canonical") and alternative ("non-canonical") pathways.

The classical signaling pathway is initiated by a large set of different extracellular signals: the pathogen-associated molecular structures that implement their effects with the help of TLR on epithelial cells, macrophages, neutrophils and pro-inflammatory cytokines: TNF-α, IL-1β, and many other factors.

An alternative signaling pathway is induced by LT-α, LT-β, CD40L (differentiation cluster ligand 40), BAFF (factor activating B – lymphocytes), RANKL (RANKL ligand activating NF-κB receptors), and apparently other factors (Figure 7).

Figure 7. Signal activation pathways of the NF - kBs family



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These two pathways of the signal transmission from receptors to DNA differ not only in the set of inducing factors, but also in the mechanisms of degradation of members of the IκBs family. Probably, this degradation can be enhanced by SARS-CoV-2.

ROLES OF CYTOKINES IN COVID-19 INFECTION

COVID-19 disease is accompanied by an extremely high level of production of pro-inflammatory cytokines (IFN-α, IFN-γ, IL-1β, IL-6, IL-12, IL-18, IL-33, TNF-α, GM-CSF) and chemokines (CCL2, CCL3, CCL5, CXCL8, CXCL9, CXCL10) (38-41). Such increase in the production of pro-inflammatory cytokines and cytokine reaction observed in SARS-CoV-2 infected patients were called "cytokine storm". These cytokines and chemokines recruit effector immunocytes, causing the development of a local inflammatory response. The characteristic feature of severe forms of disease is a decrease in IL-10 production. The "cytokine storm" is the basis for the development of ARDS and multiple organ failure, which in severe cases of SARS-CoV infection lead to death (42-44). Severe COVID-19 infection is accompanied by significantly higher serum levels of cytokines such as IL-1β, IL-6, TNF-α and CXCL8. It was found that the risk of death due to the disease is associated with a high level of IL-6 in the blood (45-47). It is assumed that the virus begins the second attack, causing the patient's condition to deteriorate about 7-14 days after the onset of the disease. From the appearance of the first symptoms of COVID-19 infection to the development of ARDS it takes about 8 days on average (48).

Ingress of SARS-Cov2 and cytokines into the blood vessels of the lungs → increased vascular permeability → exit of fluid into the lung tissue → non-cardiogenic pulmonary oedema → penetration of fluid (exudate) into the alveoli → acute respiratory failure

Lung damage is the main cause of both the severity of the course and the fatal outcomes of COVID-19 infection (44). Violations of lung perfusion that occur at the beginning of ARDS development lead to an increase in the permeability of alveolo-capillary membranes due to their hypoxic alterations. This factor, along with a deficiency of surfactant, causes the development of interstitial oedema of the lung tissue, and then the accumulation of fluid in the alveolar space. In addition, after penetration of the SARS-CoV-2 virus into the human body, the production of ACE2 protein is inhibited, which leads to a decrease in the level of ACE2 protein representation, especially in the lung tissues. The imbalance of ACE2 and ACE causes an increase in the concentration of Ang II, which over-activates AT1a receptors in the lungs, leading to an increase in the capillary permeability and development of pulmonary oedema (49,50). Subsequently, in the late period of disease development, an extremely high level of production of pro-inflammatory cytokines (IL-6, IL-1β, TNF-α, etc.) by these cells provides an influx of a large number of monocytes and neutrophils, which increase the phenomena of inflammation and contribute to the development of pulmonary oedema in patients with COVID-19. IL-1β and TNF-α induce the activity of hyaluronan synthase 2 (HAS2) in endothelial CD31+ cells, alveolar epithelial EpCAM+ cells of the lungs and fibroblasts, which leads to an excess of hyaluronic acid production and fluid accumulation in the alveolar space (51). The overexpression of hyaluronan plays a key role in the development of inflammation and oedema (52).

Virus (SARS-Cov2) → activation of macrophages and neutrophils → development of oxidative stress → increase in NF-κB activity → increase in cytokine synthesis → ROS and cytokines → damage to lung tissue → pneumonia → acute respiratory failure

OXIDATIVE STRESS IN COVID-19 INFECTION

There is a clear correlation between the markers of oxidative stress and severity of many viral diseases, such as hepatitis C (HCV), but there is very little clinical data for SARS-CoV. However, the results obtained on preclinical material indicate that the ROS generation increases and the antioxidant protection decreases, which plays a major role in the pathogenesis of SARS-CoV, as well as in the progression and severity of this respiratory disease. An experimental model on animals with the severe acute respiratory syndrome revealed the increased levels of ROS and reduced the antioxidant protection under the influence of SARS-CoV (53). Some authors suggest that the onset of severe lung damage in patients infected with SARS-CoV depends on the activation of the mechanism of oxidative stress, which is associated with the innate immunity, and activates transcription factors such as NF-κB, which leads to the inflammatory response in the body (54). Lin and colleagues (55) have shown that SARS-CoV 3CLpro (viral protease) causes a significant increase in ROS production in HL-CZ cells, which in turn is associated with apoptosis caused by 3CLpro. In this study, the authors demonstrated that SARS-CoV 3CLpro activates the NF-κB-dependent reporter gene, which correlated with an increase in ROS levels in HL-CZ cells. NF-κB sites exist in promoters of apoptosis-related genes and pro-inflammatory genes. Thus, the authors suggest that the NF-κB ROS-activated signal pathway induced by SARS-CoV 3CLpro may be considered a key player in the pathogenesis of SARS-CoV infection. In addition, another SARS-CoV protease, protein 3a, has been associated with the activation of mitochondrial cell death pathways. The proposed mechanism involves oligomerization of Bax and higher levels of p53 in 3a-expressing protein Huh7 cells, which depends on the activation of p38 mitogen-activated protein kinase (MAPK) in these cells (56). The activated (phosphorylated) forms of all MAPK members were found in the cells infected with SARS-CoV (57).

The oxidative stress-NF-κB-TLR (mainly TLR4) signaling pathways triggered by the viral pathogens such as SARS-CoV can further enhance the host's inflammatory response, ultimately leading to the acute lung damage. TLR4-TRIF-

TRAF6 signaling has been identified as a pathogenic pathway that can mediate the severity of acute lung injury. Oxidized phospholipid produced by the lung macrophages can cause a large cytokine formation and lung damage with TLR4-TRIF. Oxidized phospholipids were previously identified in the lungs of humans and animals infected with the SARS virus. In *in vivo* models, the loss of TLR4 or TRIF expression protected mice from the acute lung damage caused by H5N1. In addition, deletion of Neutrophil Cytosolic Factor 1 (NCF1), which can regulate ROS generation, improves the degree of acute lung damage. Thus, these authors suggest that oxidative stress and innate immunity play a key role in the severity of acute lung damage caused by respiratory viruses (58).

Shao and his colleagues (59) followed the increased regulation of mitochondrial genes and genes that respond to oxidative stress mononuclear cells in the peripheral blood of patients with convalescent SARS-CoV. Some of these genes, including PRDX1, FTH1, and FOS, which are sensitive to oxidative stress, were significantly elevated. These results confirm the relationship between oxidative stress, inflammation, and pathogenesis of SARS-CoV infection.

The severity and risk of death from SARS-CoV-2 or COVID-19 disease were age-related (60). It is well known that the reduction of antioxidant protection and the intensification of free radical processes occur in the aging process (61,62). It has been shown that older mice compared to young adult mice have, with pro-inflammatory cytokines, more severe lung lesions caused by SARS-CoV. It is assumed that the age-related accumulation of free radicals and reduced antioxidant protection cause a violation of the redox balance, which leads to the increased lung damage. Therefore, aging is associated not only with changes in the adaptive immune response, but also with the pro-inflammatory status. The aged macaques monkeys had a stronger body response to a viral infection than young adult macaques, with an increase in differential expression of inflammation-related genes, with NF-κB as a key factor (63).

Virus (SARS-Cov2) and cytokines → damage to endothelial cells of blood vessel (SARS-Cov2 is similar to damage of bronchial epithelial cells but cytokines are directly affected) → endothelial dysfunction → formation of endothelial cells and release into the blood a large number of procoagulants and proagregants → massive thrombosis → blockage of small vessels of the lungs, kidneys, heart, liver, and other organs with blood clots → violation of microcirculation in these organs → violation of their function → development of shock → multiple organ failure

VIRAL DISRUPTION OF ENDOTHELIAL FUNCTIONS

COVID-19 disrupts the function of the endothelium (64). In a patient with COVID-19, it is common to find the arterial hypertension (65-69), thrombosis (70-72), kidney illnesses (73,74), emboli in the lungs (75,76), cerebrovascular and neurological disorders (77,78) which proves that the virus disrupts the function of the endothelium (79), on the most important organs in the human body (80,81). Furthermore, cases of Kawasaki illness in children with COVID-19 with developed vasculitis (82), only prove this point of view.

The endothelium is not only the main structural component of intima, which acts as a barrier between the blood and the basal membrane of the vascular wall, but also an active regulator of many vital processes (83). The variety of targeted effects of the "hormonal response" of endothelial cells is based on their ability to synthesize a wide range of biologically active substances that are, for the most part, functional antagonists. The set of these substances includes vasoconstrictors and vasodilators, proaggregant and antiplatelet agents, procoagulants and anticoagulants, mitogens and antimutagenic (84). The "hormonal" activity of the intact endothelium promotes vasodilation, prevents hemocoagulation and thrombosis, and limits the proliferative potential of the vascular wall cells (85). On the other hand, in the case of alterations, such as pathogenetically significant changes in the endothelium, its "hormonal" response, on the contrary, contributes to vasoconstriction, hemocoagulation, thrombosis, proliferative process (86).

Generally, it is possible to distinguish 3 main groups of factors that initiate the "hormonal response" of the endothelium:

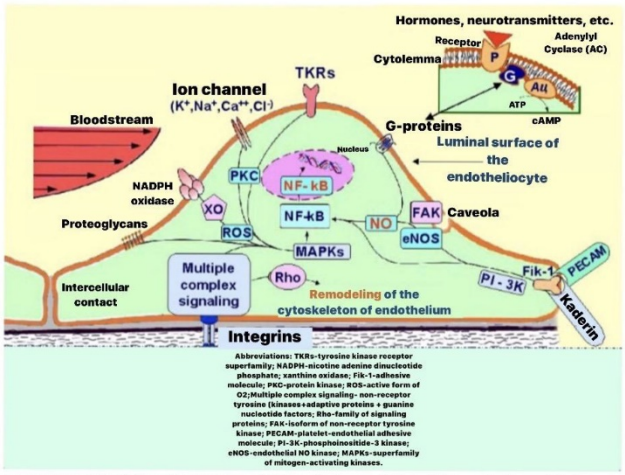
a. Hemodynamic factor - the influence of this factor on the functional activity of the endothelium depends on the speed of blood flow, its nature, and the values of blood pressure that cause the development of the so-called "shear stress" (87).
b. "Cellular" (locally formed) biologically active substances, having an autocrine or paracrine type of action. These include degranulation and lysis factors ("release reactions") of adhered and aggregated platelets (thromboplastin, fibrinogen, von Willebrand factor, platelet growth factor, fibronectin, serotonin, ADP, acid hydrolases), products of marginal, wall-mounted leukocytes, primarily neutrophils (adhesive molecules, lysosomal proteases, ROS, leukotrienes, prostaglandins E, etc.), activated mast cells (histamine, serotonin, leukotrienes C4 and D4, platelet activation factor, heparin, proteolytic enzymes, chemotactic and other factors) (88-91).
c. Circulating (distant-formed) biologically active substances that have an endocrine type of action. These include catecholamines, vasopressin, acetylcholine, bradykinin, adenosine, histamine, and many others (92).

The action of mediators and neurohormones is carried out through specific receptors located on the surface of endothelial cells. A number of substances are able to act on endothelial cells bypassing the receptors, directly through the cell membrane.

ACE2 receptors are also expressed by endothelial cells (93,94). It should be noted that all factors involved in the penetration of SARS-CoV-2 into the cell such as sialic acid, transmembrane serine protease 2 (TMPRSS2), inducer of extracellular matrix metalloproteinase (CD147) and cathepsins B and L are also expressed in endothelial cells (95-100).

It can be assumed that SARS-CoV2 entering the endothelial cell causes the activation of NF-κB(directly or through the increased amount of ROS caused by it). In addition, cytokines have a damaging and stressful effect on the endothelial cell, and it is perceived by mechanosensors located on its luminal surface. These include ion channels (K^+, $Ca2^+$, Na^+, Cl^-), caveoles, NADPH oxidases, cell membrane proteoglycans and G-proteins. G-proteins are not only mechanosensors, but, first of all, universal intermediaries in the transmission of hormonal and neurotransmitter signals from the cell membrane receptors to effector proteins that cause targeted responses. In addition, stress signals can be transmitted through the mechanosensory complex, including PECAM and Flk-1, as well as - mediated by the adhesive molecules - integrins and cadherins. The activated sensors transmit signals further along various intracellular pathways ("signal cascades") (Figure 8).

Figure 8. Main endothelial mechanosensors of stress factors and intracellular mechanisms of their implementation



The main endothelial mechanosensors of stress factors and intracellular mechanisms of their implementation.

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In recent years, there has been an increased interest in the problem of stress signal transmission, since there is evidence of the existence of specialized intracellular stress-activated signaling pathways, which include the stress-activated protein kinases. Such enzymes can be activated by a variety of factors, including oxidative stress, UV radiation, osmotic stress, integrins, TNF-α, and others. Particularly important in the transmission of stress signals are SAPK (stress-activated protein kinases) of the MAPKs, namely: JNK-N - terminal kinases (JNKs) and p38 kinase. Stimulated integrins, and many other factors exercise their influence through this multi-component signaling complex. MAPKs performs regulatory phosphorylation of the nuclear transcription factors, cell cytoskeleton proteins, and signal transfer proteins at the last stages of this process. Thus, the activity of NF-κBincreases sharply (87). In the final accounts , theendothelial cell begins to produce a large number of pro-aggregant and vasoconstrictors, which leads to higher mortality rates (71,100,101).

Patients with the fatal outcomes were found to have significantly higher levels of D-dimer and fibrin degradation product and a longer prothrombin time compared to those who survived the admission (102). Moreover, a significant decrease in the fibrinogen levels and antitrobin activity was observed in patients with the fatal outcome at the late stages of hospitalization, which is compatible with the clinical diagnosis of disseminated intravascular coagulation (DIC-syndrome) (103). It should be noted that when DIC syndrome is caused by a systemic infection, it is characterized by an acute systemic excessive inflammatory response closely related to the endothelial dysfunction (104).

The endothelial dysfunction induced by SARS-CoV2 leads to massive thrombosis and blockage of small vessels of the lungs, kidneys, heart, liver, and other organs with microthrombs, which causes a violation of microcirculation in these organs and, accordingly, a violation of their function. Microthrombosis of the pulmonary microvessels leads to the impaired lung perfusion. Indeed, the pulmonary endothelium is a fundamental barrier between the blood and interstitial tissue and performs vital regulatory functions. In particular, endothelial cells make up one-third of the lung cell population (105), but the violation of pulmonary endothelium even increases the severity of influenza-like illness (ILI) (106). COVID-19 patients with massive microthrombosis and impaired microcirculation experience a state of shock with multiple organ failure, which can ultimately lead to death.

Virus (SARS-Cov2) and cytokines → damage to endotheliocytes of blood vessels → endothelial dysfunction → formation of endothelial cells and release into the blood of a large number of procoagulants and proagregants → massive thrombosis → blockage of large vessels of the brain (development of strokes), heart (development of heart attacks), lower extremities (development of gangrene of the lower extremities) → multiple organ failure

The endothelial dysfunction can be an independent cause of circulatory disorders in the organ, since it often provokes angiospasm or vascular thrombosis (108,109), which in particular, is observed in some forms of ischemia. Deep vein thrombosis and/or pulmonary embolism have previously been described in patients with ILI (110-114) as well as the cases of thrombosis in patients with influenza associated with pneumonia (115-117).

When the endothelium is damaged in patients with COVID-19, its surface turns from antithrombotic to prothrombotic. In the case of exposure of the proadgesive surface of the subendothelial matrix, its components-adhesive proteins (von Willebrand factor, collagen, fibronectin, thrombospondin, fibrinogen, etc.) are immediately included in the formation of the primary (vascular-platelet) thrombus and consequently hypercoagulation. The endothelium is able to produce and secrete a number of substances, such as catecholamines, endotheliin-1, Ang-2 that have a vasoconstrictive effect. Ang-2 increases the permeability of microvessels (118,119), induces the transcription of tissue factor in endothelial cells (120-122) and activates thrombocytes (123-125). Moreover, Ang-2 can induce the outgo of few components of the complement system in endothelial cells (126-129) which additionally proves the main role of endothelium in the pathogenesis of venous and arterial thrombosis in patients with COVID-19 (130,131). Thus, patients with COVID 19 may be complicated by stroke, heart attack or thrombosis of the lower limb arteries with the development of multiple organ failure.

Virus (SARS-Cov2) → interacts with Toll receptors of the body's cells → the formation of a huge number of cytokines (cytokine "storm") → cytokine sepsis → multiple organ failure

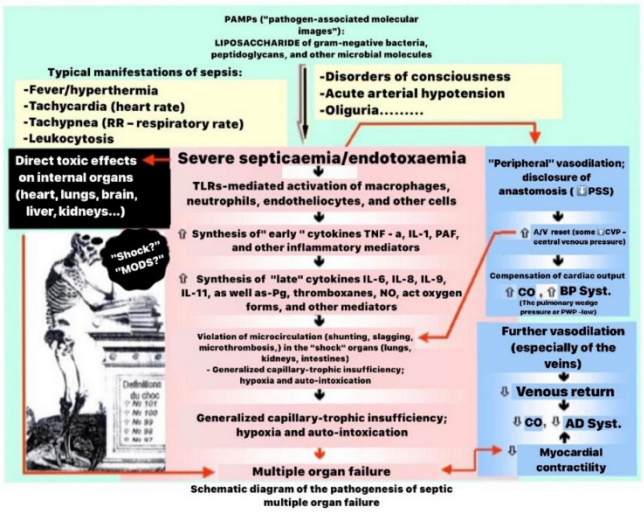
MULTIPLE ORGAN DYSFUNCTION SYNDROME (MODS) INDUCED BY COVID-19

In general terms, the pattern of sepsis-induced MODS development in COVID-19 is shown in Figure 9. When SARS-CoV-2 enters the body, it is recognized by a family TLRs that trigger the production of cytokines. The key cytokines that mediate the septic form of MODS are TNF-α, IL-1 and IL-6, whose massive release from monocytes, macrophages, neutrophils and other cells is provoked by SARS CoV-2. The isolated introduction of at least one of these cytokines into the body without the participation of any microorganisms gives a complete clinical picture characteristic of sepsis. It is noteworthy that by binding or blocking the excess TNF-α and IL-1, for example, with specific antibodies, it is possible to remove most of the phenomena of acute infectious damage and prevent the development of septic shock. Cytokines mediate their action by activating NF-κB. It was noted above that currently NF-κB is considered as the main transcription factor for genes that determine the development of a wide range of diseases of an immune and inflammatory nature, gerontological diseases, tumors, viral infections. Subsequent to the activation of NF-κB and other transcription factors, changes in the genetic program determine the stimulation of synthesis of "early" cytokines, and then "late" cytokines and other mediators of the systemic inflammatory response. Mediators cause various metabolic and functional changes in the body, manifesting the development of septic, as well as other, i.e. aseptic forms of MODS. In the genesis of acute vascular insufficiency - one of the main pathogenetic components of this syndrome, a special role is assigned to nitrogen oxide, the concentration of which can increase tenfold as a result of stimulation of macrophages TNF-α, IL-1 and other factors. It was found that the introduction of NO to experimental animals can lead to a condition that simulates sepsis. One of the main pathogenetic components of this form of pathology is a violation of microcirculation, which first leads to the development of systemic capillary-trophic insufficiency, and then, to a large extent, determines the formation of MODS.

First of all, the "shock wave" of inflammatory mediators takes over the lungs – the "biochemical filter" of blood on the way to the brain, which leads to their damage or, according to modern nomenclature, to the development of acute lung injury syndrome, which is considered as a "typical pacemaker" of MODS.

The complex of factors that makes up the pathogenetic basis of various clinical symptoms of MODS includes the acute respiratory failure (generalized hypoxia), microcirculation disorders (capillary-trophic insufficiency), endothelial dysfunction (violation of the regulation of the vascular lumen and haemostasis system), enteral insufficiency (intestinal auto-intoxication syndrome, malabsorption syndrome), changes in the metabolism (hypermetabolism syndrome, autocatabolism syndrome), encephalopathy (disorders of CNS function).

Figure 9. Pathogenesis of septic multiple organ failure



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SARS-CoV-2 can cause a local organ/tissue damage or have a generalized damaging effect on the body. In the first case, the inflammation occurs – a "locally flowing" process, which can be adequate, classical: SARS-CoV-2 and altered cells/tissues are blocked, inactivated and removed from the body, which excludes the possibility of generalization of the process, or - inadequate, i.e. with severe tissue damage, insufficient restrictive function of inflammation, excessive formation and massive output of mediators of this process in the

systemic bloodstream. "Flooding" of the blood with various biologically active substances occurs not only in conditions of the inadequate inflammation, but also accompanies more or less pronounced generalized damage to the body. Based on the data on the existence of mediators between damaging factors and phenotypic changes caused by them, the body develops a "systemic inflammatory response syndrome" – SIRS (the result of the action of pro - inflammatory mediators) and a "compensatory systemic anti-inflammatory response" – CARS, as the result of the action of anti-inflammatory mediators.

The excessive content of biologically active substances in the blood is the cause of severe lung damage. This is due to the fact that along with providing the gas exchange, the lungs perform a number of non-respiratory (including metabolic) functions. Quite intensive lung metabolism is primarily due to the neural secretory activity of apudocytes (approximately 40 types of APUD cells were found in the lungs). It should be noted that the metabolic function of the lungs largely depends on the state of the endothelial cells of the small circulatory vessels. Biologically active substances synthesized by apudocytes of a peptide nature affect the vascular tone and permeability of vascular walls, heart function, activity of the gastrointestinal tract, metabolism, excitability of membranes, etc. In the lungs, there is not only synthesis, but destruction (inactivation) of a number of substances, including-norepinephrine, angiotensin-1, etc. This "cleansing" ("barrier") function of the lungs turns them into a kind of "biochemical filter" that protects various organs, primarily the brain from the excessive amount of biologically active substances that have entered the bloodstream. That is why the lungs received the status of "shock organ". In the context of MODS development, the lungs are under the "pressure" of a large number of SIRS and CARS mediators. In this case, the cells of the lung tissue occupy the first line of defence, taking on the "impact of biochemical aggression factors", which leads to a disorder of their metabolic activity, characterized, in particular, by the development of surfactant deficiency and, accordingly, the acute respiratory failure. The resulting hypoxia of various organs and tissues determines the generalized de novo synthesis of various biologically active substances and their release into the systemic bloodstream. This second "humoral wave" of active molecules, along with the neuro–endocrine response to damaging effects, causes further systemic changes in the body, which can result in the formation of MODS.

Mediators of the humoral component of the systemic response in the conditions of MODS development (their total number is in the hundreds) are: cytokines, components of the complement system, products of arachidonic acid metabolism, platelet activation factor, histamine, cellular adhesive molecules, toxic oxygen metabolites, components of the kallikrein - kinin system, and many others.

The greatest attention in the aspect of MODS pathogenesis is paid to cytokines. Strictly speaking, the pathogenetic basis of MODS is not a systemic inflammatory reaction, but an imbalance between the pro and anti-inflammatory capabilities of the cytokines: both the predominance of pro-inflammatory and anti-inflammatory potential equally determine the development of MODS. The balance of these capabilities is determined not only by the content of pro and anti-inflammatory cytokines, but also by other factors mentioned above:

a. the number of membrane-bound cytokine receptors, which is determined by the balance between their biosynthesis and use;
b. the quality of these receptors (they can be inactivated by ligand-cytokine antagonists or antireceptor antibodies);
c. the number of soluble receptors ("trap receptors") which naturally increases in conditions of the increased proteolytic activity of the blood.

In addition, the effects of cytokines depend on the reactivity of target cells: genetic defects in the synthesis of biologically active molecules in them in the presence of a cytokine signal.

One of the characteristics of MODS is the phase of development of this syndrome. Despite the absence of a single, agreed point of view on the pathogenesis of MODS, the above-mentioned concept of a "three-phase response" seems quite convincing from a pathophysiological point of view.

In fact, the initiation of MODS is associated with the natural development of the system's protective and adaptive response, which consists of two components: neuroendocrine (stress response) and humoral (mainly "cytokine" response). From the pathophysiological point of view, the selection of such stage does not cause any doubt, as well as its perfectly adequate name – "phase of induction". This system response induces the inclusion of adaptive mechanisms aimed at mobilization, redistribution, and adequate use of energy and plastic resources in order to contain the scale of alterations, creating unfavourable conditions for various pathogenic infectious factors. The pathogenetic basis of such mechanisms consists of systemic changes in the metabolism. Therefore, the next stage of MODS development is called the "metabolic response phase". At this stage, there are various changes of a functional nature, which, on the one hand, are aimed at providing the metabolic response, and, on the other, these changes are not the "central pathogenetic event" of this phase of MODS. An exception to this statement are changes (functional and organic) in the gastrointestinal tract that occur in connection with the refusal of the body from the services of the digestive system due to the transition to more accessible endogenous food reserves. The resulting damage to the gastrointestinal tract determines the development of malabsorption syndrome and intestinal auto-intoxication syndrome, which cause further development and severity of MODS. This final stage of the formation of this syndrome is called the "phase of secondary autoagression", because it is determined not so much by etiological as pathogenetic factors of MODS. This is, in its most general form, the three-phase

concept of MODS pathogenesis which occurs under the influence of SARS-CoV-2.

CONCLUSION

In general, the introduction of SARS-CoV-2 into epithelial cells (skin, lungs, trachea, bronchi, stomach, intestines, etc.), blood, and endothelium of the body causes a local and systematic inflammatory response to damage at the first stage. The key component of this phase (induction) of the response is "cytokine storm" or more precisely – cytokine imbalance.

The pathogenetic basis of this phase of SARS-Cov2 infection is a disruption of the cellular, organ tissue and system adaptive reactions of the body. The next stage is developing a related network of events (each patient individually) which results in the failure of the body systems (respiratory, blood circulation, hemostasis, endocrine and neural), with development of different periodical complications and subsequent insufficiency of the circulatory, respiratory systems (ARDS, ALI), hemostasis (DIC), digestion (malabsorption syndrome), endocrine and nervous (shock, coma) problems resulting in the MODS.

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ACUTE KIDNEY DAMAGE IN PREGNANCY: ETIOPATHOGENESIS, DIAGNOSTICS AND BASIC PRINCIPLES OF TREATMENT

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AKUTNO OŠTEĆENJE BUBREGA U TRUDNOĆI: ETIOPATOGENEZA, DIJAGNOSTIKA I OSNOVNI PRINCIPI LEČENJA

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ABSTRACT

Acute kidney damage associated with pregnancy occurs in 1/20.000 pregnancies. In developing countries, the main cause of the development of acute kidney damage is septic abortion, and preeclampsia in the developed countries of the world. Preeclampsia is defined as newly developed hypertension, proteinuria and swelling in pregnant women after the 20th week of gestation. It occurs due to disorders in the development of placenta and systemic disorders of the function of the endothelium of the mother. It is treated with methyldopa, magnesium sulfate and timely delivery. Urgent delivery is indicated if the age of gestation is ≥ 34 weeks. HELLP syndrome is a difficult form of preeclampsia. Its main characteristics are decreased platelet count, microangiopathic hemolysis anemia, increased concentration of aminotransferase in the serum and acute kidney damage. Severe HELLP syndrome is treated with emergency delivery, antihypertensives, magnesium sulfate, and in some cases plasmapheresis and hemodialysis. Acute fatty liver in pregnancy occurs because of decreased activity of the LCHAD enzyme of the fetus. Due to the reduced beta oxidation of fatty acids in the hepatocytes of the fetus, long chain fatty acids that cause damage to the mother's hepatocytes are released. Swansea criteria are used for diagnosis, and the difficult form of the disease is treated with plasmapheresis and extracorporeal liver support. Atypical HUS is due to a reduced protein activity that regulates the activity of the alternative pathway of the complement system. Its main features are thrombocytopenia, microangiopathic hemolytic anemia and acute kidney damage. It is treated with plasmapheresis, and in case of resistance with eculizumab. Thrombotic thrombocytopenic purpura is due to decreased activity of the ADAMTS13 enzyme. It is characterized by thrombocytopenia, microangiopathic hemolytic anemia, high temperature, nervous system disorders and acute kidney damage. It is treated with plasmapheresis, and severe form of disease with corticosteroids and azathioprine. Early detection and timely treatment of acute kidney damage provides a good outcome for the mother and fetus.

Keywords: *acute kidneydamage, pregnancy, preeclampsia, eclampsia, HELLP syndrome, thrombotic microangiopathies*

SAŽETAK

Akutno oštećenje bubrega povezano sa trudnoćom javlja se kod 1/20.000 trudnoća. U zemljama u razvoju, glavni uzrok razvoja akutnog oštećenja bubrega je septički abortus, a u razvijenim zemljama sveta preeklampsija. Preeklampsija se definiše kao novonastala hipertenzija, proteinurija i otoci kod trudnica posle 20. nedelje gestacije. Nastaje zbog poremećaja u razvoju placente i sistemskog poremećaja funkcije endotela majke. Leči se metildopom, magnezijum sulfatom i pravovremenim porođajem. Hitan porođaj je indikovan ukoliko je starost gestacije ≥ 34 nedelje. HELLP sindrom je težak oblik preeklampsije. Njegove glavne karakteristike su smanjen broj trombocita, mikroangiopatska hemolizna anemija, povećana koncentracija aminotransfeaza u serumu i akutno oštećenje bubrega.Težak HELLP sindrom leči se hitnim porođajem, antihipertenzivima, magnezijum sulfatom, a u pojedinim slučajevima plazmaferezom i hemodijalizom. Akutna masna jetra u trudnoći nastaje zbog smanjene aktivnosti enzima LCHAD ploda. Zbog smanjene beta oksidacije masnih kiselina u hepatocitima ploda, oslobađaju se masne kiseline dugih lanaca koje izazivaju oštećenje hepatocita majke. Za dijagnostikovanje se koriste Swansea kriterijumi, a težak oblik bolesti leči se plazmaferezom i vantelesnom jetrinom potporom. Atipični HUS nastaje zbog smanjene aktivnosti proteina koji regulišu aktivnost alternativnog puta sistema komplementa. Njegove glavne karakteristike su trombocitopenija, mikroangiopatska hemolizna anemija i akutno oštećenje bubrega. Leči se plazmaferezom, a u slučaju rezistencije ekulizumabom.Trombotična trombocitopenijska purpura nastaje zbog smanjene aktivnosti enzima ADAMTS13. Karakteriše se trombocitopenijom, mikroangiopatskom hemoliznom anemijom, visokom temperaturom, poremećajima nervnog sistema i akutnim oštećenjem bubrega. Leči se plazmaferezom, a teže bolesti kortikosteroidima i azatioprinom. Rano otkrivanje i pravovremeno lečenje akutnog oštećenja bubrega obezbeđujedobar ishod i majke i ploda.

Ključne reči: *akutno oštećenje bubrega, trudnoća, preeklampsija, eklapmsija, HELLP sindrom, trombotične mikroangiopatije*

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INTRODUCTION

Acute kidney related damage associated with pregnancy - PR-AKI is a significant cause of morbidity and mortality of mother and fetus (1, 2). In the last decade, the incidence of acute kidney damage associated with pregnancy is reduced and currently stands at 1/20.000 pregnancies. This reduction, especially in developing countries, is due to the legalization and reduction of the incidence of septic abortion, better equipment of obstetric units and better monitoring of pregnant women and fetuses (1, 2). In developing countries, the major cause of the development of acute kidney damage associated with pregnancy is sepsis, and preeclampsia in the developed countries of the world (1, 2).

Definition of acute kidney damage associated with pregnancy

The definition and classification of acute kidney damage in pregnancy is not entirely clear, due to open questions, such as: the optimal method for measuring the strength of glomerular filtration and adaptive changes in the kidney during pregnancy. The strength of glomerular filtration in pregnancy is significantly increased (approximately 50%), resulting in a low basal concentration of creatinin in serum, compared to healthy non-pregnant women (1, 2). New classifications for acute kidney damage, such as RIFLE (Risk, Injury, Failure, Loss, and End-stage kidney disease criteria - RIFLE), AKIN (Acute Kidney Injury Network - AKIN) and KDIGO (Kidney Disease Improving Global Outcomes) have not been validated in the pregnant population (1, 2). Acute kidney damage associated with pregnancy is defined as a serum creatinine concentration greater than 71 μmol/l (normal serum creatinine concentration in the third trimester of pregnancy is 62-71 μmol/l) in pregnant women in the absence of clinical data for chronic kidney disease, (1, 2). Any increase in the serum creatinine concentration of 8.8 μmol/l (0.1 mg/dl) in pregnant women can indicate the development of acute kidney damage (1, 2).

Etiopathogenesis of acute kidney damage associated with pregnancy

Depending on the etiology, we can distinguish three types of acute kidney damage associated with pregnancy: prerenal, renal, and postrenal type (1, 2). Pregnant type of acute kidney damage occurs most often in the first trimester of pregnancy and is caused by kidney hypoperfusion of the kidney: excessive vomiting (hyperemesis gravidarum), severe bleeding (separation of placenta, abortion) and septic shock (abortion complicated by sepsis) (1, 2). Renal type of acute kidney damage most commonly occurs in the third trimester of pregnancy as a result of preeclampsia, HELLP syndrome (Hemolysis, Elevated Liver enzymes and Low Platelet count - HELLP), Acute Fatty Liver in Pregnancy - AFLP and thrombotic microangiopathy - aHUS/TTP (Atypical HemolyticUremic Syndrome aHUS/Thrombotic Thrombocytopenic Purpura - TTP) (1, 2). Postrenal type of acute kidney damage occurs due to obstruction of the urinary tract: hydronephrosis due to pressure of the enlarged uterus and damage to the ureter or bladder during the caesarean section (1, 2).

Preeclampsia and eclampsia

Preeclampsia is defined as newly emerging hypertension, proteinuria and swelling in pregnant women after the 20th week of gestation (3-7). Hypertension was defined as arterial blood pressure ≥ 140/90 mmHg (systolic arterial blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg), in at least two measurements with the interval of at least 4-6h, after 20th week of gestation in women who previously had normal arterial blood pressure (3-7). Proteinuria is defined as secretion of proteins through urine in an amount of ≥ 300 mg/24h (3-7). Severe pre-eclampsia is defined as one or more ancillary criteria: systolic blood pressure ≥ 160 mmHg or diastolic blood pressure ≥ 110 mmHg in two measurements with a gap of maximum 6 hours, proteinuria ≥ 5.0 g/24h, oliguria (urine output <500 ml/24h), severe headache, altered state of consciousness, pulmonary edema, liver damage, thrombocytopenia (<100 x 109/l), HELLP syndrome, delay in fetal growth (3-7). It occurs in 3-8% of pregnancies in the developed countries of the world, and the main risk factors for its occurrence are: first pregnancy, pregnancy with multiple fetuses, anamnestic data of pre-eclampsia during previous pregnancy, kidney disease, chronic hypertension, obesity, diabetes mellitus, systemic erythema lupus, antiphospholipid syndrome, thrombophilia, mother's age (> 35-40 years) (3-7). Basically it is a disorder of the development of the placenta (placental ischemia), disorder of balance between factors that promote vascular development(Vascular Endothelial Growth Factor - VEGF and Placental Growth Factor - PIGF) and factors that block formation of new blood vessels (soluble Fms-like tyrosine kinase 1 - sFlt-1 and soluble Endoglin - sEng) and systemic endothelial dysfunction of the mother, Scheme 1 (3-7). In preeclampsia there is a disorder in the transformation of cytotrophoblasts into endothelial cells and the disorder of their inclusion in the endothelial spiral arteries of the mother's womb. Blood vessels remain small, provide great resistance to blood flow, causing decreased perfusion of placenta and its ischemia (3-7). In conditions of ischemia, the placenta increases the releasing factors which block the formation of new blood vessels (sFlt-1/sEng), causing the systemic endothelial dysfunction of the mother (decreased production of nitric oxide and prostacyclin, the release of procoagulant proteins) and insufficiency of multipleorgan systems (kidney, heart, brain, liver) (3-7). The relationship of sFlt-1/PIGF ≥ 85 indicates preeclampsia and allows the isolation of pregnant women who have an increased risk of serious complications associated with pre-eclampsia (3-7).Treatment consists in prevention of preeclampsia and timely delivery (age of gestation, maternal condition, condition of the fetus). To prevent development of preeclampsia, low molecular weight heparin, calcium preparations (1.5 g/day) and L-arginine can be used. For the treatment of hypertension, first line medicines are methyldopa, labetalol and hydralazine (3-7). Metildopa is administered per os in a dose of 0.5-3.0 g/24h, in 2-4 divided doses. Magnesium sulfate is used to prevent eclampsia (newly tone-

clonal cramps). The magnesium sulfate impact dose is 4-6 g i.v. for 15 minutes, then, it should be continued with a maintenance dose of 1.0 g/h in the form of continuous i.v. infusion (concentration of magnesium in the serum should be 480-840 mg/l) (3-7). In pregnant women with mild preeclampsia, and the period of gestation ≥ 37 weeks a delivery is indicated (delivery is effective for optimal disease control and favorable outcome of mother and fetus). If the period of gestation is less than 37 weeks, it is necessary to monitor the pregnant woman and the fetus. Monitoring of pregnant women with mild preeclampsia includes daily monitoring of vital functions, assessment of hemodynamic status, laboratory analysis once a week: blood count, platelet count, serum creatinine concentration, serum transaminases. Fetal monitoring includes a mobility test, a biophysical profile, and a serial measurement of fetal growth every three weeks (6, 7). Among the indications for delivery of pregnant women with mild preeclampsia with less than 37 weeks of gestation and in which adequate pregnancy and fetus monitoring is applied, there are: worsening of the condition of the mother, deterioration of the fetal condition, birth pain or rupture of the placenta before birth pains and achieved gestation age (≥ 37 weeks) (6, 7). In pregnant women with severe preeclampsia, whose gestation age is less than 28 weeks, a termination of pregnancy is indicated. If the age of gestation is ≥ 34 weeks, emergency delivery is indicated. If delivery is not directly indicated (age of gestation from 28-34 weeks), there is application of magnesium sulfate (prevention of eclampsia development), corticosteroids for stimulation of lung maturation for 24-48 hours (dexamethasone 6 mg/12h i.m. (four doses of the drug) and enhanced monitoring of mother and fetus. Emergency delivery is indicated in the following cases: uncontrolled severe hypertension, development of eclampsia, development of HELLP syndrome, edema of the lungs, acute kidney damage, placental depletion, slow growth of fetus, biophysical profile ≤ 4/10 in more than two consecutive six-hour periods, reversible diastolic flow through the umbilical artery (6, 7).

HELLP syndrome

HELLP syndrome is a difficult form of preeclampsia characterized by a reduced number of platelets, microangiopathichemolytic anemia, increased serum aminotransferase concentrations, and acute kidney damage (8). This syndrome occurs in 0.5-0.9% of pregnancies, and in 10-20% of pregnant women with severe pre-eclampsia (in 70% of pregnant women it occurs between 27-37 weeks of gestation, it can occur within 48 hours after delivery) (8). It is clinically manifested by headache, pain, disgust, vomiting, and pain in the upper right quadrant of the abdomen. The most important complications of the HELLP syndrome are: disseminated intravascular coagulopathy, placental abruption, acute renal failure, pulmonary edema, hepatic subcapsular hematoma and retinal detachment (8-10). Microangiopathichemolytic anemia is importantfor diagnosis of HELLP syndrome (increased number of reticulocytes and schizocytes, decreased concentration of haptoglobin in the serum, increased concentration of lactate dehydrogenase, bilirubin and aminotransferases in the serum, and reduced platelet count). It occurs due to the passage of erythrocytes through the narrowing's, which are caused by damage to the endothelium and the deposition of fibrin on its surface in small blood vessels of various organs, including the kidney (glomerular endotheliosis) (8-10). Severe HELLP syndrome is defined as a platelet count less than 50×10^9/l, the concentration of aspartate aminotransferase - AST ≥ 70 IU/l and lactate dehydrogenase - LDH ≥ 600 IU/l (HELLP class I) (8-10). Treatment of HELLP syndrome includes application of magnesium sulfate (4.0 g i.v. bolus, and then continue to 1.0 g/h during 24 hours after birth), corticosteroids (protocol for fetal lung maturation) and urgent delivery (8-10). When HELLP syndrome is diagnosed early and promptly and vigorously treated, the majority of pregnant women improve the condition within 24-48 hours after birth: there is increasing of the number of platelets and significant decrease in concentration of liver enzymes in serum (8-10). Extremely rarely, after delivery, the condition of the pregnant woman may be exacerbated by the development of multiple organ system insufficiencies. In these pregnant women, the clinical condition is corrected by the use of plasmapheresis [category III (postpartum): individual benefit/risk assessment, gradus 2C: poor recommendations] (9-12). Plasmapheresis should be administered 24-72 hours after birth, when there was no increase in platelet count ($\geq 100 \times 10^9$/l) and no reduction of the concentration of liver enzymes (ALT), and when acute renal failure, respiratory distress syndrome or neurological disordersare developed (9-12). Plasmapheresis is applied every 24-48h, 3000-4000 ml of plasma is changed, and fresh frozen plasma is used for substitution (9-12). Through plasmapheresis, procoagulant factors that are released from the platelets and endothelial cells are removed. The criteria for termination of plasmapheresis include: platelet counts greater than 100 x 10^9/l and stable condition of pregnant women (9-12).

Acute fatty liver

Acute Fatty Liver in Pregnancy - AFLP is an emergency situation in obstetrics, which may cause the progressive and fulminant hepatic failure (13-15). The incidence of acute fatty liver in pregnancy is 1/10,000 to 20,000 pregnancies, and the onset of the disease is generally between 30 and 38 weeks of gestation (13-15). The disease occurs as a result of decreased activity of LCHAD (Long-Chain 3-Hydroxyl Coenzyme A Dehydrogenase - LCHAD) of the fetus (genetic error). This enzyme is significant for the beta oxidation of fatty acids in the fetus hepatocyte mitochondria. Due to deficiencies and reduced function of this enzyme, fatty acids with long-chain are released from hepatocytes, which by means of the circulation arrive to the liver of the pregnant women and induce damage to the hepatocytes (13-15). The disease is manifested through nausea, disgust, vomiting, pain in the upper right quadrant of the abdomen, hypoglycemia, lactic acidosis, and with progression of damage acute liver failure and hepatic encephalopathy can be developed (13-15). The diagnosis is made on the basis of „Swansea" criteria: vomiting, abdominal pain, polydipsia/polyuria, encephalopathy, ascites, or light liver on ultrasound examination, increased

bilirubin levels in the serum (> 14 μmol/l), increased concentration of uric acid in serum (> 340 μmol/l), leukocytosis (> 11 x 10^9/l), hypoglycemia (glucose concentration in serum is less than 4.0 mmol/l), increased concentrations of transaminases in serum (> 42 IU/l), increased concentration of ammonia in the serum (> 47 μmol / l), impaired renal function (serum creatinine concentration > 150 μmol /l), coagulopathia (PT > 14 s, aPTT > 34 s), microvesicular steatosis demonstrated by liver biopsy. Acute fatty liver in pregnancy is diagnosed if ≥ 6 criteria are present, and in the absence of another cause of liver damage (13-15). After delivery, a gene mutation screening is advised (the gene responsible for the synthesis of LCHAD) in neonates and pregnant women with AFLP and HELLP syndrome (13-15). Treatment consists of the optimal control of glycemia and coagulopathy (fresh frozen plasma, cryoprecipitate). Hepatic encephalopathy is treated with a hypoprotein diet and lactulose (per os). The last clinical experience indicates the benefit of plasmapheresis and MARS (*Molecular Adsorbent Recirculating Systems - MARS*) modality of extracorporeal support of liver (13-15).

Atypical haemolytic-uremic syndrome - aHUS

Pregnancy-Related atypical Hemolytic-Uremic Syndrome- P-aHUS is an emergency situation in obstetrics, which may cause progressive acute renal failure (16-19). The incidence of apregnancy-related atypical hemolytic-uremic syndrome is 1/25,000 pregnancies, and basically it is a disorder of regulation of the alternative pathway of the complement system (increased activity results in damage to the endothelial cells and the formation of clots in the small blood vessels of the kidney) (16-19). The increased activity of the alternative pathway of the complement system is caused by mutations in the genes encoding three major proteins (blockers) which regulate the activity of the alternative pathway of the complement system: factor H - Complement Factor H - CHF, factor I - Complement Factor I - CFI and Membrane cofactor protein - MCP. In addition to mutations of genes, important role in the pathogenesis of aHUS CDs have acquired autoantibodies against factor H of the complement system (anti-CFH antibodies) (16-19). aHUS associated with pregnancy generally occurs after delivery (postpartum period). Diagnosis of atypical hemolytic uremic syndrome includes: measuring the level of C3 and C4 components of complement in serum, the test for demonstrating C3 nephritic factor - C3NF, measuring the concentration of CFH, CFI, MCP in serum, analysis of gene mutation for CFH, CFI, MCP, and determination of the anti-CFH antibody titre (16-19). For the differential diagnosis of aHUS, a concentration of liver enzymes (HELLP syndrome), ADAMTS13 activity (thrombotic thrombocytopenic purpura - TTP) and tests for the diagnosis of autoimmune diseases (trigger for the development of aHUS) should be made: antinuclear antibodies - ANA and anti-dsDNA antibodies (systemic lupus erythematous), antibodies against antigens in the cytoplasm of neutrophils - ANCA (vasculitis) antiphospholipid antibodies: lupus anticoagulant (LA), and cardiolipin antibodies (aCL), anti-β2-GPI antibodies (antiphospholipid syndrome), anti-Scl-70 antibodies (Systemic sclerosis) (16-19). In patients with aHUS a first line of treatment is plasmapheresis (16-20). Through plasmapheresis, the non-functional proteins which regulate the activity of the alternative pathway of the complement system (CFH, CFI, CFB, C3) are removed, as well as autoantibodies to the CFH (anti-CFH antibodies) (16-20). Plasmapheresis should be administered within 24 hours of clinical manifestation of aHUS, daily for 3-5 days, changing to 1.0-1.5 volume of plasma (60-65 ml/kg) and for substitution is used fresh frozen plasma (16-21). During the treatment with plasmapheresis, daily are determined the number of platelets, the concentration of lactate dehydrogenase and concentration of creatinine in the serum. The aim of the plasmapheresis is to achieve remission of aHUS: normal platelet count (≥ 150 ^{x} 109/l), normal concentration of lactate dehydrogenase in the serum (< 250 IU/l) and a reduction of creatinine concentration in serum by > 25% compared to the concentration of creatinine in serum prior to plasmapheresis (16-21). In patients with aHUS who are resistant to plasmapheresis (persistent haemolysis, thrombocytopenia, absence of lactate dehydrogenase concentration reduction in serum, absence of creatinineconcentration reductionin serum after five consecutive daily plasmapheresis), a C5 complement component blocker is used (eculizumab: anti-C5 humanized monoclonal antibody) (22-26). Because of the risk of meningococcal infection (Neisseria meningitidis) is necessary to apply meningococcal vaccine at least two weeks prior to the first dose of eculizumab (US Advisory Committee and the Immunization Practices - USACIP) (in pregnant women in postpartum period) or prophylaxis for meningococcal infection (ciprocinal in postpartum period or rifampin in the partal period) in patients that require direct administration of eculizumab (22-27). The treatment consists of two phases: the initial and the maintenance phase. In the initial phase, eculizumab is administered at a dose of 900 mg, in the form of i.v. infusion for 25-45 minutes, once a week for four weeks. In the maintenance phase, eculizumab is administered at a dose of 1200 mg, in the form of i.v. infusion for 25-45 minutes, on the fifth week, and then every other week until reaching remission (22-26). Evaluation of response to therapy includes monitoring of platelet count, determination of the concentration of creatinine and the lactate dehydrogenase in serum on every two weeks. Remission is defined as the realization of the normal number of platelets, the normal concentration of lactate dehydrogenase and a normal creatinine level in the serum at least in two successive measurements, with a gap of at least four weeks. After administration of eculizumab and achieving complete remission, monitoring is required to detect signs and symptoms of aHUS for a time period of at least 12 weeks (eculizumab provides improved recovery of renal function in relation to plasmapheresis) (22-26).

Thrombotic thrombocytopenic purpura - TTP

Thrombotic thrombocytopenic purpura (TTP) is a clinical syndrome that is characterized by thrombocytopenia, microangiopathic hemolytic anemia, high body temperature, disorders of the central nervous system and acute renal insuficiency (28-30). It is caused by decreased activity of ADAMTS13 metalloproteinase, an enzyme that ctears apart

macromolecules ofvon Wilebrand's factor (vWF) secreted by endothelial cells of small blood vessels, and this results in the formation of clots (vWF + platelets), and the narrowing of lumen of the small blood vessels of the brain and kidney (28-30). Depending on the causes leading to reduced ADAMTS13 activity, we distinguish congenital (mutation of gene for ADAMTS13 synthesis) and acquired (anti-ADAMTS13 IgG antibodies) form of TTP (28-30). In congenital TTP (cTTP), ADAMTS13 activity is ≤ 5%, and for aquired TTP (sTTP) ≤ 10% (28-30). The incidence of congenital thrombotic thrombocytopenic purpura in pregnancy is 1/200,000 pregnancies. In pregnant women, thrombotic thrombocytopenic purpura is clinically represented by headache, confusion, visual impairment, proteinuria, pre-eclampsia, kidney damage, abdominal pain, embolization of the lungs and acute respiratory distress syndrome (28-30). The congenital form of TTP associated with pregnancy most commonly occurs in the postpartum period (monitoring for cTTP in the postpartum period). Treatment begins with plasmapheresis (plasmapheresis is the first line of therapy) (28-30). It is applied on a daily basis for five days, at least one plasma volume (2800 ml) is changed, and fresh plasma is used to substitute the separated plasma. In patients who are diagnosed with acute acquired form of TTP (anti-ADAMTS13 antibodies) corticosteroids are applied in addition to the plasmapheresis (1.0 mg/kg/day per os) and azathioprine (28-30). The aim of the plasmapheresis is to achieve disease remission [number of platelets > 150 ^{x} 10^9/l in the course of two consecutive days, normal or nearly normal concentration oflactate dehydrogenase in the serum (LDH < 250 IU/l), the stabilization or repair of neurological disorders]. The refractory form of TTP (resistance to plasmapheresis) is defined as the lack of response after five daily sessions of plasmapheresis (the absence of increase in the number of platelets and stabilization of the clinical condition of the patient).With pregnant women with refractory form of TTP, that are clinically unstable and have neurological symptoms, pulse doses of methylprednisolone are applied (Methylprednisolone: 1.0 g i.v. inf/day during three consecutive days), through plasmapheresis plasma volume of 1.5 is hanged and rituximab is given (i.v. inf.: 375 mg/m^2/week, for 4 weeks) (life-threatening thrombotic thrombocytopenic purpura) (28-31). In pregnant women who have been previously diagnosed with TTP plasma treatment infusions should be administered between 8-10 weeks of gestation, initially every two weeks, and frequency of infusions of fresh frozen plasma increases every week during the second and early start of the third trimester of pregnancy, or if the platelet count falls below 150 ^{x} 10^9/l or the concentration of lactate dehydrogenase increases in serum (elective use of fresh frozen plasma), combined with small doses of aspirin (75-100 mg/day) and low molecular weight heparin (28-30). Infusion of fresh frozen plasma is administered every week for six weeks after delivery (postpartum period) (prevention of development of acute episode of cTTP) (28-30). In the acquired form of TTP associated with pregnancy (positive anti-ADAMTS13 IgG antibodies), prior to conception rituximab should be applied at a dose of 375 mg/m^2/week, in 6 doses [level of anti-ADAMTS13 IgG is reduced below the normal value (normal concentrations of anti-ADAMTS13 is less than 12 IU/ml), and the ADAMTS13 activity is increased) (28-30). Twelve months after the administration of rituximab, conception (planned pregnancy) is advised (28-30). For prevention of acute episode of sTPP in pregnancy, infusion of fresh frozen plasma is applied (10 ml/kg) every other week from 8-10 weeks of gestation until childbirth (when ADAMTS13 activity drops below 10%, and normal ADAMTS13 activity is 50-100%), in combination with low doses of aspirin and heparin of low molecular weight [plasmaphresisis used in the acute phase of the disease (acute episodes cTTP/STPP), and for the prophylaxis the infusion of fresh frozen plasma is used] (28-30).

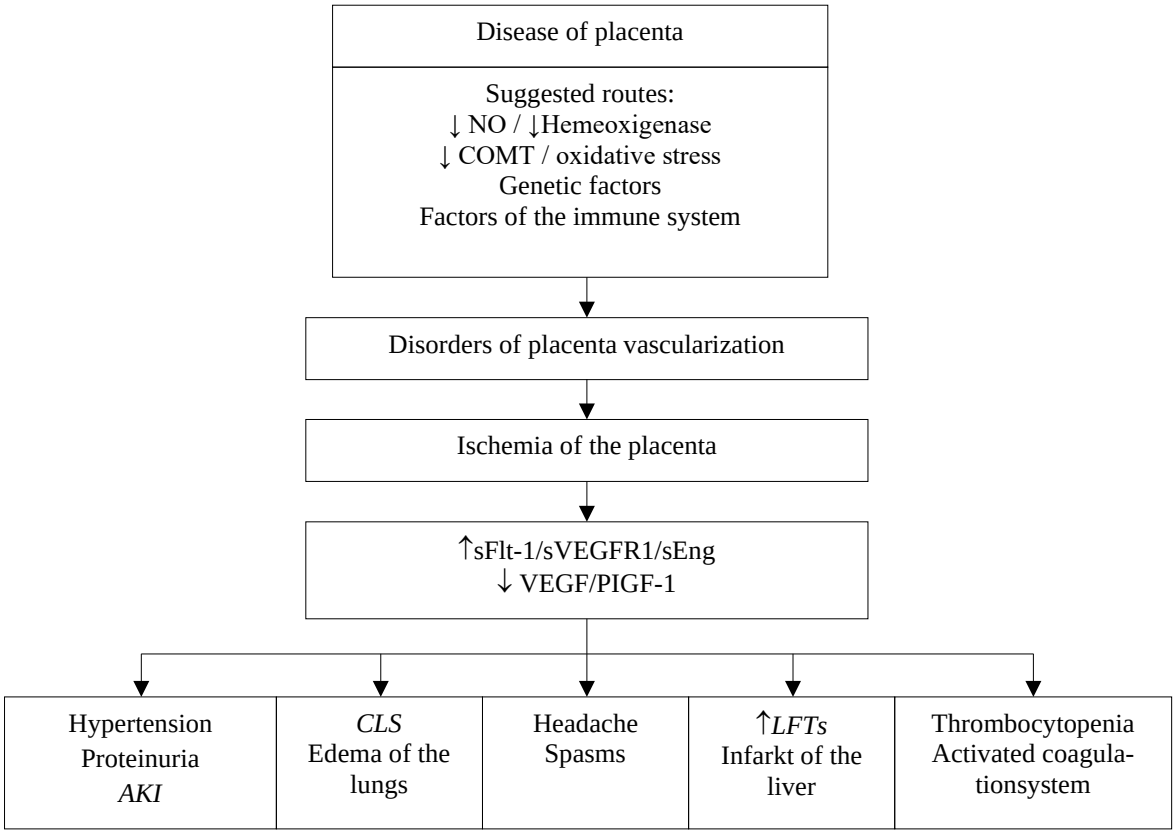
ACUTE DIALYSIS DURING PREGNANCY

Treatment of acute kidney damage associated with pregnancy consists in the elimination of the cause (volume resuscitation, urgent delivery, timely application of kidney function replacement therapy) (32-34). Acute kidney damage in pregnancy that requires treatment with dialysis occurs in 1.0 at 10.000-15.000 pregnancies. Indications for acute dialysis during pregnancy include: the presence of signs and symptoms of uremia (encephalopathy, pericarditis and neuropathy), volume overload (excess fluid in the body), hyperkalemia and metabolic acidosis that do not respond to the starting/initial therapy (32-34). Daily intermittent hemodialysis is applied (> 20 h/week), with a slight ultrafiltration (500 ml/dialysis session) in order to prevent the development of hypotension and placental hypoperfusion (net ultrafiltration for each session of hemodialysis should be ≤ 500 ml) (34). Every day hemodialysis (> 20 h/week) improves the removal of uremic toxins, provides hemodynamic stability for the pregnant woman and optimal placental perfusion. Bicarbonate solution is used for hemodialysis (the concentration of bicarbonate 25 mmol/l), with the concentration of sodium of 135 mmol/l. Unfractionated heparin is used for anticoagulation of extracorporeal circulation(that does not cross the placenta) (34). With haemodynamically unstable pregnant women in a critical condition, with acute renal failure and insufficiency of multiple organ systems, as a supportive dialysis therapy, continuous dialysis modalities are used (34).

CONCLUSION

Acute kidney damage associated with pregnancy is a factor in the risk of an adverse outcome for the mother and fetus. Early detection and timely appropriate treatment of acute kidney damage requires good equipment of obstetric units, precise monitoring of mothers and fetus, team approach, enhanced cooperation between obstetrician and nephrologist, all in order to correct the outcome for the mother and fetus during pathological pregnancy.

Sheme 1. Pathogenesis of preeclampsia



AKI - Acute Kidney Injury, CLS - Capillary Leak Syndrome, NO - Nitric Oxide, COMT - Catechol-O-methyltransferase, LFT - Liver Function Test, PIGF-1 - Placental Growth Factor-1, sEng - soluble Endoglin, sFlt-1 - soluble Fms-like tyrosine kinase-1, sVEGFR1 - soluble Vascular Endothelial Growth Factor Receptor-1 - VEGF - Vascular Endothelial Growth Factor Modified in accordance to reference [3].

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COMPARISON OF THYROGLOBULIN CONCENTRATIONS MEASURED BY TWO IMMUNORADIOMETRIC ASSAY

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KORELACIJA KONCENTRACIJA TIREOGLOBULINA ODREĐENIH KORIŠĆENJEM DVA IMUNORADIOMETRIJSKA TESTA

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ABSTRACT

Circulating thyroglobulin measurements is a highly specific test in the management of patients affected by differentiated thyroid cancer after total thyroidectomy, followed by radioiodine ablation. The aim of our study was to compare two thyroglobulinimmunoradiometric assays (INEP, Serbia and Cisbio Bioassays, France). Study included 42 patients of both genders with DTC. The subjects were on suppres¬sive doses of levothyroxine and followed up. Results showed concordance between the two assay methods for determining serum thyroglobulin for 39 (92.85%) patients. Statistical analysis showed that there was a direct correlation between two IRMA tests, with a positive correlation coefficient r=0.613 (p 0.05). We concluded that there is good agreement between the two thyroglobulin assays compared in this study.

Keywords: *differentitated thyroid carcinoma, thyroglobulin, immunoradiometric assay.*

ABSTRACT

Merenje serumskog tireoglobulina je visokospecifičan test koji se koristi u praćenju pacijenata obolelih od diferentovanih karcinoma štitaste žlezde nakon totalne tireoidektomije praćene radiojodnom ablacijom. Cilj studije je poređenje koncentracija tireoglobulina dobijenih primenom dva imunoradiometrijska dijagnostička kompleta (proizvođača INEP, Srbija i Cisbio Bioassays, Francuska). Ispitivanjem su obuhvaćena 42 pacijenta oba pola koji su nakon totalne tireoidektomije zbog diferentovanog karcinoma štitaste žlezde lečeni radiojodnom terapijom. Svi pacijenti su bili na supresivnoj terapiji levotiroksinom i redovno su kontrolisani. Izvođenje obe analize je sprovedeno u skladu sa prospektom proizvođača. Rezultati su pokazali da postoji korelacija između dva imunoradiometrijska testa za 39 (92,85%) pacijenata. Statistička analiza je pokazala da postoji korelacija između oba imunoradiometrijska testa sa pozitivnim koeficijentom korelacije r=0,613. Na osnovu rezultata može se zaključiti da postoji značajan stepen slaganja u rezultatima dobijenim primenom dva imunoradiometrijska testa.

Ključne reči: *diferentovani karcinom štitaste žlezde, tireoglobulin, imunoradiometrijski test.*

INTRODUCTION

Differentiated thyroid cancer (DTC) is the most common endocrine malignancy (1, 2). Primary method for treatment of DTC is total or near-total thyroidectomy (1, 2). Radioiodine (^{131}I) ablation after total thyroidectomy is a usual procedure in patients with DTC, which is used to ablate the thyroid tissue remnants, the residual or recurrent thyroid cancer, and/or its local or distant metastases (1, 2). Since DTC originate from follicular thyroid cells, the malignantly transformed cells retain some functional characteristics of thyrocytes, depending on the degree of differentiation. Thus, they have receptors for thyroid-stimulating hormone (TSH) and they can produce thyroglobulin (Tg) (1, 2).

After total or near total thyroidectomy, followed by radioiodine ablation serum thyroglobulin is a suitable marker for DTC following (1-4). Measurements of the serum concentration of thyroglobulin in patients with DTC serve as tumor marker, to evaluate the effectiveness of the applied treatment, detect residual disease and recurrence of disease in these patients (1-5).

Serum thyroglobulin is now generally measured by two-antibody "sandwich" immunometric assays (IMA), the antigen is sandwiched between the two antibodies, in which the capture antibody is bound to a solid support and the detection antibody is labeled with either an isotopic (immunoradiometric assay, IRMA) or non-isotopic (usual-

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ly immunochemiluminescent assay) marker (3,6). Second-generation) thyroglobulin immunometric assay measurements, have an higher functional sensitivity (≤0.10 μg/l) than older (first-generation) tests (functional sensitivity ~1.0μg/l) (3, 4, 6). During follow-up low risic patients with DTC, blood samples are common taken for thyroglobulin measurement while the patient is taking L-thyroxine suppression (1-4). Therefore, thyroglobulin assays need second-generation functional sensitivity in order to monitor the low basal (non-TSH stimulated) thyroglobulin concentrations (3-4).

Unfortunately, the thyroglobulin autoantibodies (TgAbs) present in 25% to 30% of patients with DTCs can interfere with thyroglobulin measurement (3, 5). All of the immunometric assay methods were prone to underestimate serum Tg in the presence of TgAb, whereas the radioimmunoassays methods appeared resistant to TgAb interference (6, 7). Radioimmunoassays are still in use because this competitive methodology appears to convey more resistance to TgAbs interference than other IMA-class tests, although some interfering TgAbs undoubtedly cause some falsely high or low serum thyroglobulin values (7, 8-10).

Considering the large differences in concentrations thyroglobulin that can be measured by different tests for the same serum samples, the aim of our study was to compare the concentrations thyroglobulin obtained using two radioimmunoassays of various manufacturers: Cisbio Bioassays (France), which has been used for several years in the Department of Nuclear Medicine, Clinical Center Kragujevac and new assay from INEP, Serbia which is the first time used in our laboratory.

PATIENTS AND METHODS

This study included 42 patients of both genders with DTC. All patients underwent total thyroidectomy and were treated with ^{131}I in order to ablate the remaining thyroid tissue. The subjects were on suppressive doses of levothyroxine (TSH<0.15 mIU/L) and followed up. The study was conducted at the Department of Nuclear Medicine, Clinical Center Kragujevac. All blood samples were originally obtained for diagnostic purposes and studied in accordance with national ethical principles and in compliance with the Helsinki declaration. Blood (10 mL) from each patient was taken by venipuncture, and the serum separated by centrifugation at 2000 rpm for 15 minutes. The sera were stored frozen at -20°C and then thawed and all assayed together.

Serum concentrations of thyroglobulin were measured by immunoradiometric sandwich assay (IRMA) (THYROGLOBULINE, Cisbio Bioassays, France) according to the manufacturer's instructions. This assay uses an IRMA technique based on the following principle: five monoclonal TgAbs are used for the sandwich. The first four monoclonal antibodies are adsorbed onto the tube walls. Fifth TgAb (^{125}I-labeled) recognizing an epitope different from those recognized by the other four is used as the tracer. According to the manufacturer, the lower limit of detection was 0.2 μg/L and the calibration range was up to 500 μg/L. The functional sensitivity was 0.7 μg/L. Intra-assay CV was <7.0%, and inter-assay CV was <14.6%. The assay was standardized against Certified Reference Material 457 (CRM 457).

Manufacturer of the second assay is INEP, Serbia. Concentration of thyroglobulin is determined by a immunoradiometric method using two clones of monoclonal antibodies specific to different epitopes on the thyroglobulin molecule. First monoclonal antibody is attached to the bottom of the tube and the second monoclonal antibody is labeled with the radioactive isotope of iodine ^{125}I. According to the manufacturer, the lower limit of detection for this new assay was 0.1 μg/L and the calibration range was up to 200 μg/L. Intra-assay CV was <5.6%, and inter-assay CV was <5.9%.

After incubation is completed, the contents of the tube are aspirated to remove unbound labeled antibodies, and the radioactivity in the bound complex is measured in a gamma counter. The amount of measured radioactivity is directly proportional to the concentration of Tg in the sample.

Serum thyroglobulin results were considered concordant if they were undetectable or detectable by both methods. Discordance was defined as being present when serum thyroglobulin was greater than 1 μg/L by one, but undetectable by the other assay.

The concentration of TgAbs was determined by a competitive "one-step" radioimmunoassay (TgAb I step) which has been used for several years in our department (Cisbio Bioassays, France). It is based on the competition type principle and carried out in human serum. In TgAb radioimmunoassay TgAbs in calibrators and diluted patient sera are allowed to interact with ^{125}I labelled thyroglobulin. After a incubation and aspiration of the supernatant, the tubes are counted on a gamma counter. According to the manufacturer, the intra- and inter-assay precisions were less than 8.3% and 12.8%, respectively. The method was calibrated against the World Health Organization (WHO) First International Reference Preparation CRM 65/93 and had an analytical detection limit of 6.0 IU/mL. The manufacturer made no declaration about possible interference of thyroglobulin on antithyroglobulin measurements. The measured TgAb values were analyzed toward the value of 30 IU/mL (cut-off for healthy subjects without thyroid disease as recommended by the manufacturer of the assay). Autoantibody concentrations higher than 30 IU/mL were considered "enhanced."

For statistical analysis we use the correlation analysis method (Pearson coefficient of correlation), the number of detectable Tg levels were compared by the chi-square test (SPSS 10.0 program). Results are graphically presented using the MS Excel application.

RESULTS

In our study serum thyroglobulin concentrations were tested in 42 patients with DTC. Within regular follow-up a blood sample was taken for the determination of the thyroid hormones, thyroglobulin and antithyroglobulin antibodies in the radioimmunological laboratory of the Department of Nuclear Medicine, Clinical Center Kragujevac. Concentration of thyroglobulin is determined using two different radioimunoassay, the concentrations of the remaining laboratory parameters were determined by radioimmunoassays that are in regular use in our laboratory. The characteristics of the tests taken from the manufacturer's brochure are given in Table 1.

According to the manufacturer's brochure, the obtained thyroglobulin concentrations are expressed in μg/L for both assays. The measured concentrations were within the range < 0.1 to 87.0 μg/L (INEP), and < 0.2-46.7 μg/L (Cisbio). The thyroglobulin concentrations measured by Cisbio Bioassays and INEP assays were different in serum samples of 3 patients. In first case, high thyroglobulin values (87.0 μg/L) was measured by the INEP assay, while the Cisbio assay also detected an elevated thyroglobulin value, but almost twice less (43.7 μg/L). In the second case, a high concentration of thyroglobulin (57.1 μg/L INEP) significantly differs from the concentration obtained for Cisbio thyroglobulin (0.2 μg/L). In the third case, Cisbio radioimmunoassay detected a high concentration of thyroglobulin (46.7μg/L), while the concentration measured by the INEP radioimmunoassay was below the the limit of detection. The one patient had low, but measurable level of thyroglobulin in the range 0.2-0.6 μg/L.

Concordance between the two assay methods for determining serum thyroglobulin levels was noted for 39 (92.85%) patients. Statistical analysis showed that there was a direct correlation between two IRMA tests, with a positive correlation coefficient r=0.613 (p<0.01). Using assays we have found that 38 patients had serum thyroglobulin levels below the limit of detection by both methods and 2 patient had elevated serum thyroglobulin level by one of methods. Concentrations of thyroglobulin are given in Table 2. Statistical analysis of the results showed that the assays do not differ by separation of the pathological from the normal values (χ^2= 3.841, p> 0.05).

Table 1. The characteristic of two assays according to the manufacturers

Parameters	IRMA Tg (*INEP*)	THYROGLOBULINE IRMA (Cisbio Bioassays)
Principle of the assay	IRMA	IRMA
Tracer	^{125}I	^{125}I
Antibodies	monoclonal	monoclonal
Measuring range	0.1-200 μg/L	0.2-500 μg/L
Detection limit	0.1 μg/L	0.2 μg/L

Table 2. Agreement of serum thyroglobulin between the two assays

	Thyroglobulin			
	<0.2 μg/L	0.2-1 μg/L	>1 μg/L	Number of patients
Thyroglobulin INEP	39	1	2	42
Thyroglobulin, CIS Bioassays	39	1	2	42

In our study population two patients (4.33%) had elevated levels of thyroglobulin antibodies (57.7 IU/mL and 1049 IU/mL). In the first patient (TgAbs 57.7 IU/mL), both thyroglobulin assays found a low level of thyroglobulin, below the first standard concentration (<0.2 i.e. <0.1 μg/L). In the second patient, the concentration of TgAbs was high (1049 IU/mL), in this case, the Cisbio assay detected the high concentration of thyroglobulin 46 μg/L and INEP assay measured low concentration (<0.2 μg/L).

Statistical analysis showed a statistically significant positive correlation between the concentration of TgAbs and thyroglobulin Cisbio p<0.01, the Pearson coefficient was 0.717, but there is no statistically significant correlation between the concentration of TgAbs and second thyroglobulin assay.

DISCUSSION

Measurement of serum thyroglobulin is a highly specific test in the management of patients with DTC after surgical treatment and radioiodine ablation (1, 2). According to the guidelines (1, 2) serum thyroglobulin should be measured using a sensitive IRMA assay (functional sensitivity <1.0 ng/ml) standardized on the European reference standard (CRM 457) (3). Presence of TgAbs in the circulation may interfere with the assay, leading to false negative serum thyroglobulin determination. Increased concentrations of TgAbs are manifested in 20-30% of patients with DTC (4-6), so the presence of elevated TgAbs must be confirmed or ruled out by determining their concentration (4-6). Thyroglobulin is produced by both normal and neoplastic thyroid cells and its production is under TSH control (1, 2). Serum TSH should always be measured at the time of thyroglobulin determination (1-2). According to the guidelines, in the serum of our patient we measured concentration of thyroglobulin, TgAbs, thyroid hormones and TSH.

In clinical work with patients, the results are expressed as normal, low or high values, when we take into account the acquired values and the reference range. The highest percentage of our patients had a low concentration of thyroglobulin, below the lower limit of the measuring ranges. When comparing two assays, results are consistent for 92.85% patients. In literature data, the coefficient of correlation thyroglobulin measured by different tests is from 0.68 to 0.792 (5, 9). In our work the coefficient of correlation is slightly lower than in the previously mentioned literature and it is 0.613.

Measurement of thyroglobulin in serum is technically difficult, associated with methodological problems, that

can reduce the clinical significance of the obtained result. We find that the thyroglobulin concentrations measured by Cisbio Bioassays and INEP assays were quite different in serum samples of three patients (7.15%). (87.0μg/L INEP vs. 43.7μg/L Cisbio, 57.1 μg/L INEP vs. 0.2 μg/L Cisbio, <0.2 μg/L INEP vs. 46 μg/L Cisbio). Many studies have shown that measured values for thyroglobulin differ depending on the test employed (4, 5, 10-12). Epitope mapping on the thyroglobulin molecule has shown the existence of six different antigenic regions to which different TgAbs can bind (9, 10). In our first assay (Cisbio) four monoclonal antibodies are adsorbed onto the tube walls and fifth antibody (^{125}I-labeled) recognizing an epitope different from those recognized by the other four is used as the trace. In the second assay first monoclonal antibody is attached to the bottom of the tube and the second monoclonal antibody is labeled with the radioactive isotope ^{125}I. So, this differences in individual concentrations of thyroglobulin measured in our study could be due to the different specificity of the antibodies directed at thyroglobulin used in assays. Subtle variations thyroglobulin concentration was observed in one case (i.e. 0.3 μg/L using the INEP vs 0.6 ng/mL using the Cisbio). These differences were clinically acceptable. No reference values have been established for measuring thyroglobulin during substitution therapy with thyroid hormones. Group of authors suggested that for patients who have undergone total or near-total thyroidectomy and radioiodine ablation and have no clinical evidence of residual tumor serum thyroglobulin should be below 1 μg/L during thyroid hormone suppression (17).

Before our results were interpreted, it should be noted that all the values for thyroglobulin and TgAbs in sera from DTC patients were obtained and we considered our results for thyroglobulin concentration in parallel with the concentration of TgAbs. One patient had significantly elevated level of TgAbs (1049 IU/mL), second patient had slightly elevated level od TgAbs (57.7 IU/mL). In the first patient, with slightly elevated TgAb (57.7 IU/mL), both thyroglobulin assays found a low level of thyroglobulin, below the first standard concentration (<0.2 i.e. <0.1 μg/L). On the contrary, in the second patient with the high concentration of TgAbs (1049 IU/mL), the Cisbio assay showed a considerable elevation of serum ratio of thyroglobulin but INEP assay measured low concentration thyroglobulin (46 μg/L vs. <0.2 μg/L). Two different results can be explained by the effect of elevated concentration of anti-thyroglobulin antibodies. The presence of TgAbs leads to over- or underestimation of thyroglobulin concentrations with different degrees among assays (7, 8, 18). Radioimmunoassays methods appears more resistant to TgAbs interference than IMA-class tests (7, 19, 20). RIA is relatively resistant to interference from TgAbs (4, 7, 8), and some authors have observed good correlation among different immunoradiometric assays (15).

CONCLUSIONS

There is good agreement between the two thyroglobulin assays compared in this study. The new INEP IRMA thyroglobulin is a sensitive assay for thyroglobulin measurement, and the results were highly correlated with those obtained with the Thyroglobulin Cisbio Bioassays.

Conflicts Of Interest

The authors declare no conflict of interest.

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HEALTH LITERACY IN FEMALE – ASSOCIATION WITH SOCIOECONOMIC FACTORS AND EFFECTS ON REPRODUCTIVE HEALTH

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ZDRAVSTVENA PISMENOST ŽENA – UDRUŽENOST SA SOCIOEKONOMSKIM FAKTORIMA I EFEKTI NA REPRODUKTIVNO ZDRAVLJE

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ABSTRACT

The aim of the study is to assess the health literacy of women who are using health services within the Gynecology Obstetric Clinic "Narodni Front" in Belgrade. Testing of health literacy was conducted as a cross-sectional study in the period October- November 2012. As instruments of research the following questionnaires are used: Short Test of Functional Health Literacy in Adults and General information questionnaire of respondents who referred to the demographic, social and economic characteristics of respondents, self-assessment of health, use of health services, health knowledge and behavior in the area of reproductive health. Inadequate health literacy level is registered in every ten respondents. The education level of the respondents proved to be a significant predictor of health literacy. Demographic and socio-economic characteristics of the patients (age, occupation, marital status) as well as self-evaluation of the health status were not significantly related to the health literacy. Health literacy respondents did not significantly dependent on risk behaviors related to reproductive health. The level of health literacy is consistent with the knowledge of subjects in the field of protection of reproductive health. Health literacy as the ability to function within the health care system is equally certain by individual characteristics and skills, characteristics of the health and education systems as well as a wide range of social and cultural factors. Health literacy is more systematic than individual problem, so it requires a broader social action.

Keywords: *health literacy, women, reproductive health*

SAŽETAK

Cilj rada je procena zdravstvene pismenosti žena koje koriste zdravstvene usluge u okviru Ginekološko akušerske klinike „Narodni front" u Beogradu. Ispitivanje zdravstvene pismenosti sprovedeno je kao studija preseka u periodu oktobar- novembar 2012. god. u Ginekološko-akušerskoj klinici „Narodni front" u Beogradu. Kao instrumenti istraživanja korišćeni su sledeći upitnici: Test za ispitivanje funkcionalne zdravstvene pismenosti kod odraslog stanovništva i upitnik o opštim podacima ispitanica koji se odnosio na demografske, socijalne i ekonomske karakteristike ispitanica, samoprocenu zdravlja, korišćenje zdravstvene službe, zdravstveno stanje i znanje i ponašanje u oblasti reproduktivnog zdravlja. Neadekvatan nivo zdravstvene pismenosti registrovan je kod svake desete ispitanice. Nivo obrazovanja ispitanica se pokazao kao značajan prediktor zdravstvene pismenosti. Demografske i socioekonomske karakteristike ispitanica (životna dob, radni status, bračni status) kao i samoprocena zdravstvenog stanja nisu bili značajno povezani sa zdravstvenom pismenošću. Zdravstvena pismenost ispitanica nije značajno zavisila od rizičnih oblika ponašanja u vezi sa reproduktivnim zdravljem. Nivo zdravstvene pismenosti je u skladu sa znanjem ispitanica iz oblasti očuvanja reproduktivnog zdravlja. Zdravstvena pismenost kao sposobnost funkcioniranja unutar sistema zdravstvene zaštite jednako je određena pojedinim karakteristikama i vještinama, karakteristikama sistema zdravstva i obrazovanja, kao i širokom spektru društvenih i kulturnih faktora. Zdravstvena pismenost je sistematičnija od pojedinačnog problema, pa zahteva širu društvenu akciju

Ključne reči: *health literacy, women, reproductive health*

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INTRODUCTION

Health literacy as a form of literacy has become very important for social, economic, and health development (1). The World Health Organization has defined health literacy as "the cognitive and social skills and capacity needed to access, understand and use information in a way that promotes and protects good health" (2). Health literacy enables people to increase control over their health and improve it. Health literacy includes cognitive and social skills which determine the motivation and ability of individuals to obtain, understand and use information in a way that will promote and preserve good health. Health literacy refers to the skills and competencies of people to meet the complex requirements of health in modern society Division into basic functional, interactive and critical health literacy shows that in the health system patients are required to have at least a satisfactory level of functional health literacy in order to participate in the realization of their own health care (3-5). Health literacy as the ability to function within the health care system is equally certain by individual characteristics and skills, characteristics of the health and education systems as well as a wide range of social and cultural factors. Numerous studies have shown that low levels of health literacy causes more frequent use of emergency combat, often needs for hospital treatment, frequent drug use, less use of preventive services, which contributes to higher costs for the health system (6,7). Previous studies have shown a positive effect of education and literacy on the health of the population. Previous studies have shown a positive effect of education and literacy on the health of the population. Reports from "State of the World's Mother" by the organization Save the Children (8) estimated the rate of literacy of the adult women, as one of ten key indicators to determine the "well-being of women." Literature data also suggest an association between health literacy and prevention behaviors among women (9,10). The World Health Organization has defined women's health literacy as a "cognitive and social skills which determine the motivation and ability of women to access, understand and use information in ways which promote and preserve their health and the health of their children." (11,12)

The aim of the study is to assess the health literacy of women who are using health services within the Gynecology Obstetric Clinic "Narodni Front" in Belgrade.

METHOD

Testing of health literacy was conducted as a cross-sectional study in the period October- November 2012. in Gynecology Obstetric Clinic "Narodni front" in Belgrade.

As instruments of research the following questionnaires are used:

- An abbreviated version of the questionnaire TOFHLA (STOFHLA population - Short Test of Functional Health Literacy in Adults)) - test of functional health literacy among the adult. TOFHLA original version of the questionnaire is available in two forms (standard and abbreviated - STOFHLA) (13).
- General information questionnaire of respondents who referred to the demographic, social and economic characteristics of respondents, self-assessment of health, use of health services, health knowledge and behavior in the area of reproductive health.

The subjects of research were the beneficiary of tertiary health care of Gynecology and Obstetrics Clinic "Narodni front" in Belgrade. The criteria for entering the respondents in the survey were:

- The age group (over 18 years)
- The willingness to voluntarily participate in the study
- Literate person
- Possession of visual skills to complete the questionnaire
- A medical condition that allows them to be able to complete the questionnaire.

Before the start of the research respondents were familiar with the objectives and procedures of research.

An abbreviated version of the questionnaire TOFHLA (STOFHLA) consists of 36 parts which assesses the ability to read and understand information from health care environment.

Scoring is done as like in the standard version of TOFHLA questionnaire in the section pertaining to the understanding of reading, so that the total number of points obtained by a shortened version of the questionnaire was 36. TOFHLA has already adapted to the Serbian language and showed good internal consistency (Cronbach's alpha = 0.94). Evaluation was as following:

- Inadequate health literacy implied between 0 and 16;
- The marginal was between 17 and 22 and
- Adequate health literacy between 23 and 36 points.

In standard and shortened version of the questionnaire, the categories of health literacy included the following:

- Inadequate health literacy implied the impossibility of reading and understanding the text related to health;
- Marginal health literacy refers to the difficulty in reading and understanding the text referred to health and
- Adequate health literacy is the ability to read and understand most of the texts related to health (13,14).

The obtained results were analyzed using the methods of descriptive and inferential statistics. For statistical analysis, we used the software package program Statistical Package for the Social Sciences, SPSS 17.0. Following descriptive statistics were used: measures of central tendency (mean and median), the measures of variability (standard deviation) for continuous variables and the absolute frequencies and percentages for categorical variables. For testing the significance of differences we were using chi square test (contingency tables). We tested the differences in health literacy in relation to demographic, social and economic characteris-

tics, the self-assessment of health, use of health services, risk behavior and knowledge in the field of protection of reproductive health. In all cases of differential testing, the statistical test is accepted if the probability of the null hypothesis was equal to or less than 5%.

RESULTS

The results showed that from the complete number of subjects, 121 (90.3%) had adequate level of health literacy, while the 8 (6%) had a marginal, and the inadequate level of health literacy show up in 5 (3.7%) of the respondents. The largest number (95.1%) of respondents with adequate health literacy is in the category aged 18 to 29 years, while one fifth of inadequate is from category 40 to 49 years.

In relation to the marital status of respondents, it is noted that most of them (96.4%) with adequately literacy are single subjects, and at the highest (12.6%) with inadequately literacy are married ($\chi^2 = 2.488$; $p = 0.288$). A statistically significant difference in the level of health literacy has been observed in relation to the level of education of the patients ($\chi^2 = 8.627$; $p = 0.013$). Most of them (94.5%) with adequate health literacy were with a college education, and half (50.0%) with inadequately literacy were in an incomplete or primary school. It can be seen that the 5.5% inadequately literacy were the patients with a college education, and every tenth was with finished high school.

Unemployed women were with an adequate level of health literacy as like the 90.2% of women from the category of employed. Nearly one-fifth of inadequately is from group of the housewife, students / high school student, unable to work and retired ($\chi^2 = 3.595$; $p = 0.166$). No statistically significant difference was observed in the level of health literacy in relation to the self-assessment of financial situation. The largest number of adequate health literate women was in category that assessed their financial situation as well.

An adequate level of health literacy had the highest number (93%) of women who assess their health as good, while 15.4% of women with inadequate health literacy assessed its health as average. Also, an adequate level of health literacy was recorded in 87.5% of respondents who perceived their health as poor ($\chi^2 = 2.223$; $p = 0.329$).

It was not observed association between examined factors of risk behavior and health literacy. The largest number (96.9%) of patients with an adequate level of health literacy use contraception, while 12.8% with inadequate literacy don't use any form of protection from sexual transmitted diseases and unwanted pregnancy. An adequate level of health literacy was observed in 91.7% of the respondents which use preservative as a mechanical contraceptive. Almost in every tenth respondent who do not use condoms reveals inadequate levels of health literacy. In 90.4% of respondents who correctly answered the question, to what purpose is performed Pap test, there was an adequate level of health literacy. However, almost one in ten patients with inadequate levels of health literacy knew that the Pap test is performed to detect premalignant and malignant changes in the cervix. It can be noticed that in the category of patients with inadequate health literacy, the greater number was from those who gave the correct answer than from those who gave incorrect answers to the question ($\chi^2 = 0.575$; $p = 0.490$), table 1.

DISCUSSION

Studies worldwide show that health literacy is the strongest predictor of health status of an individual, even before the age, education, income, employment or ethnicity (15). A higher level of health literacy is significantly associated with higher social status, a higher frequency of monitoring health emissions but it is also associated with a younger age. It turned out that health literacy is also linked to health status, health behavior, availability and use of health care services (16).

A lot of factors have been identified as factors related to different levels of health literacy. Studies are showing correlation between level of health literacy and social determinants, so that the groups with proportionally higher risk limited literacy are defined. Research conducted in eight countries of the European Union has registered an inadequate general health literacy by 12% of respondents, and more than one third of respondents had a problematic level of health literacy, so that almost every second respondent had limited health literacy. The results of this study varied between countries. The highest degree of health literacy was recorded in the Netherlands, where the 72% of subjects were registered with excellent and satisfactory level of health literacy, and a high level of health literacy is recorded in Ireland, too (61.3%). In both countries, less than 10% of respondents registered an inadequate level of health literacy (17).

Many studies have shown that a large percentage of the adult population (53%) have health literacy at the secondary level, 22% at primary level and 14% below the basic level of health literacy, which leads to conclusion that they don't have the ability to accept, understand and use useful information for their health (18). Research conducted in Canada showed that 60% of adult Canadians (aged 16) has a problem to accept and understand health information and to have difficulty in making the correct medical decisions (19). The low level of health literacy was recorded in research in Australia, where 59% of respondents had low or very low levels of health literacy (20).

Studies of health literacy in South America show that more than one-third (32.4%) healthy individuals who have used the services of the University Hospital and other hospitals in Sao Paulo (Brazil) had inadequate or marginal health literacy level (21) 19 and 30 , 1% of inadequate health literacy in Argentina by research (22). Research results in a health insurance literacy Ireland showed that 19.9% of them had a high probability limited functional

Tabel 1. Health literarcy relative to demographic and socioeconomic caracteristics

Characteristics of female subjects	Adequate health literacy N (%)	Inadequate health literacy N (%)	p value*
Age of life			
18-29 years	39 (95.1)	2 (4.9)	0.201
30-39 years	55 (91.7)	5 (8.3)	
40-49 years	19 (79.2)	5 (20.8)	
50 and more years	8 (88.9)	1 (11.1)	
Marital status			
married	76 (87.4)	11 (12.6)	0.288
unmarried	27 (96.4)	1 (3.6)	
other	18 (94.7)	1 (5.3)	
Education			
faculty	52 (94.5)	3 (5.5)	**0.013**
high/higher or middle school	67 (89.3)	8 (10.7)	
uncompleted or elementary school	2 (50)	2 (50.0)	
Employment			
employed	83 (90.2)	9 (9.8)	0.166
unemployed	19 (100)	0 (0.0)	
other	19 (82.6)	4 (17.4)	
Financial situation			
good	42 (97.7)	1 (2.3)	0.138
average	67 (87.0)	10 (13.0)	
bad	12 (85.7)	2 (14.3)	
Self-assessment of health			
good	80 (93.0)	6 (7.0)	0.329
average	33 (84.6)	6 (15.4)	
bad	7 (87.5)	1 (12.5)	
Use of contraception			
yes	31 (96.9)	1 (3.1)	0.334
no	90 (87.15)	12 (12.85)	
Use of condoms			
yes	44 (91.7)	4 (8.3)	0.91
no	76 (89.3)	9 (10.7)	
Purpose of using Papa test			
correct	103 (90.4)	11 (9.6)	0.575
incorrect	17 (94.4)	1 (5.6)	

• relative to chi square test

health literacy, 22.5% of contained functional literacy and 57.6% of an adequate level of health literacy (23). The study of health literacy in Israel showed that 29.4% of respondents have inadequate literacy, 12.6% and 58.0% marginal adequate level of health literacy (24).

Some research has identified sex as an important factor of health literacy, but there are also studies in which this relationship is not clearly established (25-27). No statistically significant difference was found in the level of health literacy between married, unmarried, and others, so that the marital status of respondents could not be identified as a factor that has an impact on the level of health literacy. Although age was not significantly associated with the level of health literacy, it can be seen that the largest number of respondents had proper literacy in the category up to 39 years while almost half of respondents older than 40 years had an inadequate level of health literacy. The results of European studies have shown a negative correlation between health literacy and age, except in the Netherlands (17). The results of research in the US shows that respondents aged 65 years and older have a lower average health literacy in relation to adult literacy at a young age. Adults aged 25 to 39 years had a higher average health literacy compared to other age groups. The part of adults with average health literacy, except in the group 65 years and over, ranged from 53% to 58%, and among adults aged 65 years and over 38% (18). The results of studies of health literacy in South Australia also showed that the propor-

tion of people with inadequate health literacy levels, older than 65 years was higher than 50%, while the number of inadequate literacy among the younger respondents were less (11%). In addition to age, the level of education proved to be a significant predictor of health literacy in the available literature. The level of health literacy in a number of studies, irrespective of the population and the health care environment, dependent on the level of education, namely people with higher education levels showed significantly better results in health literacy (20). The results of research in the US shows that health literacy is directly dependent on the level of education. More than three-quarters of the adult population with a level of education with not completed high school had basic or below basic health literacy levels, and the percentage is significantly decreased as the level of education that is growing namely level of health literacy has been growing with the level of education (18). The results of our study do not differ with the available literature considering that the statistically significant difference level of health literacy in relation to the education level of respondents is shown.

Employment status of respondents, according to research, is also important for the health literacy. However, the results of our study showed that all the respondents who are not employed, had an adequate level of health literacy, while one in five respondents in category (housewife, student of / student, unable to work and retired) had inadequate levels of health literacy. Data from previous studies shows that the poor financial situation is contributing factor in lower levels of health literacy (17,25). The results of our study showed that there is a significant difference in the level of health literacy among respondents who assess their financial situation as good, average or bad.

A study conducted in Iran was aimed to assess health literacy of women on the subject - cervical cancer in reproductive age and related factors. Almost half (47.2%) of participants had limited health literacy, and health literacy was associated with education, employment, income, search for counseling. Low level of education, unemployed woman and lower income had a significantly and less counseling affected on the reproductive health of woman. There was no relationship between health literacy and age. Adapting health education interventions in relation to the different levels of the health literacy of women may increase the frequency of use of screening examinations for cervical (28).

Evaluation and research on health literacy of women in Taiwan and its links with health behavior has shown that an adequate level of health literacy is often associated with positive health behavior, but it is not so when it comes to women smokers (29).

The health status of the respondents, namely self-assessment of health proved to be a factor that was not significantly associated with the level of health literacy. Data from other studies suggest a link between health literacy and self-assessment in health namely self-assessment of the health conditions are often associated with the functional capacity, morbidity and is an important determinant of the state of health (25,26).

CONCLUSION

The right to health literacy should exist as there is a universal right to health care. Although health literacy is the product of individual capacity, it is also conditioned by the complexities of the health care system. Therefore, it is necessary to create political awareness of health literacy, with the aim of shared responsibility witch should include politicians, professionals and whole society. Health literacy should be understood as a key determinant of health and effective strategies and actions for improvement should be created.

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ANTIAPOPTOTIC PROTEINS MCL-1 AND BCL-2 AS WELL AS GROWTH FACTORS FGF AND VEGF INFLUENCE SURVIVAL OF PERIPHERAL BLOOD AND BONE MARROW CHRONIC LYMPHOCYTIC LEUKEMIA CELLS

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ANTIAPOPTOTSKI PROTEINI MCL-1 I BCL-2 KAO I VASKULARNI ENDOTELNI FAKTOR RASTA (VEGF) I FIBROBLASTNI FAKTOR RASTA (FGF) UTIČU NA PREŽIVLJAVANJE ĆELIJA HRONIČNE LIMFOCITNE LEUKEMIJE U PERIFERNOJ KRVI I KOSTNOJ SRŽI

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ABSTRACT

Apoptosis inhibition in chronic lymphocytic leukemia (CLL) is one of the most important mechanism in the disease onset, progression and therapy response and is dependent of interaction with different microenvironments.

Aim of our paper is to determine expression of antiapoptoic proteins mcl-1 and bcl-2 in CLL cells isolated from two different compartments (peripheral blood and bone marrow) and its relation to percent of apoptotic cells and concentration of growth factors (FGF and VEGF).

Our results showed that peripheral blood CLL lymphocytes have lower apoptotic rate then those isolated from bone marrow, though bone marrow CLL lymphocytes express higher levels of antipoptotic proteins bcl-2 and mcl-1. In bone marrow FGF concentration is 10-fold higher then in patients plasma but has an limited impact on mcl-1 expression. In contrary, VEGF concentration is higher in peripheral blood and corelate with percent of apoptotic cells and mcl-1 expression in this compartment.

CLL cells derived from two different microenvironmets acts differently when tested for apoptosis „ex vivo". In peripheral blood apoptosis is strongly connected with expression of antiapoptoic proteins (mcl-1 and bcl-2) and growth factors, but not in bone marrow.

Keywords: *CLL, apoptosis, mcl-1, VEGF, FGF*

SAŽETAK

Inhibicija apoptoze u hroničnoj limfocitnoj leukemiji (HLL) predstavlja jedan od najvažnijih mehanizama kako nastanka bolesti, tako i progresije ali i odgovora na primenjivanu terapiju i zavisi od interakcije malignog limfocita sa različitim mikrosredinama.

Cilj našeg rada je odrediti ekspresiju antiapoptotskih proteina mcl-1 i bcl-2 u HLL limfocitima izlovanim iz dve različite mikrosredine (periferne krvi i kostne srži) i njihovu povezanost sa procentom apopotičnih limfocita kao i koncentracijom faktora rasta (VEGF i FGF).

Naši rezultati su pokazali da HLL limfociti izolovani iz periferne krvi imaju manji procenat apoptoze nego oni izolovani iz kostne srži, iako im je ekspresija antiapopotskih proteina bcl-2 i mcl-1 niža. U mikrosredini kostne srži koncentracija FGF je 10 puta veća nego u plazmi pacijenata, ali je njen uticaj na ekspresiju mcl-1 minimalan. Sa druge strane koncentracija VEGF je veća u perifernoj krvi i korelira sa procentom apoptotskih limfocita kao i ekspresijom mcl-1 u HLL limfocitima izolovanim iz periferne krvi.

Limfociti hronične limfocitne leukemije izlovani iz dve različite mikrosredine pokazuju značajnu razliku u procentu „ex vivo" testirane apoptoze. U perifernoj krvi procenat apoptotskih ćelija snažno je povezan sa ekspresijom antiapopototskih proteina (mcl-1 i bcl-2) kao i koncentracijom faktora rasta, dok u kostnoj srži ove povezanosti nema.

Ključne reči: *HLL, apoptoza, mcl-1, VEGF, FGF*

ABBREVIATIONS

Bcl-2 - B cell lymphoma 2 protein
mcl-1- myeloid leukemia cell 1 protein
VEGF - vascular endothel growth factor
FGF - fibroblast growth factor
CLL - chronic lymphocytic leukemia

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INTRODUCTION

Apoptosis inhibition in chronic lymphocytic leukemia (CLL) cells stands as one of the most important mechanisms in the disease onset, but as well as in progression and therapy response. Multiple mechanisms of CLL cells apoptosis resistance have been discovered and described so far, but most important is definitely intrinsic pathway represented through bcl-2 family members (bcl-2, mcl-1, bcl-XL, Bax, Bad) (1). These antiapoptotic pathways are potent and keeps the cells long living. The bcl-2 protein is important for maintenance of B lymphocyte population in adults (long living memory cells) in normal hematopoesis while mcl-1 predominantely follows hematopoetic stem cell and B cell differentiation process (2 - 4). In CLL cells concentrations of these proteins are elevated due to enhanced syntesis, but also their reduced clevage. It is mostly due to hypomethylation of the promoter bcl-2 region as well as lack of microRNA 15, 16 and 29, which leads to accumulation of bcl-2 and mcl-1 in the CLL cells, resulting in the prolonged survival (1,5). Yet, when cultivated in vitro in monocultures, CLL cells undergo apoptosis in the higher percent than healthy B lymphocytes, which imposes a conclusion that prolonged survival is not characteristic od CLL cell itself, but is a product of interaction of CLL cell with protective microenvironment(6). The bone marrow and lymph node are two most important microenvirontems for proliferation, apoptosis inhibition and drug resistance of CLL cells. Predominantly stromal „nurse like" cells, but also endothelial cells in these microenvironments, through cell-cell interaction and soluble molecules modulate CLL cells in their apoptotic and proliferation signals. Pathogenesis of CLL is also impacted by angiogenesis in these protective microenvironments. Vascular endothelial growth factor (VEGF) is pro-angiogenic factor with multiple roles proved in CLL. Its stimultion of angiogenesis is associated with an advanced stage of the disease, resistance to apoptosis and cell motility. (7,8,9). Fibroblast growth factor (FGF) in tumors promotes angiogenesis, has important role in proliferation of stromal cells and thus modify microenviroment (10,11).

Aim of our paper is to determine expression of antiapoptoic proteins mcl-1 and bcl-2 in CLL cells isolated from two different compartments (peripheral blood and bone marrow) and its relation to percent of apoptotic cells and concentration of growth factors (FGF and VEGF) in these microenvironments.

PATIENTS AND METHODS

Study population and sample collection

In our study we evaluated 60 samples (30 peripheral blood and 30 matching bone marrow samples) from 30 patients diagnosed with chronic lymphocytic leukemia. Patients were at least 6 months without any chemotherapy and not suffering from other acute and chronic condition which could impact tested parameters. The study was approved by the Ethical Committee of the Clinical Center Kragujevac. All patients gave their written informed consent according to the Declaration of Helsinki. Democraphic data of our patients group refers to typical CLL patients, average age 67 years (53 – 87) with male predomination (22 males and 8 women). For study purpose we collected 5ml of peripheral blood and 4ml of bone marrow aspirate from each patient. Native samples were used for determination of expression of mcl-1 and bcl-2 in the CLL cells, while from the rest we isolated mononuclear cells and plasma and bone marrow supernatant. Plasma and bone marrow supernatant were quickly frozen to -70C and collected for determination of VEGF and FGF concentration. The mononuclear cells were isolated using comerrcial gradient LymphoPrep and used for the apoptosis detection.

Detection of antiapoptotic proteins

Peripheral blood and bone marrow aspirate samples of patients were analyzed using 5-color flow cytometry. Analyzing sample was prepared using 50 µl of whole blood, incubated 15min on room temperature with surface markers, and then dyed for intracellular markers (mcl-1 and bcl-2) using commercial intarcellular kit *IntraPrep* (*Beckman Coulter 2389*). Antibody sources were as follows: CD19 fitc Beckman Coulter (Cat.No A07768), CD5 PE-Cy7 Beckman Coulter (Cat.No A21690), *bcl-2* pe (*Invitrogen MHBCL04*), *mcl-1 8C6D4B1* (*Abcam ab31948*) and *Goat anti mouse IgG pe* (*Abcam ab97041*). Cells were analysed in the CD19/CD5 gate and expression was determined as postive/negative and low/high according to the isotypic control and MFI values. Samples were analysed on BC FC 500.

Growth factors mesaurments

Concentration of VEGF and FGF in plasma and bone marrow supernatant were determined using a commercial flowcytometric kit Human VEGF-A Flowcitomix Simplex Kit (e-bioscience, BMS80277FF), Human FGF-2 Flowcitomix Simplex Kit (e-bioscience, BMS82074FF) on a FC500 Beckman Coulter Flow Cytometer according to the manufacturer's instructions. Collected data were analyzed using FlowCytomix™ Pro 3.0 Software.

Detection of apoptotic lymphocytes

Apoptotic cells were detected on flow cytometer on isolated mononulclear cells, dyed using commercial kit Annexin V-FITC/7-AAD kit (BC IM3614) according to the manufacturer's instructions. Finally, cells were analyzed on an FC500 Beckman Coulter flow cytometer to the number of 20000 events, gating lymphocytes and CD19+ cells. In the analysis Annexin V negative and 7-AAD negative cells are viable, Annexin V positive and 7-AAD negative cells are in the early stages of apoptosis, Annexin V positive and 7-AAD

Table 1. Median values of tested parametrs in peripheral blood and bone marrow

	Peripheral blood	Bone marrow	p value
% of CD19+ cells in early apoptosis	0,03 (0,0001-0,22)	0,155 (0,025-0,38)	**0,068**
% of CD19+ cells in late apoptosis	0,001(0,0001-0,18)	0,13 (0,03-0,58)	**0,005**
% of cells with high bcl-2 expression	12,29 (0,71-52,74)	1,36 (0,15-66,36)	0,323
% of cells with mcl-1 expression	52,06 (20,9-65,8)	37,22 (15,7 - 58,39)	0,13
VEGF (pg/ml)	24,44 (21,16-58,05)	21,69 (17,14 - 25,10)	0,791
FGF (pg/ml)	103,75 (86,67-119,51)	1106,24 (299,64 - 2360,87)	**<0,005**

positive cells are in late stages of apoptosis, while Annexin V negative and 7-AAD positive cells are necrotic. The percentages of early and late apoptotic cells, as well as necrotic cells were determined using CXP Cytometer software.

RESULTS

We determined percent of apoptotic CD19+ cells in peripheral blood (PB) and bone marrow (BM), as well as expression of bcl-2 and mcl-1 in CD19+/CD5+ CLL cells and concentration of VEGF and FGF in both patients plasma and bone marrow supernatnt. These results comparing values from both compartments were presented in the table 1.

Peripheral blood lymphocytes show lower percentage of cells in both early and late apoptosis when tested „ex vivo" compared to the lymphocytes isolated from bone marrow, though the cells does not have significant difference in expression of antiapoptotic proteins (bcl-2 and mcl-1). (Figures 1 and 2). Concentration of VEGF in PB and BM does not differ significantly, while concentration of FGF in BM is 10-fold higher then in PB.

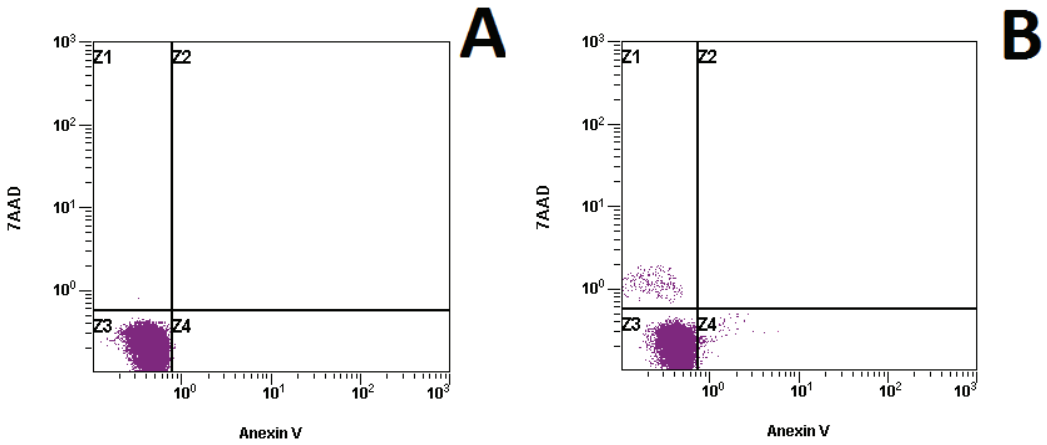

Figure 1. Dot plots showing apoptotic rate of lymphocys derived from peripheral blood (A) and bone marrow (B)

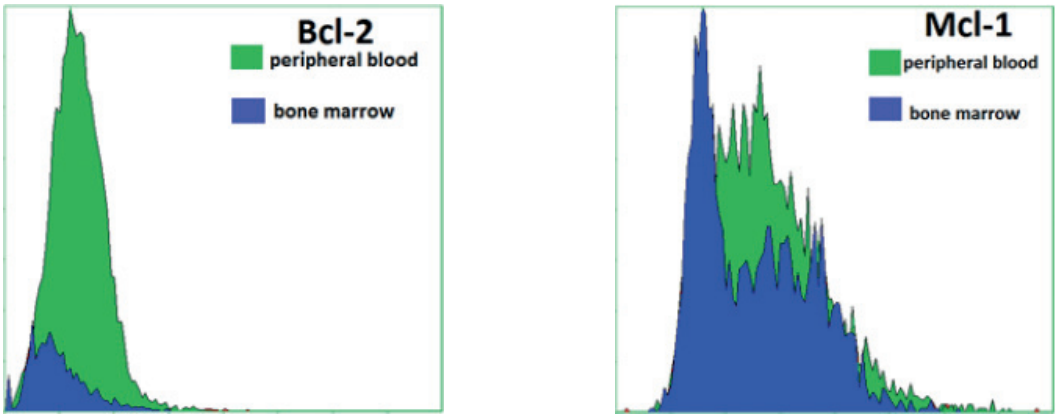

Figure 2. Overlay histograms showing expression of bcl-2 (left) and mcl-1 (right) in the CLL lymphocytes derived from peripheral blood and bone marrow

Expression of mcl-1 in bone marrow derived CLL cells correlate positivly with percent of both early and late apoptotic cells in bone marrow r^2= 0,453 p= 0,016, while it does not correlate with percent of apoptotic cells in peripheral blood r^2=0,191 p=0,313.

Early and late apoptosis in bone marrow are in strong positive correlation with concentration of VEGF r^2= 0,704 p< 0,0001 and r^2=0,554 p=0,002 respectivly. Also there is a strong positive correlation between FGF concentration in bone marrow with CLL cells early apoptosis in bone marrow r^2=0,611 p=0,001, as well as with CLL cells late apoptosis in bone marrow r^2= 0,620 p<0,0001

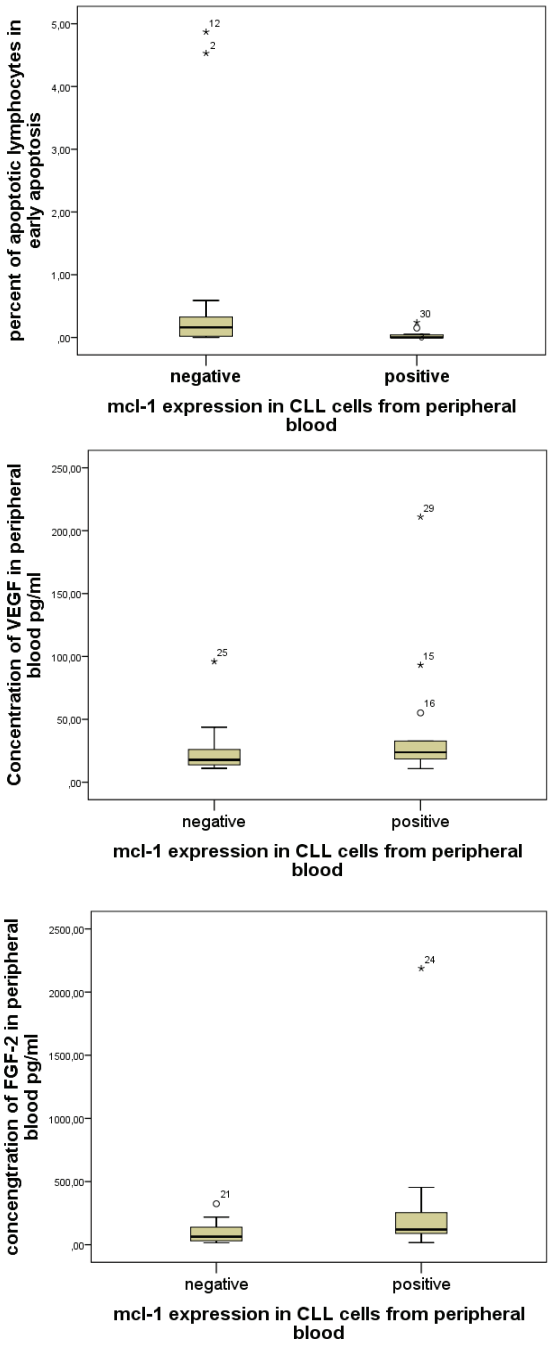

Figure 3. Apoptosis of CLL cells, VEGF and FGF concentration in peripheral blood depending on positivity of CLL cells to mcl-1

Results showed that when CLL cells in peripheral blood exprime mcl-1 molecule we have higher concentration of VEGF in peripheral blood (24,8±21,14 versus 42,98±52,73 pg/ml, p=0,077), higher concentration of FGF in peripheral blood (98,66±88,1 versus 309,4±552,84 pg/ml, p= 0,031), and lower early apoptotic rate (0,73±1,56% versus 0,038±0,07%, p=0,006) (Figure 3). There is no statisticaly significant difference in late apoptotic rate or bcl-2 expression in these cells.

There is no statisticaly significant difference in bcl-2 molecule expression, rate of early and late apoptosis, or concentration of VEGF and FGF in bone marrow supernatant between the mcl-1 positive and negative CLL cells.

Though there has been detected significant medium to strong correlation between mcl-1 expression in peripheral blood and bone marrow samples, r^2= 0,524 p=0,004.

DISCUSSION

Microenviroment studies in CLL has been in progress in the last decades thanks to which our view to CLL patients and its treatment has been changed. Protective role of microenvironments are the reasons for this disease to have so many faces.

CLL cells undoubtly have lower apoptotic rate comparred to lymphocytes isolated from peripheral blood of healthy subjects (12). But unlike most other studies that promote better survival of CLL cells in the protective microenvironments (bone marrow and lymph nodes), our results show that bone marrow derived CLL lymphocytes have higher apoptotic rate then those isolated from peripheral blood. Those results indirectly points that protective microenvironment acts in site, but does not make a permanent change in CLL cell which make it more prone to apoptosis when taken away from microenviromnent signals. So Siekluska at al, and Witkowska et al showed that there is a greater apoptotic rate when CLL cells are tested „ex vivo", just as our work showed, and that it can be correlated with the disease progression (13,14).

Main mechanism of CLL cell survival is apoptosis inhibition. All known apoptotic pathways were investigated in CLL, with conclusion that most impact deffinitely belongs to bcl-2 family proteins (15). This mechanism is widely used in the field of introducing novel therapies (15). Mcl-1 protein together with Act and in lot less percent other members of bcl-2 family (bcl-2 and bcl-XL) are the main regulators of apoptosis resistance in CLL cells. (16) Mcl-1 can be upregulated by several mechanisms, most of which include B cell receptor activation, which suggest that extrinsic as well as intrinsic factors cooperate in disease onset, progression and therapy resistance (16). All these mechanisms depends on stromal-mediated increase in RNA synthesis which could be a result of activation of transcription factors such as c-myc and NFκB (17). Since mcl-1 is an early responder and a fast turnover molecule it is amplified more then other proteins. Being a short living

molecule, there is a constant equlibrium between stabilisation and degradation of mcl-1, but in CLL cell it outweigh in favor of mcl-1 accumulation (17). Our results show that peripheral blood CLL lymphocytes have higher expression of mcl-1 then those derived from bone marrow, which suggest that mcl-1 expression is more important in antiapoptoic effect in peripheral blood.

Answer to the question of origin of VEGF in CLL is not simple. While Kay et al, and Chen et al came to the conclusion that CLL cells themselves secrete VEGF, Gehrke et al proved that only stromal cell derived VEGF have protective effect on CLL cells (18 - 20). Our results showing that plasma concentration of VEGF is higher that in bone marrow supernatant, give advantage to the fact that CLL cells themselves produce VEGF. Our results also showed that *ex vivo* apoptosis is accelerated in bone marrow comparing to peripheral blood CLL cells. CLL cells have receptors for VEGF on the cell membrane, expriming both VEGFR1 and VEGF R2 (13). When binded to these receptors VEGF increase apoptotic resistance of cells interact with STAT1 and STAT3 which ends up in upregulation of XIAP and mcl-1 expression (8,21,22). Also VEGFR expression in CLL cells are stimulated by endothelin -1 receptor signaling through hypoxia inducible factor 1, suggesting the impact of endotel cells in CLL survival (23, 24). Our results show greater concentration of VEGF in peripheral blood then in bone marrow, as well as a strong connection between mcl-1 expression and concentration of VEGF in peripheral blood, which also suggest that VEGF is secreted in peripheral blood and not overflow from bone marrow. Also VEGF mRNA levels in CLL cells are in strong positive correlation with mcl-1 expression (25).

Basic FGF (FGF-2) has an important role in early hematopoesis in proliferation of hemangioblasts, common progenitor cells for hematopoetic and endotel cells (26). For those reasons FGF is mostly investigated for its effect on endothelial cells. Concerning survival of endothelial cells in culture, adding FGF promote longer survival in the early phase because of the better cell adherence, and latter due to activation of MAP kinase, FGF promotes upregulation of antiapoptotic proteins (27). Out of bcl-2 protein family, FGF is proven to upregulate only bcl-2 protein (27). In our work we have proved that there is a relatively high concetration of FGF in patients plasma, but not significantly different from those in healthy subjects, but even 10-folder higer in bone marrow supernatant. (12) Several investigator proved that FGF is mostly secreted by stromal and endotel cells, and that its major role is in proliferation of bone marrow stromal cells (28). Also its concentration in CLL patients is higher than in the healthy control subjects (26,29,30). Similary to its effect in endothelial cells it is proven that FGF have a protective role in CLL cell survival, and that it has an impact on bcl-2 upregulation, but no effect on levels of mcl-1. (30). Beside longer survival, higher levels of FGF and presence of upregulated FGF-2 receptors on CLL cells are connected with resistance to standard fludarabine therapy (28). Our results show that levels of FGF in bone marrow strongly correlates with percent of apoptotic CLL cells, as well as with level of mcl-1, and not level of bcl-2 which is not in concordance with other results. Our results indirectly point to conection between FGF level in bone marrow and mcl-1 expression, which could be possible mechanism of FGF effect on CLL cell. Though the FGF effect should be further investigated towards its connection to mcl-1 molecule.

CONCLUSION

CLL cells derived from two different microenvironmets show different rate of apoptosis „ex vivo". In peripheral blood CLL cells apoptosis is strongly connected with expression of antiapoptoic proteins (mcl-1 and bcl-2) and growth factors, but not in bone marrow. However, these mechanisms are just a part of complex regulatory system.

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EXERCISE TREADMILL TEST IN PATIENTS WITH DIABETES MELLITUS TYPE 2

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TEST FIZIČKOG OPTEREĆENJA NA TREDMILU KOD PACIJENATA SA DIJABETES MELITUSOM TIPA 2

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ABSTRACT

There is a concern regarding the high incidence of coronary heart disease (CHD) among patients with diabetes mellitus (DM) type 2 since it is a leading cause of mortality in those patients. Exercise treadmill test (ETT) is proposed as a suitable, non-invasive method for identifying asymptomatic patients with ischemic changes, who would benefit from pharmacological treatment, thus contributing to a reduction of adverse cardiovascular events. Therefore the objective of our study was to evaluate myocardial ischemia in asymptomatic patients with DM type 2 by performing ETT. The present investigation was conducted in Health Center Pozega during the year 2018. 40 insulin-dependent, aged 33.05 ± 2.01 years, with DM type 2 were included in the study. They had nor history nor symptoms of cardiac disease. All patients underwent ETT according to Bruce protocol, while 12-lead ECG was recorded and blood pressure was monitored. All patients had negative ETT results. Also no ST segment depression, no signs of insufficiency of peripheral circulation, no changes in heart rhythm, no symptoms by the central nervous system were observed. Additionally response of heart rate and blood pressure to exercise was within physiological range. These promising findings indicate that diabetes didn't alter myocardial integrity and function, thus suggesting that coronary reserve in examined patients was preserved.

Keywords: *Diabetes mellitus type 2, Exercise treadmill test, Coronary heart disease*

SAŽETAK

Postoji zabrinutost zbog visoke stope incidence koronarne bolesti srca (KBS) kod pacijenata sa dijabetes melitusom (DM) tip 2, jer su one vodeći uzrok smrtnosti kod tih pacijenata. Test fizičkog opterećenja na tredmilu (TFOT) predložen je kao pogodna neinvazivna metoda za identifikaciju asimptompatskih pacijenata sa ishemijskim promenama, koji bi imali korist od farmakološkog tretmana, čime bi se doprinelo smanjenu neželjenih kardiovaskularnih dogadjaja. Stoga je cilj naše studije bio da ispita prisustvo ishemije miokarda kod asimptomatskih pacijenata sa DM tip 2 izvodjenjem TFOT. Istraživanje je sprovedeno u Domu zdravlja Požega tokom 2018. godine. U studiju je bilo uključeno 40 insulin zavisnih pacijenata, starosti 33,05 ± 2,01 iогина, sa DM tip 2. Nisu imali istoriju ni simptome bolesti srca. Svi pacijenti su podvrgnuti TFOT po Bruce protokolu, uz snimanje elektrokardiograma (EKG) u 12 odvoda i merenje krvnog pritiska. Svi pacijenti su imali negativan test opterećenja. Takodje uočeno je odsustvo ST depresije, znakova insuficijencije periferne cirkulacije, promena u srčanom ritmu i simptoma od strane centralnog nervnog sistema. Dodatno odgovor srčane frekvence i krvnog pritiska na fizičko opterećenje je bio u fiziološkim granicama. Ovi obećavajući rezultati ukazuju na to da dijabetes nije izmenio integritet i funkciju miokarda, što ukazuje da je koronarna rezerva kod ispitivanih pacijenata očuvana.

Ključne reči*: dijabetes melitus tip 2, test fizičkog opterećenja na tredmilu, koronarna bolest srca*

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INTRODUCTION

Diabetes mellitus remains one of the most rapidly growing chronic diseases, with a prevalence increasing at an alarming rate (1). It presents a group of metabolic disorders characterized by inadequate fasting or postprandial hyperglicemia, as a result of lack of insulin secretion or diminished tissue responses to insulin. Consequently metabolism of carbohydrates, fats and proteins is impaired (2). In DM type 2 insulin resistance and/or progressive decrease in the insulin secretion occurs, thus preventing insulin from reaching target tissue (2, 3). Condition of chronic hyperglicemia is associated with numerous microvascular and macrovascular complications, leading to a dysfunction of various organs, involving heart and blood vessels, muscle, skin, eyes, kidneys, nerves etc (4). Management of diabetic complications represents a great challenge of the 21st century, both for physicians and scientists, especially when it comes to cardiovascular disorders. There is a great concern regarding the development of coronary heart disease among patients with DM type 2 since it's the main cause of death in those patients. Furthermore numerous data has confirmed that patients with DM type 2 have 2-4 fold higher risk for CHD in comparison to the age-adjusted general population, thus indicating the urgent need for obtaining the correct *diagnosis* in an asymptomatic stage (5-7).

Regular physical exercise exerts myriad health benefits, involving a regulation of blood pressure, glycemia, lipidemia. Therefore it's been proposed as one of the most efficient non-pharmacological treatment modalities in control of DM type 2. Hypoglycemic effect may be reached by increased need for glucose utilization by skeletal muscle during different training protocols (8, 9). Nevertheless, long-term hyperglycemia alters structural and functional properties of the heart, with an emphasis on endothelial dysfunction (10, 11). Exercise tolerance test, as non-invasive test, which involves treadmill and bicycle ergometer exercise, is proposed a great method for assessment of physical capacity of persons with DM type 2 if considering to start with moderate or vigorous fitness program. Additionally diabetic patients are strongly recommended to undergo this test for early detection of ischemic changes in myocardium, taking into consideration the high incidence of CHD (12-14).

Regarding all above mentioned data, the aim of our study was to establish ischemic heart changes in asymptomatic patients with diabetes type 2 by performing exercise treadmill screening test.

SUBJECTS AND METHODS

Study design

This study was conducted in the year 2018. The study protocol was approved by the Medical Ethics Committee of The Health Center Požega and was carried out according to the Declaration of Helsinki. All the participants were informed about the research protocol before giving their written consent to participate in the study.

Study population

In total 40 patients suffering from DM type 2 were included in the study. They were insulin-dependent patients, whose therapy included long- and rapid-acting insulin, in combination with metformine and sulfonylurea derivatives or other oral hypoglycemic drugs. A dose of long-acting insulin or insulin analogue was 14 units daily and rapid-acting insulin or its analogue was 24 units daily.

Procedure

All patients were subjected to physical examination, routine biochemical analysis, electrocardiography and echocardiography. Body weight, height and waist circumference were measured. All patients were instructed to prepare for exercise testing by excluding beta blockers from their therapy on the testing day. It was not necessary to exclude coronary dilators since there were no patients receiving this group of drugs, as well as amiodaron and propafen. Three hours before testing, patients had light meals, smokers (10 patients) didn't smoke and they received a dose of insulin reduced for 4 and regular dose of oral hypoglycemic medications.

Excluding criteria were reflected in the following: presence of aortic stenosis, inflammatory conditions such as pericarditis, myocarditis or endocarditis, blood pressure higher than 135/85 mmHg during resting and relaxation, rapid uncontrolled atrial fibrillation or other disorders of heart rhythm and cardiac decompensation. Exercise treadmill test (ETT) was conducted according to Bruce protocol. Maximum predicted heart rate was calculated by subtracting the person's age from 220 (210 for women).

12-lead ECG was used for continuously monitoring during the test and blood pressure was measured at rest and every 2 minutes during exercise and recovery period. Furthermore symptoms of all patients were continuously observed. If patient reached 85% of maximum predicted heart rate it was considered as satisfactory myocardial response. ETT test was considered positive if there is a horizontal (planar) or downsloping ST-segment depression of > 1mm. The value of BP up to 225 mm Hg is interpreted as normal. If patients had experienced fatigue and breathlessness, we would have interrupted test (16, 17).

Statistical analysis

IBM *SPSS* Statistics *19.0* for Windows was used for statistical analysis. Descriptive statistics was used to calculate average value ± standard deviation (SD). If there were patients with positive test results, the association between

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their clinical and laboratory characteristics and test results would be assessed using *Chi-square test for categorical data.* Value of $p<0.05$ was considered to be statistically significant.

RESULTS

Clinical characteristics of patients are presented in Table 1, while laboratory characteristics are presented in Table 2. Laboratory values were within reference range. All patients had negative screening test results, with no changes in echocardiography. They were able to complete the ETT, and changes in HR were presented in Figure 1. No ST segment elevations, no signs of insufficiency of peripheral circulation, no symptoms by the central nervous system such as dizziness, presyncopa, ataxy were observed. Furthermore the test was not interrupted due to patient request for any reason. Values of BP were within referent range during whole period of measurement. Additionally there were no changes in heart rhythm during the entire duration of the test.

DISCUSSION

It's been reported that mild inflammation, increased generation of pro-oxidants, enhanced coagulability, endothelial dysfunction etc play role in occurrence of CDV in patients with DM (17). People with diabetes have six to eight years shorter life expectancy compared to general population and the main reason doesn't lie in diabetes per se, but in developed CHD (18). The complex mechanisms associating CHD and diabetes have been under extensive investigation with the aim to discover novel approach, which would decrease mortality and morbidity rate in these patients. More severe manifestations of CHD are recorded in patients with diabetes, involving multiple, more peripheral and smaller blood vessels, which is closely related to pronounced difficulties in treatment (19, 20). Additionally due to the absence of symptoms, silent ischemia delays time for diagnosis and make the prognosis unfavorable. Therefore application of certain diagnostic procedures which would detect early ischemic myocardial changes is of a great importance in management of diabetes (21).

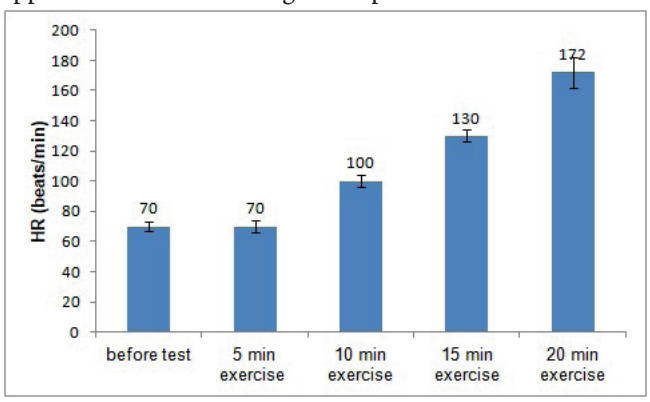

Figure 1. *The change of HR during exercise treadmill test*. Data are presented as *mean ± SD.*

Table 1. *Clinical characteristics of patients*. Data are expressed as mean ± SD.

Variables	Mean±SD
Age	33.05 ± 2.01
Sex M/F	25/10
Weight (kg)	87.5 ± 5.21
Height (cm)	177.5 ± 11.02
Waist circumference (cm)	90 ± 4.5
Duration of disease (years)	5.5 ± 1,1

Table 2. *Laboratory analysis and values of blood pressure and heart rate of patients. Data are presented as mean ± SD.* rHR- resting heart rate; rSBP-resting systolic blood pressure; rDBP- resting diastolic blood pressure; BG- blood glucose; HbA1c- glycated haemoglobin; *TC-* Total cholesterol; TG- total *triglycerides; LDL-C-* low-density lipoproteins cholesterol; *HDL-C-* high-density lipoproteins cholesterol; AST- aspartate aminotransferase; ALT- alanine aminotransferase; GGT- gamma-glutamyltransferase; TB- total bilirubin; AI- atherogenic index of plasma; CRP-C reactive protein; ESR- erythrocyte sedimentation rate; WBC- white blood cells; RBC- red blood cell; PLT- platelets; HgB- haemoglobine.

Variables	Mean±SD
rHR beats/min	77 ± 4
rSBP (mmHg)	135 ± 15
rDBP (mmHg)	85 ± 10
BG (mmol/l)	5.8 ± 0.1
HbA1c (%)	8.12 ± 1.51
TC (mmol/l)	5.9 ± 0.3
TG (mmol/l)	1.5 ± 0.1
LDL-C (mmol/l)	3 ± 0.8
HDL-C (mmol/l)	1.5 ± 0.1
AST (U/l)	30 ± 2
ALT (U/l)	25 ± 2
GGT (U/l)	25 ± 1
TB (Umol/l)	10 ± 0.6
Urea (mmol/l)	5.2 ± 1.4
Creatinine (mmol/l)	88 ± 3.1
AI	1±0.23
CRP (mg/l)	1± 0.2
ESR (mm/hr)	4 ± 1
WBC (cells /l)	8.4×10^9
RBC (cells/l)	$4{,}4 \times 10^{12}$
PLT (cells/l)	233×10^9
HGB (g/l)	126×10^{12}
K (mmol/l)	4 ± 0.2
Urine	Normal

We have chosen to apply ETT in our study since it has been proposed as a convenient test for assessing the presence of silent ischemia among patients with diabetes (22).

Our results have shown that no ST segment depression was found among observed patients and all ECG recordings were within reference range. It's been previously confirmed that not only ST-segment behavior during exercise may predict cardiovascular events, but also exercise time, heart rate and recovery of blood pressure. Moreover abnormal values of heart rate and recovery of blood pressure correlate with a greater risk for adverse cardiovascular outcome (23). Due to the presence of autonomic dysfunction it may be expected that chronotropic response to ETT in diabetic patients is deteriorated, reflected in inability of the heart to increase heart rate when exposed to physical activity (24). A drop in blood pressure after the exercise suggests poor prognosis regarding cardiovascular complications (16). In our study heart rate increased from the baseline 70 beats/min, up to 172 beats/min at the end of 20 minute exercise. We revealed physiological response of the blood pressure to exercise and the absence of progressive decline which would indicate failure of the heart muscle due to possible distinctive coronary disease. Moreover our findings clearly show that the coronary reserve in all examined patients is preserved, indicating that DM 2 doesn't progress in all persons so rapidly and duration time of about 5,5 years in our patients didn't alter myocardial integrity and function. It has been known that symptomatic patients with CHD have a longer disease history in comparison to asymptomatic patients with CHD. The link between the age and presence of CHD has also been reported, thus suggesting that higher positive predictive values of ETT for coronary artery stenosis is among patients older than 55 years (25).

On the one hand, the fact that CHD is frequently missed in the asymptomatic phase of disease emphasizes the need for performing ETT screening test in order to identify individuals who need to receive anti-ischemia therapy (26). Sensitivity of the exercise stress tests is 45–61%, while specificity is in range 70–90% (27). Beside the frequent use in clinical practice there are several limitations for the test application, such as impossibility of the patients with peripheral neuropathy, peripheral vascular diseases etc to exercise (13). Interestingly data regarding the performance of ETT in asymptomatic patients are controversial. Possibility of false positive responses to ETT in those patients require further confirmation by radionuclide imaging techniques (28). There is a recommendation for application of ETT in elderly patients with more than 10 years history of DM type 2 or shorter if the risk factors such as hypertension, hyperlipidemi, smoking are present (25).

A strong correlation between the presence of hypertension and dyslipidemia and CHD was revealed in patients with diabetes (13). However we weren't able to test the link between associated patients' characteristics and coronary artery stenosis since routine laboratory analysis, general medical examinations as well as ETT result of our selected patients proved to be in reference range. When encountering diabetes as a contemporary disease of mankind bringing so many cardiological complications (numerous forms of heart attacks, stents, by-passes etc), negative ETT results obviously indicate a hope that appropriate insulin and oral hypoglicemic therapy may preserve coronary function. However impact of novel medication, modification of nutrition intake or implementation of physical activity on maintenance of cardiovascular homeostasis shouldn't be missed.

Based on our result we may conclude that patients with DM type 2 achieved predicted heart rate during exercise stress test, without associated ST segment depression, followed by a physiological response of blood pressure. Our promissing results provide insight into the favourable prognosis of these patient in terms of cardiovascular events. Further studies are certainly necessary in order to gain more information about the role of ETT in early diagnosis of CHD in asymptomatic patients with diabetes, management of recognized patients with CHD and necessity for different treatment strategies.

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GAS TRANSPORT CHARACTERISTICS OF HEMOCORRECTORS AND PERFUSATES BASED ON PERFLUOROCARBON BLOOD-SUBSTITUTING EMULSIONS

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GASNO TRANSPORTNE KARAKTERISTIKE HEMOKOREKTORA I PERFUZATA ZASNOVANIH NA PERFLUOROKARBONSKIM EMULZIJAMA KOJE SE DODAJU U KRV

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ABSTRACT

This review summarizes the data regarding the gas transport characteristics of hemocorrection and perfusates on the basis of low concentrated drugs nano-sized perfluorocarbonic 20% Perftoran (a blood substitute, it is allowed for clinical use in Russia), 20% Ftoremulsion III (an improved blood substitute, registered in Russia), 10-20% Perfusol (a perfusion solution for perfusion of the isolated heart), 20% Ftorem (a cardioplegic emulsion for surgeries on the stopped heart) used in the biomedical field. The compensation of blood loss using traditional plasma substitutes without the gas transport function or with low gas transport characteristics leads to a decrease in the oxygen capacity of the resulting mixture and subsequently to deterioration in the oxygen transport characteristics of blood. The synthetic gas-transport blood substitutes can be used in the treatment of various forms of ischemia, such as carbon monoxide poisoning. Furthermore, recent results regarding the mechanism of COVID19 infection indicate a possible use of the synthetic gas-transport blood substitutes in the treatment and therapy of COVID19 infected patients.

***Keywords**: perfluorocarbon; synthetic gas-transport blood substitutes; ischemia treatment*

SAŽETAK

Ovaj rad sumira podatke koji se tiču gasno transportnih karakteristika hemokorekcije i perfuzata na osnovu lekova niske koncentracije perfluorokarbonskog 20% Perftoran velicine nano cestica(supstituta krvi koji je dozvoljen za kliničku upotrebu u Rusiji), 20% Ftoremulsion III (poboljšan supstitut krvi, registrovan u Rusiji), 10-20% Perfusol (perfuzioni rastvor za perfuziju izolovanog srca), 20% Ftorem (kardioplegijska emulzija za operacije na zaustavljenom srcu) korišćenih u biomedicinskoj oblasti. Kompenzacija gubitka krvi korišćenjem tradicionalnih supstituta plazme bez gasno transportne funkcije ili sa nisko gasno transportnim karakteristikama dovodi do smanjenja u kapacitetu kiseonika rezultujuće mešavine i zatim pogoršanju u transportnim karakteristikama kiseonika u krvi. Sinteticki gasno transportni supstituti krvi mogu se koristiti u lečenju različitih oblika ishemije, kao što su trovanje ugljen monoksidom, Osim toga, skoriji rezultati što se tiče mehanizma infekcije COVID-om 19 ukazuju na moguću upotrebu sintetičkih gasno transportnih supstituta krvi u lečenju i terapiji pacijenata inficiranih COVID-om 19.

***Ključne reči:** perfluorokarbon, sintetički gasno transportni supstituti krvi, lečenje ishemije*

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INTRODUCTION

One of the main pathophysiological problems in clinical practice is the elimination of hypoxia and delivery of oxygen to tissues and organs during blood loss using the oxygen-carrying blood substitutes.The compensation of blood loss using the traditional plasma substitutes without the gas transport function or with low gas transport characteristics leads to a decrease in the oxygen capacity of the resulting mixture and, accordingly, to ‘ deterioration in the oxygen transport characteristics of blood. A decrease in the blood oxygen capacity may not always be compensated by an increase in the blood flow rate and other adaptation mechanisms. Therefore, the creation and use of full-fledged hemocorrectors based on the gas-transport blood-substituting with perfluorocarbon drugs, that can compensate blood loss without reducing the oxygen capacity of the blood and its rheological characteristics, are currently becoming an urgent pathophysiological problem.

Since the last century, the gas-transport hemocorrectors based on perfluorocarbon blood-substituting emulsions-binary or multiphase systems, are actively used in the medical and biological field as multifunctional drugs, in particular, as the gas-transport substitutes for donor blood and perfusates for transplantation and preservation of organs and tissues (1,2,12,18-22).

It should be noted that the organic perfluorine compounds (PFOC) themselves dissolve any gases, including oxygen and carbon dioxide, much more than donor blood. Thus, in Russian perfluoroorganic compounds such as perfluorodecalin (PFD), perfluoromethylcyclohexylpiperidine (PFMCP) and perfluorotributylamine (PFTBA), the solubility of oxygen is about 40 vol.%, and carbon dioxide is 140-150 vol.% (16,17).

However, due to the low concentration (10-20%) of organofluorocarbon compounds (PFOS, gas carriers) in Russian perfluorocarbon preparations of the type 20% perfluorocarbon (approved for the clinical use) (9), 20% Fluoroemulsion III (registered) (11), 10-20% Perfusol (perfusion emulsion) (4), 20% Fluoroem (cardioplegic emulsion) (3,7), respectively, the preparations have a low oxygen capacity (5-7 vol.%), compared with donor blood (15-20 vol.%), which raises the question about the effectiveness of hemocorrection and these perfusates.

This review article is dedicated to the clarification of this issue.

Gas-transport characteristics of the perfluorocarbon blood-substituting drug Perftoran during compensation for 60-70% of acute blood loss in the experiment

To determine the gas transport efficiency, the perfluorocarbon blood-substituting emulsion Perftoran was studied. In an experiment on animals, blood loss was compensated with the drug Perftoran, in control experiments, blood loss was compensated with the classic plasma substitute drug Polyglucin (Dextran with mol. weight 60 thousand D). Under pressure control in dogs, in the aorta and superior vena cava, exchange replacement of 50 ml of blood per kg of body weight was performed in an isovolemic mode. During blood replacement and during the first 2 hours, pH, pO_2 and pCO_2 in arterial and venous blood were studied, the concentration of hemoglobin in the blood and the content of PFOS in the blood flow were calculated. The total content of O_2 in arterial and venous blood, chemically bound and physically dissolved oxygen was calculated.

For both groups, blood loss is characterized by a sharp decrease in the oxygen capacity of the blood (table 1). However, the content of physically dissolved oxygen in arterial blood when using the drug Perftoran is 3.5 times higher (2.94 ± 0.31) than in the group with the drug Polyglucin (0.88 ± 0.02) ($p<0.05$). This partially compensates for the decrease in the total value of the arterio-venous difference (a-vO_2 diff.) in oxygen and provides almost equal to the original (66.5±0.85) value of real oxygen transport, which is 70±0.4 ml/min.m2. In the group with Polyglucin with an equal amount of blood loss, the volume of infusion and identical values of the heart index (2.2±0.1; 2.1±0.1), this value is 37% lower and is 44±0.3 ($p<0.05$), which indicates an insufficient supply of oxygen to the tissues in the control.

As shown in studies (5.9), despite the low oxygen capacity in comparison with donor blood, the drug Perftoran during the compensation of blood loss in 60% -70% of the volume of circulating blood (BCC) successfully performs the gas transport function and maintains the normal value of oxygen transport, compared to the traditionally used hemodynamic plasma substitute – the drug Polyglucin.

Table 1. Indicators of oxygen supply of blood in the experiment on dogs when compensating for blood loss of 60-70% with Perftoran and Polyglucin

INDICATORS	Before blood substitution (control group)	After blood substitution (after 2 hours)	
		Polygluckin	Perftoran
Concentration of Hb (%)	12 ± 0,95	4,7 ± 1,2	3,6 ± 0,34
pO2 art. (mm Hg)	234 ± 13,6	259 ± 58	360 ± 42
pO2 ven. (mm Hg)	52 ± 8	53 ± 8,4	45 ± 3,4
pCO2 art. (mm Hg)	53 ± 3,1	40,5 ± 3	57 ± 7
pCO2 ven. (mm Hg)	59 ± 2,5	47,7 ± 4,1	61 ± 4,5
pH art.blood	7,35 ± 0,06	7,33 ± 0,10	7,34 ± 0,08
pH ven. Blood	7,23 ± 0,02	7,26 ± 0,10	7,24 ± 0,16
Concentration of O2 of art. blood bound to Hb (%)	16,1 ± 1,2	6,30 ± 0.7	5,06 ± 0,6
a-vO_2 diff. bound with Hb (%)	3,96 ± 0,9	1,41 ± 0,1	0,96 ± 0,05
Concentration of O_2 in art. blood in the physical solution (%)	0,86 ± 0,01	0,88 ± 0,02	2,94 ± 0,31
a-vO_2 diff. in the physical solution (%)	0,67 ± 0,01	0,69 ± 0,01	2,40 ± 0,30
Total value a-vO_2 diff. (%)	4,58 ± 0,9	2,01 ± 0,1	3,35 ± 0,3
Real transport of O_2 (ml/min m^2)	66,5 ± 0,35	44 ± 0,3	70 ±0,4 (!)

Gas-transport characteristics of the perfluorocarbon blood-substituting drug Fluoroemulsions III during compensation of blood loss in the clinic

The results of a clinical trial of the drug Fluoroemulsions III revealed its good clinical effectiveness. In most cases, patients who used the drug had severe comorbidities. Infusion of the drug to 24 patients was performed during endotracheal anesthesia under conditions of the oxygen content in the supplied gas mixture of 20-40 %. Out of the total number of patients (32 patients), only in one case, a minor adverse reaction to the drug was detected (chills, tachycardia, hypertension and hyperemia), after stopping the infusion, the patient's condition with acute intestinal bleeding normalized. The amount of intraoperative blood loss was from 0.5 to 1 liter. When evaluating the effect of the drug 20% Fluoroemulsions III, as a means with the gas transport characteristics, such indicators were important regarding the gas transfer as: Hb, pO_2, sO_2, measured before and after the surgery.

Studies have shown that the infusion of Fluoroemulsions III during the surgery, even in small doses of 200-600 ml, caused positive changes in hemostasis and acid-base state of the blood in patients. In all cases, without exception, after the drug infusion, even against the background of a decrease in hemoglobin, an increase in oxygen tension over 40% and an increase in sO_2 were observed (Table 2).

This clearly confirms the gas transport characteristics of the drug. The return of oxygen in the perfluorocarbon emulsion Fluoroemulsion III occurs more intensively and completely than in blood, since in perfluorocarbon, oxygen is physically bound and its return to tissues occurs along a concentration gradient, in contrast to blood, where oxygen is chemically bound and cleavage is completely different, more difficult. In the control group (table 3), where the traditional blood substitutes such as Polyglucin were used, the gas transport and hemodynamic indicators were significantly worse than in the experimental group. This occurred due to the fact that traditionally used drugs and salt solutions are ineffective gas carriers. So, if 20% perfluorocarbon emulsion Fluoroemulsion III at pO_2=760 mm Hg dissolves about 7 vol.% oxygen, then traditional drugs, under the same conditions, dissolve oxygen at the water level, about 2.3 vol.%, which is almost 3 times lower.

It is necessary to note an important fact, that, in the group of patients who received the perfluorocarbon preparation in the post-operative period, despite the fact that the level of hemoglobin significantly decreased due to blood loss from 142±2.5 (before the surgery) to 126 ± 2.4 g/l (after the surgery), the oxygen pressure of pO_2 in arterial blood significantly increased from 74.8±4.5 (before the surgery) to 131.6±5.5 mm Hg (after the surgery). This effect emphasizes the gas-transport characteristics of the drug Fluoroemulsions III. In addition, the oxygen saturation in the group of patients (who received Fluoroemulsion III in the postoperative period) increased from 90.6±3.2 (before the surgery) to 95.2± 3.4% (after the surgery), in comparison with the group on the drug Polyglucin, where the oxygen saturation in the blood of patients began to decrease from 90.1±2.4 (before the surgery) to 85.1±3.6% (after the surgery).

Table 2. Some indicators of the blood gas transfer during the intravenous administration of the drug Fluoroemulsions III in correction of blood loss in the clinic

INDICATORS	Before surgery	After surgery
Hb (g/l)	142 ± 2,5	126 ± 2,4*
pO_2 art. (mm Hg)	74,8 ± 4,5	131,6 ±5,5*
sO_2 (%)	90,6 ± 3,2	95,2 ± 3,4

*p < 0.05 compared to the indicators before the operation

Table 3. Some indicators of the blood gas transfer during the intravenous administration of the drug Polyglucin in correction of blood loss in the clinic

INDICATORS	Before surgery	After surgery
Hb (g/l)	138 ± 3,4	119 ± 3,8
pO_2 art. (mm Hg)	73,6 ± 2,6	69,2 ± 2,4
sO_2 (%)	90,1 ± 2,4	85,1 ± 3,6

As shown in studies (11), despite the low oxygen capacity of the drug Fluoroemulsion III in comparison with donor blood, clinical trials of the perfluorocarbon drug revealed its positive gas transport characteristics and clinical effectiveness.

Gas transport characteristic of the perfluorocarbon preparation 20% Perfusol emulsion (without a hemodynamic agent) for normothermic 4-hour perfusion of the isolated heart in the experiment

This section presents the gas transport characteristics of perfusates on the model of the isolated rabbit heart perfused during normothermy using the Langendorff method. Our research was conducted on 2 groups of hearts: 1-a group of control hearts perfused with Krebs-Henseleit solution (KNS); 2 - a group of experimental hearts perfused with perfusate based on 20% Perfusol emulsion.

When comparing the gas transport characteristics of the control solution and the Perfusol emulsion, it was found that the oxygen content in the arterial sample during perfusion with the Krebs-Henseleit solution was maintained at the level of 0.84 - 1.16 vol.% during 4 hours of perfusion, and in the venous - 0.55-0.38 vol.%. it was as an increase in the arterio-venous difference in oxygen, which was initially 0.3 vol.%, and at the end of the control time - 0.79 vol.%. With this oxygen supply, the heart, perfused according to Langendorff with a Krebs-Henseleit solution, consumes 1.9 ml/min*g in the initial state and 0.98 ml / min*g - after 4 hours of perfusion (table 4). A decrease in PO_2 after 4 hours of perfusion is associated with a sharp decrease in the value of the coronary flow (by 75%) and the development of edema. At the same time, the water content in the myocardial tissues increased from 78.1 ± 0.4% (intact hearts) to 84.9 ± 0.8% - by the end of the 4th hour of perfusion.

As it is known, the gas characteristics of the emulsion Perfusor are significantly higher than that of the conventional crystalloid solutions. Therefore, the oxygen content in the arterial and venous samples at an equal value of pO_2 (250-350 mm Hg) was 3 times higher than in the corresponding samples of the Krebs-Henseleit solution. Thus, the content of oxygen in the blood sample of the emulsion of Perfusal is 3,34-3,62 % during the entire perfusion time (4 hours). The arterio-venous difference in oxygen is significantly higher than in a similar indicator of a traditional solution. The oxygen consumption of the myocardium made up 5.45 and 4.6 ml/min . during the entire perfusion period. The coronary flow and degree of myocardial edema after 4-hour coronary perfusion with the Perfusol emulsion (without a hemodynamic agent) were, respectively, 5.2-3.6 ml/min . g and 81.1%, which is significantly better than in the group of hearts perfused with the traditional Krebs-Henseleit solution (KNS) (table 5).

Table 4. Oxygen supply to the myocardium during normothermic 4-hour perfusion of the isolated rabbit heart with the Krebs-Hanselate solution

INDICATORS	Result (after 15 min)	After 4 hours
pO_2 art. (mm Hg.)	269±31	370±18*
pO_2 ven. (mm Hg)	172±18	121±16*
Concentration of O_2 art. (v%)	0,84±0,1	1,16±0,07*
Concentration of O_2 ven. (v%)	0,55±0,06	0,38±0,05*
a-vO_2 diff. (v%)	0,3±0,05	0,79±0,08*
O_2 perfusion (ml/min · g)	1,9±0,3	0,98±0,2*
Coronary flow (ml/min · g)	6,1±0,72	1,5±0,26 *
Utilization of O_2 (v%)	37±7	56±6*

where *) < 0.05 in comparison with the initial indicators

As it is shown in studies (4,5),the adequate delivery of oxygen to tissues during perfusion of the isolated heart with perfusate-20% Perfusol emulsion provides a significantly better preservation of the myocardium than when using the traditional Krebs-Henseleit crystalloid solution.

Table 5. Oxygen supply to the myocardium during normothermic 4-hour perfusion of the isolated rabbit heart with the perfluorocarbon perfusol emulsion

INDICATORS	Result (after 15 min)	After 4 hours
pO_2 art. (mm Hg.)	358±41	392±64
pO_2 ven. (mm Hg)	235±39	250±34**
Concentration of O_2 art.(v%)	3,34±0,36 **	3,62±0,6**
Concentration of O_2 ven. (v%)	2,18±0,38**	2,33±0,36**
a-vO2 diff. (v%)	1,16±0,08**	1,3±0,24
O_2 perfusion (ml/min · g)	5,45±0,4**	4,6±0,8**
Coronary flow (ml/min · g)	5,2±0,6**	3,6±1,2**
Utilization of O_2 (v%)	31±6	35±8**

** p<0.05 compared to the indicators in table 2

Gas transport characteristics of the perfluorocarbon preparation of 10% Perfusol emulsion (with a hemodynamic agent) for hypothermic 24-hour perfusion of the isolated heart in the experiment

Hypothermic 24-hour perfusion of the dog heart with 10% Perfusol emulsion began immediately after isolation of the heart from the dog's body, without registering the initial level of vital activity of the graft, using the control group data as initial indicators. The heart started to be cooled by perfusing 10% Perfusol emulsion through the coronary arteries at temperature of 18-20 ℃. Then, the preservative was connected to a perfusion system located in a household refrigerator, and perfused retrograde with a peristaltic pump in a recirculating mode at temperature of 4-8 ℃.

Studies have shown (6) that the oxygen consumption of the myocardium during the heart preservation with 10% Perfusol emulsion was 0.31-0.37 ml / min.*100g. In the recovery period, the functional capabilities of the myocardium were measured after 24-hour hypothermic perfusion with 10% Perfusol emulsion on the stand. The heart activity was restored independently in all cases. The attention is drawn to the fact that before the heterotopic attachment to the neck vessels of the recipient dog, all the hearts were soft to the touch, and after switching to the stand, they contracted rhythmically during the entire observation period (6 hours). In total, the perfusion of the isolated heart was 30 hours, including 24 hours of the hypothermic perfusion on 10% of the Perfusol emulsion, 6 hours of perfusion after blood-feeding on the recipient dog.

Within 6 hours after the hypothermic perfusion, the isolated heart was switched to perfusion on a stand to load samples. So, 6 hours after the recovery, there was no difference between the control data and similar indicators in the experiment.

Table 6. Changes in the hemodynamic parameters, contractile function of the dog's myocardium and oxygen consumption by the myocardium after 24-hour hypothermic perfusion with 10% Perfusol emulsion

INDICATORS	Control group (without perfusion)	Experimental group after 6 hours of 24-hours perfusion
Heart rate (u./min)	121,5 ±8	132±4
BP system. (mm Hg)	127±4	120±4
BP diastolic. (mm Hg)	61,6 ±8	83,2±7
BP average (mm Hg)	94±3	100,5±4
Pressure in left ventricle (mm Hg)	125,3 ±3	117,2±4
End-diastolic pressure in left ventricle (mm Hg)	4,2 ±1,3	7,5± 1,3
Coronary flow (ml/min·100g)	104,3 ±5	102,5±6
Pressure in right atrium (mm H20)	100*	100*
Cardiac output (ml/min·100g)	612 ±69	575± 61
Utilization of O_2 (ml/100 g)	5,2 ±0,7	4,2± 0,4
CVR (mm Hg ·min ·100 g/ml)	0,9± 0,03	0,96± 0,05
Work of left ventricle (kg· m/min·100g)	0,77± 0,05	0,76± 0,08
+ dp/dt (mm Hg/sec)	2149± 182	2097± 231
- dp/dt (mm Hg/sec)	2126± 132	2306± 262
V_{max} (sec^{-1})	4,6± 0,4	4,4± 0,4
VCE (sec^{-1})	2,95± 0,3	2,97 ±0,2
O_2 perfusion (ml/min·100g)	7,07± 0,3	6,65± 0,5

Thus, the evaluation of functional capabilities of the heart with loading tests revealed a statistically significant difference between different blood flow to the left atrium (volume burden) and immediate response in the aorta and left ventricle, while there were no differences with similar indicators of the control group, which indicates a full recovery of the heart activity after 24-hour hypothermic perfusion of the isolated heart with 10% Perfusol emulsion.

Gas transport characteristics of the perfluorocarbon preparation 20% of the Ftorem - cardioplegic emulsion for perfusion-free preservation of the isolated heart in the experiment

A promising area of application of the perfluorocarbon emulsions is the cardioplegic solution for heart operations, which does not enter the major blood flow, but is used only when filling the coronary vessels.

Studies have shown (7) that use of the cardioplegic composition based on the perfluorocarbon - Ftorem emulsion while preserving the rabbit heart for 6 hours (14-16 °C) has a significant advantage compared to the crystalloid hyperkalic solution. Thus, after 6 hours of cardioplegia with the Ftorem emulsion (with 3 reperfusions), the cardiac activity was restored independently after the beginning of coronary perfusion. The amplitude of heart contractions in 30 minutes after the recovery was 89±6% of the initial level.

In the study of the Ftorem emulsion in cardioplegia on isolated hearts of dogs (3), in comparison with the traditionally used crystalloid solutions, under the same conditions, the restoration of heart activity on the traditional solutions was not observed, only single muscle contractions or ventricular fibrillation were recorded. As a comparison, 6-hour cardioplegia was used on the standard solutions: hyper potassium / hypertonic and novocain-containing. After initial assessment of the functional state of the isolated dog heart, the studied cardioplegic compounds were once introduced into the mouth of the aorta. In group I, where the hypertonic solution was used, it was not possible to restore the heart activity. In group II, when using the novocain-containing solution, only sluggish idiopathic muscle contractions of the myocardium were registered. In group III, the Ftorem emulsion was used in 5 out of 10 cases, spontaneous recovery of the heart contractions with the preservation of sinus rhythm was observed, and in the remaining cases, the rhythm was restored after 1-

2 defibrillator discharges. After switching to the stand, despite the burden, the hearts continued to contract rhythmically without conduction disturbances during the entire observation period. After the restoration of electrical and contractile activity, the majority of hemodynamic parameters did not differ from the control values. The cardiac output, blood pressure, and coronary blood flow were kept at the stable level. The heart rate decreased by 13%, and the speed indicators of myocardial contractility were reduced by the 15th minute (Vmax by 27%, VCE by 31%). +dp/dt did not differ from the original. Of course, the diastolic pressure in the left ventricle increased, but remained within the normal range, as well as the pressure in the left atrium. A comparative evaluation of the results of approbation of three cardioplegic solutions shows clear advantages of a solution based on the fluorocarbon emulsion- Ftorem.

The analysis of the conducted research allows us to identify the following positive effects of the perfluorocarbon emulsions of the Ftorem type on the myocardium:

- reducing the loss of intracellular potassium ions;
- inhibition of the entry of calcium ions into the cell;
- reducing the frequency of arrhythmias in the recovery period;
- reducing the sensitivity of the myocardium to catecholamines;
- slowing down the development of acidosis in the stopped myocardium;
- increasing the degree of relaxation of myofibrils;
- reduction of the tissue edema.

All the listed effects of the perfluorocarbon emulsion and its gas-transport characteristics provide the following advantages in comparison with the traditional cardioplegic solution: a single infusion of the perfluorocarbon emulsions stops the development of ischemic myocardial contracture, while the hypertonic crystalloid cardioplegic solution extends this period for only 10-15 minutes. In addition, the perfluorocarbonic emulsion type Ftorem:

- reduces the depolarization of the cell membrane and stabilizes it during cardioplegia;
- increases twice the oxygen supply of the myocardium after infusion of the cardioplegic solution;
- contributes to better preservation of the myocardium;
- stabilizes the pH of the myocardium;
- supports the aerobic and anaerobic metabolism;
- slows down the development of tissue edema even without a colloidal component;
- eliminates the risk of serious heart rhythm disorders;
- reduces the reperfusion reoxygenation damage;
- reduces the coronary vascular resistance;
- creates the additional relaxation, which facilitates free manipulation of the open heart;
- reduces the damaging effect of endogenous catecholamines at the beginning of cardioplegia and in the reperfusion period.

All this helps to preserve better the state of the myocardium after cardioplegia.

Table 7. Oxygen supply to the myocardium during the cardioplegic arrest of the isolated rabbit heart with the perfluorocarbon 20% Ftorem emulsion

INDICATORS	Before cardioplegie	1 hour after 6-hours cardioplegic heart failure
pO_2 art. (mm Hg)	158±29	190±39
pO_2 ven. (mm Hg)	22±1,8	31±3,9
Concentration of O_2 art. (v%)	12,5±1,2	15,4±1,1
Concentration of O_2 ven. (v%)	7,9±1,5	10,6±1,1
a-vO_2 diff. (v%)	4,6± 0,5	5,57±0,7
O_2 perfusion (ml/min·g)	6,4±0,7	5,78±0,5
pH art.	7,7± 0,08	7,6± 0,09
pH ven.	7,64±0,06	7,57±0,03

Thus, the pronounced protective effect of anoxia of the perfluorocarbon emulsion of the Fluoroem type, allows us to advise introduction of the cardioplegic perfluorocarbon solution for use in the cardiac surgery practice during the surgical treatment of severe patients with the acquired heart leaflet's defects.

Gas transfer and oxygen dissolution in the perfluorocarbon emulsions

According to calculations, contribution to the transport of O_2 of various blood components after an infusion of 10 ml/kg of the perfluorocarbon emulsion is: red blood cells carry 98.3% of total blood O_2, plasma-1.29%, PFOS emulsion-0.5% (10). It is clear that the role of PFOS in O_2 dissolution is small in comparison with red blood cells. However, if we take into account that the direct exchange of gases between cells, their environment and blood is carried out by free O_2 and CO_2 molecules, then the circulation of emulsion particles in the vascular flow significantly increased the oxygen capacity of plasma with physically dissolved oxygen (by 38%), especially its buffer capacity in relation to the consumed O_2, firstly. Secondly, the PFOS emulsion improves the oxygen supply of tissues by enhancing the extraction of oxygen by emulsion particles from erythrocyte hemoglobin. Third, an increase in mass transfer of O_2 to the presence of PFOS is much greater than in plasma, due to the accelerated diffusion of O_2 to PFOS. This is due to the fact that the Krog diffusion constant for O_2 and CO_2 is an order of magnitude greater in PFOS than in plasma. Fourth, an increase in O_2 mass transfer in the presence of PFOS occurs due to a higher rate of O_2 saturation in PFOS, since the rate of PFOS oxygenation is an order of magnitude greater than the oxygenation of red blood cells. Fifth, an increase in O_2 mass transfer in the presence of PFOS is associated with a large gas exchange surface in submicron particles of the PFOS emulsion. It is known that when the oxygen voltage decreases, the total amount of diffusion is preserved by increasing the gas exchange surface. In the PFOS emulsion with a relatively high oxygen voltage (pO_2 up to 100 mmHg), the total surface of the PFOS emulsion particles, for example, in 100 ml, is 1200 m^2, which allows to maintain the necessary amount of diffusion.

In addition, contribution of the PFOS emulsion to the model of anaemic hypoxia in the presence of perfluorocarbon particles in the bloodstream significantly affects the state of the blood-tissue gas balance, increasing the total flow of mass of oxygen from the blood to the tissues and carbon dioxide in the opposite direction. This is due to the fact that the rate of return and addition of O_2 and CO_2 by PFOS particles are not factors that limit the transport of gases. Thus, it was noted that when a small dose of the PFOS emulsion was monitored, the total CO_2 content in venous blood of experimental animals was significantly higher compared to the control. It turned out that the absolute amount of CO_2 dissolved in the PFOS particles (mm/l) at the pCO_2 of venous blood is 3 times lower than the values of total carbon dioxide. Therefore, the difference in the total carbon dioxide content between the experimental and control animals cannot be explained by a simple increased solubility of CO_2, but it can be attributed to the accelerated diffusion of CO_2 in the presence of its carrier (14,15). The main reason for this phenomenon is a change in the total mass transfer of blood gases.

CONCLUSION

It should be noted that the effective use of synthetic gas-transport blood substitutes is in various forms of hypoxia, caused, for example, by carbon monoxide poisoning, when CO binds with hemoglobin of red blood cells, forming carboxyhemoglobin and blocks the transfer of oxygen to organs and tissues; anemia, caused by the autoimmune acute intravascular hemolysis in severe infectious disease such as babesiosis (piroplasmosis, babesiasis) when there is a rapid destruction of both, its own and donor red blood cells with the release of large amounts of hemoglobin into plasma. With all these pathologies, the correction of hypoxia is not possible with the help of donor blood or red blood cell mass.

With the current situation in the world when there is the spread of coronavirus infection, which is accompanied (as noted by some authors) (23) by the penetration of the virus proteins into red blood cells and their binding with hemoglobin in such ways that "knocked out" iron ion from the blood plasma, hemoglobin loses its ability to bind enough oxygen. The use of synthetic gas perfluorocarbonic hemocorrectoring drugs like the Perftoran and Ftoremulsion III would be, as we believe, advisable for the treatment and correction of hypoxia.

In conclusion, it can be emphasized that hemocorrectors and perfusates based on the synthetic nano-sized perfluorocarbon emulsions are effective and safe for the gas transport in the medical and biological field for anti-ischemic protection of isolated organs and relief of various forms of hypoxia, when the use of donor blood is not possible due to any kind of reasons.

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ROLE OF COMBINING COLOUR DOPPLER AND GREY SCALE ULTRASOUND IN DIFFERENTIATING BENIGN FROM MALIGNANT OVARIAN MASSES

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ULOGA KOMBINOVANE COLOR-DOPPLER I GRAY-SCALE ULTRAZVUČNE METODE U DIFERENCIJACIJI BENIGNIH OD MALIGNIH OVARIJALNIH PROMENA

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ABSTRACT

The aim of this study was to evaluate ovarian masses with conventional grey scale ultrasonography and colour Doppler flow imaging and to assess the diagnostic reliability of these methods in differentiating benign and malignant ovarian masses.

We assessed 56 patients with an ovarian mass. Morphological characterisation of the mass was performed utilising the Sassone score. Colour Doppler parameters were recorded for each patient, and the Caruso vascular score was also applied. The results were compared with surgical/pathological and/or follow-up scans.

Using the Sassone score, overall reliability in differentiating ovarian masses had a sensitivity of 89.5% and a specificity of 78.4%. Using the Caruso score alone, we found a sensitivity of 89.5% and a specificity of 86.5%. Using the Sassone and Caruso scores together, we found a sensitivity of 94.7% and a specificity of 89.1%.

Combining both morphological and colour Doppler scores in the evaluation of ovarian masses obtained higher specificity, sensitivity, and accuracy than was obtained using a single score only.

Keywords: *Ovarian mass, Ultrasonography, Vascular score, Malignant, Benign.*

SAŽETAK

Cilj ove studije bila je evaluacija ovarijalnih tumora konvencionalnom crno-belom ultrasonografijom kao i kolor-dopler metodom sa ciljem diferentovanja benignih i malignih osobina tumora.

Uključili smo 56 pacijenata sa tumorima ovarijuma. Morfološka karakterizacija tumora je urađena pomoću Sasson skora. Za svakog pacijenta određeni su parametri pomoću kolor-dopler metode kao i Karuzo vaskularni skor. Ovi rezultati su upoređivani sa hirurškim i/ili patološkim nalazima.

Korišćenjem Sasson skora, opšta pouzdanost u proceni vrste ovarijalnih tumora ima senzitivnost od 89,5% i specifičnost od 78,4%. Korišćenjem Karuzo skora, pronašli smo senzitivnost od 89,5% i specifičnost od 86,5%. Upotrebom oba skora, Sassone i Karuzo, senzitivnost je bila 94,7% a specifičnost čak 89,1%.

Kombinovanjem morfoloških i kolor-dopler skorova u evaluaciji i dijagnostici ovarijalnih tumora dobijamo veću specifičnost, senzitivnost i tačnost dijagnoze u odnosu na pojedinačno upotrebljene metode.

Ključne reči: *Ovarijalni tumor, ultrasonografija, vaskularni skor, maligni, benigni*

INTRODUCTION

The diagnosis of ovarian masses is a frequent dilemma in clinical work. Most ovarian masses are benign (1,2). The most crucial step following identification of the ovarian mass is the perception of a level of malignancy; determining the level of malignancy will have a great impact on patient survival. It is the danger of malignancy that drives us to reliable and immediate diagnosis to decrease morbidity and mortality. Ovarian cancer represents a principle surgical difficulty in that it requires exhaustive and usually complicated therapies, and it greatly affects the patient's psychological and physical state. It has the greatest case fatality rate of all the gynaecological malignan-

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Table 1. Criteria used in Sassone score for sonomorphological characterisation (7)

Inner wall structure	Smooth	Irregularity less than 3 mm	Papillarities more than 3 mm	Not applicable	-
Septae	No septa	Thin less than 3 mm	Thick more than 3 mm	-	-
Wall thickness	Thin less than 3 mm	Thick more than 3 mm	Not applicable mostly solid	-	-
Echogenicity	Sonolucent	Low echogenic	Low echogenic with echogenic core	Mixed echogenicity	High echogenicity

Benign: <9; Malignant: ≥ 9

cies (3). Therefore, it is necessary to have a diagnostic tool for its immediate discovery and conventional treatment and to increase survival. Therefore, we want diagnostic means that allow proper classification of ovarian masses before surgery; hence, it is essential to identify the nature of the tumour before surgery. Ultrasonography (USG) is regarded as the basic imaging modality for recognising the nature of the ovarian mass as benign or malignant (4). USG morphologic assessment is still the most common modality for detecting ovarian cancer (5). USG relates morphologic images with gross macroscopic pathologic characteristics of ovarian masses. However, when morphologic characteristics only are used to predict the ovarian malignancy, there is a tendency to over-diagnose malignant tumours because of a large overlap between malignant and benign masses. Accordingly, the addition of colour Doppler imaging with pulsed Doppler spectral analysis enhances the characterisation of ovarian masses by means of quantitative blood flow measurements obtained from tumour vessels and thus improves sensitivity and specificity of the characterisation of ovarian masses (1-4). High operator dependence and extreme variability in the characteristics of ovarian tumours make a definite diagnosis still difficult. To overcome these limitations, applying scoring systems has been promoted. These scoring systems, joining various parameters of USG and colour Doppler, raise the sensitivity and specificity of diagnosis with excellent accuracy (6).

The purpose of this study was to evaluate the ovarian masses with conventional grey scale and colour Doppler flow imaging and to assess the diagnostic reliability of these methods in differentiating benign and malignant ovarian masses.

MATERIALS AND METHODS

This prospective study was carried out between August 2015 and January 2017. The study included 56 patients who were clinically suspected to have ovarian neoplasm and referred for USG and Doppler examinations. Ethical clearance for the study was obtained from our local institutional scientific and ethics committee with approval number 32 / 2015 before the commencement of the study. Informed consent for all participating patients was obtained. All patients were examined on GE Voluson E6 Color Doppler Machine with 3.5–5 MHz convex and 7.5 MHz transvaginal transducers and with grey scale, power, and spectral Doppler. The detailed history of all patients was studied, and complete examination was performed. USG preferably was performed during the proliferative phase of the menstrual cycle in premenopausal women. The same radiologist evaluated all the cases. Scanning was performed in the supine position. The whole of the abdominal cavity was scanned in longitudinal and axial plane with particular reference to the pelvic cavity. The ovaries were recognised. Ovarian masses in either ovary, if seen, were assessed. In uncertain cases of ovarian masses on transabdominal USG, transvaginal USG was done to exclude extra-ovarian masses. The Sassone scoring system on the basis of morphological parameters was applied where a score ≥9 is considered to be probably malignant. Table 1 shows the Sassone scoring system, which is based on the visualisation of the inner wall structure, wall thickness, septae and solid part echogenicity. Subsequently, power and Doppler flow imaging and spectral analysis were performed. Doppler parameters were optimised for detection of flow and calculation of impedance indices. Flow results were recorded as being absent or present and further characterised as normal or increased. Normal flow was characterised by fine branching vessels, no evidence of "hot-spots"/aliasing, and presence of peripheral flow. The flow was classified as increased if di-

Table 2. Criteria used in Caruso score (8)

Vessels location	Absent	0
	Present	1
	Peripheral	0
	Septal	1
	Central	2
Arrangement of vessels	Regular	0
	Random	2
Waveform pattern	Sharp with diastolic notch	0
	Smooth without notch	2
Lowest RI	More than 0.43	0
	Less than 0.43	2

*Benign: <5; Malignant: ≥ 5. RI: Resistive index

Table 3. Distribution of ovarian masses according to age and parity.

		Histopathology		
		Malignant %	Benign %	P value
Age	<30	21.1	29.7	0.287
	30---39	15.8	32.4	
	40---49	15.8	13.5	
	=>50	47.4	24.3	
	Mean±SD (Range)	45.0±15.5(20-67)	38.2±12.2(20-67)	
Parity	Nulliparous	26.3	16.2	0.016
	P1	10.5	37.8	
	P2	10.5	29.7	
	P3	26.3	10.8	
	P4 & more	26.3	5.4	
	Mean±SD (Range)	2.3±1.8(0-5)	1.5±1.1(0-4)	

SD: Standard deviation, P: Para.

lated prominent parenchyma vessels were present; "hot-spots" and aliasing were seen in colour flow mapping. The vessel location (peripheral, central, and septal), arrangement (regular/random) and morphology (normal-fine tapering vessels versus abnormal dilated prominent vessels, focal stenosis, aneurysms, blind-ending lakes and dichotomous branching) were also noted. Spectral Doppler study including RI (Resistivity index), PI (Pulsatility index), PSV (Peak systolic velocity) and presence or absence of dicrotic notch were recorded in each patient. Caruso score (Table 2) was applied for further characterisation of the mass where a score ≥5 was supposed indicative of malignancy. Benign and malignant classification of the ovarian masses was done depending upon the grey scale and colour Doppler USG. The results were correlated with the histopathological findings.

Table 4. The histopathological diagnosis of the studied ovarian masses.

	Histopathological diagnosis	No. (%)
Benign	Serous cystadenoma	8 (14.29)
	Mucinous cystadenoma	4 (7.14)
	Mature teratoma	13 (23.21)
	Haemorrhagic cyst	5 (8.93)
	Fibrothecoma	2 (3.57)
	Serous cystadenofibroma	2 (3.57)
	Endometriosis	2 (3.57)
	Epidermoid cyst	1 (1.79)
	Total	37(66.07)
Malignant	Serous cystadenocarcinoma	5 (8.93)
	Mucinous cystadenocarcinoma	3 (5.35)
	Endometroid adenocarcinoma	2 (3.57)
	Immature teratoma	7 (12.50)
	Brenner cell	1(1.79)
	Fibrosarcoma	1(1.79)
	Total	19 (33.93)

Statistical analysis

Statistical Package for the Social Sciences version 20 (SPSS 20) was used for both data entry and data analysis. Discrete variables were displayed as a number (%). Chi-square test (or Fisher's exact test when appropriate) was used to test the significance of the relationship for the discrete variable. P-value of < 0.05 was regarded as significant.

RESULTS

Fifty-six patients were included in this study. Thirty-eight (68%) patients were pre-menopausal, and 18 (32%) were postmenopausal women. The mean (± SD) age of patients included in the study was 45.0 ±15.5 years (range 20-67 years) for malignant masses and 38.2±12.2 years (range 20-67 years) for benign masses. In correlation with the parity, the malignant masses were significantly noted more among nulliparous and para 3 women, while the benign masses were more among para 1 women. Table 3 shows the distribution of the ovarian masses according to age and parity. Table 4 summarises the histopathological diagnosis of 56 ovarian masses studied where 37 (66.07%) were benign and 19 (33.93%) were malignant.

Table 5 shows the distribution of 56 patients according to the Sassone, Caruso and combined scoring systems and its correlation to the finally confirmed histopathological diagnosis. Out of 37 benign cases, the Sassone scoring system alone was able to diagnose 29 (78.3%) cases, the Caruso scoring system alone was able to identify 32 (86.4%) cases, and the combined scoring system was able to identify 33 (89.1%) cases. Out of 19 malignant cases, the Sassone scoring system alone was able to diagnose 17 (89.4%) cases, the Caruso scoring system alone was able to diagnose 17 (89.4%) cases, and the combined scoring system was able to diagnose 18 (94.7%) cases. These findings, regarding the Sassone scoring system alone, had a sensitivity of 89.5%, a specificity of 78.4%, a positive predictive value (PPV) of

Table 5. Comparison between Sassone, Caruso and combined scoring systems and histopathology

	Benign	Malignant	Total
Sassone score			
Benign (0-8)	29	2	31
Malignant (≥9)	8	17	25
	37	19	
Caruso score			
Benign (<5)	32	2	34
Malignant (≥5)	5	17	22
	37	19	
Combined score			
Benign	33	1	34
Malignant	4	18	22
	37	19	

86.0%, a negative predictive value (NPV) of 93.5% and an accuracy of 82.1%; the findings of the Caruso scoring system alone had a sensitivity of 89.5%, a specificity of 86.5%, a PPV of 77.3%, an NPV 94.1% and an accuracy of 87.5%. Using both the Sassone and Caruso scores together, we found a sensitivity of 94.7%, a specificity of 89.1%, a PPV of 81.8%, an NPV of 97.0% and an accuracy of 91.0%.

Table 6 gives comparative efficacy of Sassone, Caruso, and combined scoring systems in differentiating benign from malignant ovarian masses and shows that the combined scoring system is a better performing scoring system.

DISCUSSION

Today, the commonly applied means for distinguishing between malignant and benign ovarian masses are the physical examination, serum tumour markers, and grey scale and colour Doppler USG (9). Colour and pulsed Doppler can improve preoperative diagnosis of ovarian tumours when compared to transvaginal sonography alone or tumour marker assessment (10). Although grey scale USG is sensitive in identifying ovarian carcinoma, its reliability has not been enough to preclude further invasive methods, such as laparoscopy and laparotomy. Colour Doppler imaging and spectral Doppler imaging have been reviewed as potential means of increasing the specificity of grey-scale USG in differentiating benign from malignant masses (11,12).

Timmerman D et al.(13) in their prospective validation study, which was conducted in 19 USG centres in

Table 6. Statistical comparison between two scoring systems

Statistical parameter	Sassone scoring system %	Caruso scoring system %	Combined scoring system %
Sensitivity	89.5	89.5	94.7
Specificity	78.4	86.5	89.1
PPV	68.0	77.3	81.8
NPV	93.5	94.1	97.0
Accuracy	82.1	87.5	91.0

PPV: Positive predictive value, NPV: Negative predictive value

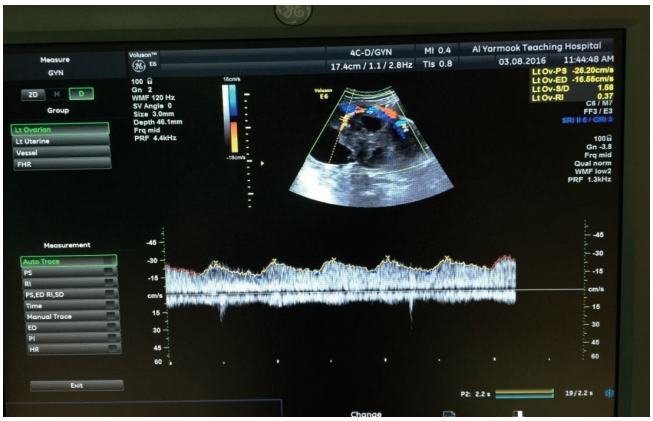

Figure 1. Spectral Doppler USG of 47 year-old patient shows complex ovarian mass with internal vascularity and low RI=0.37 diagnosed as serous cyst adenocarcinoma on histopathology

eight countries, concluded that the use of the simple USG rules (shape, size, solidity, and results of colour Doppler examination) to distinguish benign from malignant ovarian masses has the potential to improve the management of women with an ovarian mass.

Characteristics that raise the suspicion of malignancy in USG include the presence of thick septa, papillary projections, heterogeneous echotexture, and septa greater than 3 mm in thickness or which have flow on colour Doppler USG (14,15). Neovascularisation in the tumour always offers lower resistance to blood flow in malignant neoplasms (Fig 1).

Benign tumours have been characterised as being unilocular, with thin septae, homogenous iso echogenicity and thin wall capsule (16).

In our study, using only a grey scale Sassone scoring system, out of 37 benign tumours, 29 were correctly diagnosed and 8 were misdiagnosed. Out of 19 malignant tumours, 17 were correctly diagnosed as malignant and 2 were misdiagnosed as benign. Using Caruso scoring system, out of 19 malignant masses, 17 were correctly diagnosed. Using the combination of both scoring systems, out of 19 malignant cases 18 were correctly diagnosed. The only case, which was not diagnosed, was of immature teratoma. In this case, the tumour was of mixed echogenicity without solid mass or vascularisation. Accordingly, out of 37 benign masses, 33 were correctly diagnosed as benign and 4 were misdiagnosed as malignant; these were 2 cases of fibrothecoma and 2 cases of serous cystadenofibroma. In these cases, the tumours were encountered as unilocular cysts with solid areas and central flow.

In our study, Colour Doppler results showed predominantly peripheral localisation of vessels in benign masses (65%) and predominantly central or septal vessel localisation (81.8%) in malignant masses. This agrees with the results of Jokubkiene et al. (17) who found that 57% of benign masses showed peripheral vascularisation versus 70% of malignant masses that showed central vascularisation.

In our study, the RI alone was an insufficient discriminating parameter, as there was overlap between benign and

malignant masses. The RI cut-off value of <0.43 used had a significant p-value (<0.0005). Pulsatility index <1.0 had a sensitivity of 73.6% and a specificity of 64.9%, and there was a significant overlap between malignant and benign masses. Ueland et al. (16) reported sensitivity and specificity of 52.8% and 77.6%, respectively, using the cut-off value of PI < 1. In spite of that, Abbas et al. (18) reported that PI < 1 was an important feature of malignancy (80.4%), but PI < 1 was also found in 15.7% of benign masses. Thus PI alone cannot be a reliable parameter to detect malignancy. Shah D et al. (19) reported sensitivity (97.5 %) and specificity (84.1 %) with *PI* and *RI* values of <1.0 and <0.6, respectively, in their multi-parameter analysis utilising B-mode USG along with Colour Doppler and Spectral Doppler to differentiate between malignant and benign ovarian tumours. These findings are correlated with our result.

In the present study, B mode USG along with Doppler showed a sensitivity of 94.7%, a specificity of 89.1%, a PPV of 81.8%, an NPV of 97.0% and an accuracy of 91.0%. These results agreed with those of Abbas et al. (18) who were using a new scoring model (Assiut Scoring Model {ASM}), in which they used two-dimensional USG and Doppler features and showed a sensitivity of 93.5%, a specificity of 92.2%, a PPV of 82.7% and an NPV of 97.3%, with overall accuracy of 92.6%. Our results also agree with the results of Dhwani et al. (20), who conclude that using the combination of both grey scale and colour Doppler in differentiating benign from malignant ovarian masses gives results with more accuracy. Furthermore, our results agree with those of Malhotra A et al. (21), who conclude that grey scale USG combined with Colour and Spectral Doppler is superior to grey scale USG alone in differentiating benign and malignant adnexal masses. Gagandeep et al. (22) evaluated 30 patients with ovarian mass in their study, and they showed a sensitivity of 91.7% and a specificity of 77.7% when using the Sassone score alone, a sensitivity of 83.3% and a specificity of 88.9% when using Caruso score alone, and a sensitivity of 90.9% and a specificity of 93.3% when using both scores together. These findings are well correlated with our results.

Based on the results of our study, patients with masses score < 5 can be managed in the gynaecological unit by a gynaecologist, either conservatively or surgically, according to their features. Patients with masses score ≥ 8 must be referred to a gynaecological oncologist and be managed in specialised oncology centres. Patients with masses score 4-6 are suspicious with high possibility of malignancy if score ≥ 6, so further investigations may be ordered such as MRI.

CONCLUSION

There is significant overlap in the morphologic features of different ovarian masses. The combination of grey scale USG with colour and spectral Doppler is recommended as the leading diagnostic modality in patients with an ovarian tumour. This combination gives better diagnostic achievement than an individual method and accordingly will establish the definite diagnosis of malignancy early in the course of the disease.

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THE PREVALENCE OF DEPRESSION AND ANXIETY AND THEIR LIFESTYLE DETERMINANTS IN A LARGE SAMPLE OF IRANIAN ADULTS: RESULTS FROM A POPULATION BASED CROSS-SECTIONAL STUDY

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PREVALENCIJA DEPRESIJE I ANKSIOZNOSTI I NJIHOVIH DETERMINANTI ŽIVOTA U VELIKOM UZORKU IRANACA: REZULTATI STUDIJE PRESEKA

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ABSTRACT

Association of lifestyle-related factors and mental health has been less studied in Middle Eastern countries. This study aimed to examine the prevalence of two common mental health problems, i.e., depression and anxiety, and their lifestyle determinants in a large sample of Iranian population.

This study was conducted within the framework of SEPAHAN population based cross-sectional study (N=4763(. The General Practice Physical Activity Questionnaire (GPPAQ) was used to assess physical activity and the Iranian-validated version of Hospital Anxiety and Depression Scale (HADS) was applied to screen for anxiety and depression. Logistic regression was used as the main statistical method for data analysis by SPSS version 16.0. A P-value <0.05 was considered to be statistically significant.

The risk of anxiety and depression was 2.5 (OR=2.56,95% CI: 1.97-3.33) and 2.21(1.83-2.67) times higher in women than men, respectively. With every one-year increase in the age, the risk of anxiety decreased by 2% (OR=0.98,95% CI:0.97-0.99). Individuals with higher education had 56% lower risk of anxiety (OR=0.44,95% CI: 0.36-0.55) and 46% depression (OR=0.54,95% CI: 0.46-0.64) than the undergraduate group, and the risk of depression in the inactive (less than one hour of activity per week) group was 27% higher than the active group (OR=1.27,95% CI: 1.06-1.51). The risk of anxiety in the non-smoker group was 65% (OR=0.35,95% CI: 0.20-0.59) and depression was 64% lower than among smokers (OR=0.34,95% CI:0 .22-0.53). In the ex-smoker group, the risk of anxiety was 60% (OR=0.40,95% CI:0.19-0.85) and depression was 59% lower than for the smoker group (OR=0.41,95% CI: 0.24-0.73).

SAŽETAK

Udruženje faktora vezanih za način života i mentalnog zdravlja u zemljama Bliskog istoka je dosada malo istraživano. Ova studija imala je za cilj da se ispita prevalencija dva česta mentalna zdravstvena problema, tj. depresiju i anksioznost, i njihove determinante u velikom uzorku iranske populacije. Studija je sprovedena u okviru SEPAHAN-a studije preseka (n = 4763). Upitnik opšte fizičke aktivnosti (GPPAQ) je korišćen za procenu fizičke aktivnosti i iranska validirana verzija skale bolničke anksioznosti i depresije (HADS) primenjena je za praćenje anksioznosti i depresije u populaciji. Logistička regresija je korišćena kao glavni statistički metod za podatke u programu SPSS verzije 16.0. Statistički značajnom je smatrana p vrednost <0,05.

Rizik od anksioznosti i depresije bio je 2,5 (OR = 2.56,95%) CI: 1,97-3,33) i 2,21 (1,83-2,67) puta više nego kod žena muškaraca. Sa svakim jednogodišnjim povećanjem starosti, rizik od anksioznosti se smanjio za 2% (OR = 0,98, 95% CI: 0,97-0,99). Pojedinci sa visokim obrazovanjem imali su 56% manji rizik anksioznosti (OR = 0,44, 95% CI: 0,36-0,55) i 46% depresije (OR = 0,54, 95% CI: 0,46-0,64) nego grupa studenata, i rizik od depresije kod neaktivnih (manje od jednog sata nedeljno) ispitanika je bila 27% veća od aktivne grupe (OR = 1,27 95% CI: 1,06-1,51). Postoji rizik od anksioznosti u grupi nepušača i bila je 65% (OR = 0,35 95% CI: 0,20-0,59) i depresije, koja je bila 64% niža nego kod pušača (OR= 0,34 95%) CI: 0,22-0,53). U grupi bivših pušača, rizik od anksioznosti je bio 60% (OR = 0,40 95% CI: 0,19-0,85) i depresija je bila zastupljena 59% manje nego u grupi pušača (OR = 0,41 95% CI: 0,24-0,73). Rezultati ove studije pokazali

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This current study's results demonstrated significant associations between unhealthy lifestyle factors and increased risk of anxiety and depression. Hence, special attention must be paid to preventive intervention programmes aiming to enhance healthy lifestyle among at-risk populations.

Keywords: *Anxiety, Depression, Life Style, Prevalence*

su značajne povezanosti između nezdravih faktora načina života i povećanog rizika od anksioznosti i depresije. Dakle, mora se posvetiti posebna pažnja preventivnim programima sa ciljem poboljšanja zdravog načina života među rizičnim populacijama.

Ključne reči: *anksioznost, depresija, životni stil, prevalencija*

INTRODUCTION

Mental health is increasingly being considered as one of the main components of public health in society. Mental health problems are common and their prevalence has increased in many countries around the world (1, 2). About half of American and 40% of European populations met the criteria for psychiatric disorders throughout their lives (3, 4). The prevalence of mental health problems in Iran has been reported to range from 10% to 20% (5, 6). Depression and anxiety are the most common chronic mental health problems among the general population, which negatively affects the quality of life, performance and productivity of individuals as well as society. As a result, these illnesses have been considered to be a public health priority. (7-9).

Mental health is not only influenced by personality traits, living conditions and important life event stressors, but much evidence also suggests that the routine behaviours in the framework of an individual's life style can also have an effect (10). Prospective studies revealed a bidirectional association between mental health and lifestyle factors, in which healthy lifestyle has a positive impact on enhancing mental health, including reducing depression and anxiety symptoms. Better mental health was associated with greater physical activity, normal body mass index, and non-smoking (10,11,12). The association of different lifestyle dimensions including physical activity (13), smoking (14), body mass index (15), and diet (16) with various physical illnesses such as cancer, heart disease and stroke was also shown (10). The relationship between lifestyle and mental health has been shown in various studies. People with depressive symptoms have been found to have an unhealthier lifestyle than people without depressive symptoms. For example, anxiety and depression were significantly lower in both genders among those who were physically inactive, had an unhealthy diet and were smokers (17-19).

People with mental health problems require not only clinical treatments but also behavioural interventions for promoting lifestyle factors in order to enhance mental health. In fact, the need for health promotion, prevention programmes and treatment of mental disorders is certainly the primary health challenge of the 21st century.

If a population-based study shows that lifestyle factors are associated with anxiety and depression, general education in the field of lifestyle can be effective in reducing these disorders and their outcomes.

Most of the previous studies in this field to date have focused on one aspect of lifestyle and primarily on physical illness. AdditionallyIn addition, these studies have been conducted in specific instead of general populations. The evaluation of the simultaneous impacts of multiple lifestyle factors on mental health problems provides more reliable conclusions. This study is the first large population-based study among Iranian adults aimed at investigating the association of multiple lifestyle and demographic factors with anxiety and depression.

METHODS

Study design and participants

The current cross-sectional study was conducted in the framework of the Study on the Epidemiology of Psychological, Alimentary Health and Nutrition (SEPAHAN), describing the epidemiological aspects of functional gastrointestinal disorders and their association with lifestyle and psychological factors in 2010. Details about SEPAHAN have already been published (20). The studied population was selected from among 4 million people living in 20 counties across the Isfahan Province, Iran. Multistage random cluster sampling was performed by geographical regions to select participants from each region. The participants were selected from healthy people who lived in Isfahan Province. The questionnaires were issued to the participants in their home and workplace, and the participants answered the questionnaires in their free time. All data were collected anonymously and confidentially. Participation in this study was completely optional. To increase the accuracy of data and response rate, data were collected in two separate waves. The first wave was implemented in April 2010, and participants returned completed questionnaires within 7 days, and the second wave was implemented in mid-May 2010. In the first wave, 8,691 questionnaires out of 10,087 distributed questionnaires were returned (response rate: 86.16%). Data regarding gastrointestinal and mental health disorders were gathered in the second phase (response rate: 64.6%). In

the end, 4,763 questionnaires obtained in the second wave were matched with their equivalent questionnaires in the first wave. In this study, we used the data from 4,763 adults. The protocol of study was approved by the ethics committee of Isfahan University of Medical Sciences (IUMS) and was clarified for all the participants before participating in the study. Written informed consent was obtained from all participants.

Study instruments and variables assessment

The physical activity of study participants was assessed using the General Practice Physical Activity Questionnaire (GPAQ). This questionnaire is a simple validated screening tool for ranking the physical activity of adults by focusing on current general activities (21). Participants were classified into 4 categories: active (>3 h/week), moderately active (1–3 h/week), moderately inactive (<1 h/week), and inactive (no physical activity), based on the type and intensity of their physical activities in work hours and during the weekends. Finally, participants were categorized into two major groups: active and inactive. Participants were classified as active if they engaged in physical activity for more than one hour per week and inactive if they engaged in less than one hour of physical activity per week. The validity of the GPAQ for the assessment of habitual physical activity levels has been shown in earlier studies (21).

In SEPAHAN, information about mental health disorders, including depression, anxiety and psychological distress, was obtained from the validated Iranian version of Hospital Anxiety and Depression Scale (HADS). HADS includes two discrete parts assessing the severity of anxiety and depression. There are 7 questions with a four-point rating scale in each part. Higher scores demonstrate a greater severity of anxiety or depression. The possible scores ranged from 0 to 21 for both disorders. According to the values suggested by earlier studies assessing the validity and reliability of HADS in Iranians, scores of 8 or higher in either part were considered to indicate the presence of anxiety or depression, while scores of 7 or less were considered normal (22).

Assessment of other variables

Demographic data were collected regarding age (years), sex (male, female), marital status (single, married), educational level (less than diploma, diploma based on 12 years of formal education, university graduation). Participants who had a diploma or less than a diploma were considered to be undergraduates and those who had university educational levels were considered to be university graduates. Health data regarding smoking (current smoker, ex-smoker, non-smoker), weight (kilograms) and height (centimetres) were obtained using a self-reported questionnaire. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in metre. In our validation study of 200 participants from the same population, we found that the correlation coefficient between self-reported and the measured weight and height were 0.95 (P<0.001) and 0.83 (P<0.001), respectively. The correlation coefficient for the computed BMI from self-reported values and from the measured values was 0.70 (P<0.001). Participants were classified into three categories based on their BMI: normal weight (18.5–24.9 kg/m^2), overweight (25–29.9 kg/m^2) or obese (more than 29.9.0 kg/m^2).

Statistical analyses

Continuous and categorical data were presented as the mean±SD and frequency (percentage). Continuous data were compared between depressed and anxious and non-affected participants using independent samples t-test and categorical data using the chi-square test. Multiple logistic regression analysis was used for evaluating the association between demographic and lifestyle determinants of anxiety and depression, and the associations were depicted as odds ratio (OR) and 95% confidence interval (95% CI for OR). All statistical analysis was conducted using SPSS version 16 (SPSS Inc, Chicago, IL, USA).

RESULTS

Table 1 presents the basic characteristics of study participants and the prevalence of anxiety and depression in categories of demographic and life style variables. Among the participant samples, 2106 (44.2%) were male and 2657 (55.8%) were female. The mean age was 36±8.09 years and the mean BMI was 25±4.64.

The results showed that the prevalence of depression and anxiety was 28.8% and 14% in the total population, respectively. The prevalence of both anxiety and depression disorders in women was significantly higher than men (P = 0.001). Age was significantly associated with anxiety (P = 0.02), in which the prevalence of anxiety was higher among younger than the older people (older than 50) (14% vs. 7%). Findings also showed that the prevalence of depression was significantly higher in single people (32.2%) than the married people (27.9%). (p = 0.01). Both anxiety and depression were less prevalent in those people with a higher level of education (p = 0.001).

The prevalence of anxiety in physically inactive people was significantly higher than among physically active people (15% vs. 11%); similar results were also observed regarding and the depression (31% vs. 23%) (for both, P = 0.001).

The study of smoking status in the total population showed that only 3.6% were current smokers, 4.7% were ex-smokers and the remaining population never smoked; among smokers 18.4% were anxious compared to 13.6% of those who had never smoked and 12.0% of ex-smokers who were suffering from anxiety. However, these differences were not statistically significant. Similar results were found regarding the prevalence of depression

Table 1. Prevalence of depression and anxiety in categories of demographic and lifestyle variables.

	Total sample Frequency (%)	Prevalence (%) of anxiety	p_value	Prevalence (%) of depression	p_value
Male	2106 (44.2)	10.0	0.001	22.2	0.001
Female	2657 (55.8)	17.2		33.9	
Age (Yrs)					
34-19	1782 (43.9)	14.6	0.021	27.8	0.387
50-35	2085 (51.4)	14.1		29.8	
>50	191 (4.7)	7.3		28.8	
Single	3776 (81.2)	14.1	.6290	27.9	.0120
Married	874 (18.8)	13.5		32.2	
Undergraduate (Diploma and less than Diploma)	1986 (42.8)	17.9	0.001	33.6	0.001
Graduate (University)	2650 (57.2)	11.1		24.9	
Physically inactive (More than one hour per week)	2855 (65.2)	15.1	0.001	31.4	0.001
Physically active (Less than one hour per week)	1522 (34.8)	11.2		23.3	
non-smoker	3856 (91.7)	13.6		27.6	
ex-smoker	196 (4.7)	12.0	.2010	27.2	.0040
smoker	153 (3.6)	18.4		40.1	
Normal-weight(<25Kg/m^2)	228 (5.0)	16.8		28.2	
Overweight (25-29.9)	2199 (48.2)	13.0	.1740	27.8	0.320
Obese (>30)	2133 (46.8)	14.4		32.4	

P-values resulted from Chi-square test.

in BMI categories (32.4% among smokers, 27.8% among those who never smoked and 28.2% among ex-smokers) (p = 0.32). (Table 1).

To investigate the multivariable association of demographic and lifestyle variables with anxiety and depression, two logistic regression models were separately fitted (Table 2). The results of the logistic regression analyses showed that gender, age, education and smoking were significant determinants of suffering from anxiety and gender, education, physical activity and smoking were significant predictors of being depressed.

The risk of anxiety in women was 2.56 times higher than men (OR=2.56; P<0.0001). Women were also more than twice as likely to be affected by depression than men (OR=2.2; P<0.0001). Older age was inversely associated with a risk of anxiety; with a one-year increase in age, the risk of anxiety decreased by approximately 2% (OR=0.98; P= 0.02), but depression was not significantly associated with age (P= 0.35).

Level of education was significantly associated with anxiety and depression disorders. Among those with a higher level of education, the risk of anxiety was 56% lower than among less educated people (OR=0.44; P <0.0001). The odds of being affected by depression among people with a higher level of education was 46% less than people with a lower educational attainment (OR=0.54; P <0.0001).

The results of the regression logistic analysis showed no significant association between anxiety and physical activity, while the risk of depression was significantly higher among inactive people (OR=1.27; P = 0.01). In our multivariable logistic regression analyses we observed a significant association between a smoking habit and depression

Table 2. The results of logistic regression analysis for the association demographic and lifestyle determinants of anxiety and depression

	Anxiety		Depression	
	OR (95% CI)	p_value	OR (95% CI)	p_value
Female	2.56 (1.97-3.33)	<0.0001	2.21 (1.83-2.67)	<0.0001
Male (Reference)	1		1	
Age	0.98 (0.97-0.99)	.020	1.01-(0.99-1.02)	.3540
Single (Reference)	1		1	
Married	0.86 (0.65-1.15)	.310	1.22 (0.98-1.51)	.0740
Undergraduate (Reference)	1		1	
graduate	0.44 (0.36-0.55)	<0.0001	0.54 (0.46-0.64)	<0.0001
Physically inactive	1.18 (0.93-1.50)	.170	1.27 (1.06-1.51)	.0090
Physically active (Reference)	1		1	
non-smoker	0.35 (0.20-0.59)		0.34 (0.22-0.53)	
ex-smoker	0.40 (0.19-0.85)	<0.0001	0.41 (0.24-0.73)	<0.0001
smoker (Reference)	1		1	
BMI	0.99 (0.97-1.02)	.810	0.99 (0.97-1.01-)	.430

and anxiety. The risk of anxiety (OR=0.35; P<0.001) and depression (OR=0.34; P<0.001) was lower among people who had never smoked than among participants who were current smokers; also, the risk of experiencing both disorders was lower among ex-smoker than smokers.

DISCUSSION

The current study was conducted with a large sample of Iranian adults to investigate the association between lifestyle and demographic variables with two common mental health problems, i.e., depression and anxiety, in Iran (23) and as well as in the world (9, 24).

In this study, the prevalence of depression and anxiety was found to be 28.8% and 14% among the total population, respectively. Among the studied variables, female sex, younger age, lower education level, and a smoking habit were significantly associated with suffering from anxiety. Additionally, female sex, lower education level, less physical activity, and a smoking habit were significantly related to an increased risk of depression.

These findings show that the prevalence of depression and anxiety among the population of Iranian adults was higher compared with the reported rates in other studies in world. The observed variations may be attributed to the socio-demographic background of the studied population, to the differences in methodology and tools for screening, or to classification based on various cut of values and misclassification.

The results of this study showed a higher risk of anxiety and depression in women, which was similar to the results of previous studies (5, 9, 25). Also in line with the results of previous studies, our study showed a positive and significant relationship between a lower education level and an increased risk of anxiety and depression (8, 26). Men and women showed differences in disease presentation and course. In addition to a higher prevalence of mental disorders that meet the full diagnostic criteria, subclinical anxiety and depression symptoms are also more common in women. Understanding the differentiated biological and socio-cultural backgrounds of men and women in their experiences of affective disorders such as mental health problems is likely to be a useful perspective into the mechanisms of these illnesses.

Our study showed that as age increased, the level of anxiety decreased, but there was no significant association between age and depression. Several previous studies also showed a higher prevalence of anxiety in younger age groups (9, 27). The findings of the current study are consistent with the findings of community-based epidemiologic surveys, showing an age-related decline in the prevalence of current anxiety disorders; although in some studies, the prevalence of psychological problems increased with age (5, 23), and some studies did not show any significant difference (25). Some people have challenged these findings, arguing that epidemiologic surveys may underestimate the prevalence of mental health disorders in older persons due to the reluctance on the part of older people to acknowledge emotional and psychological symptoms, reduced sensitivity of screening survey instruments for older persons, as well as recall bias (28).

The results of previous studies showed a lower prevalence of depression and anxiety among married participants and indicated a better mental health status than among single participants (10); (29, 30). However, in the current study, marital status did not show any significant relationship with any of the mental health disorders. This inconsistency likely reflects a variation across studies in several key methodological features, including that the majority of studies of marital disruption exclude the remarried. This exclusion may exaggerate the effect of marital disruption on mental health, especially among women. Additionally, most previous studies have used depression symptom scales rather than standardized diagnostic instruments and the degree of control for history of prior psychopathology varies across studies (31).

In the present study, there was no significant association between BMI and anxiety and depression. By contrast, data from 46,704 participants in a study in Australia revealed a nonlinear relationship between BMI and mental health, wherein obese women were shown to have a higher odds of experiencing mental illness (32). Other studies also showed an increased risk of mental disorders among obese people (10, 33). There is a question as to the potential effect of the severity of obesity. There was evidence that depression increases with the severity of obesity (33, 34). Another important question that can be posed is whether or not this relationship is causal. Previous studies have shown that depression is a predictor of obesity (35, 36), although these findings are shown only for women, and not men (37). The results of a meta-analysis with the aim of determining the relationship between obesity and depression on prospective studies showed that depression predicts obesity and the risk of obesity among depressed people was higher than among non-depressed subjects (38). There is growing evidence that depressive and anxiety disorders share common health problems with obesity such as cardiovascular disease and type II diabetes, which increases the risk of early death. These health problems could partly be explained by an unhealthy lifestyle, which is found to be more common among depressed, anxious and obese people (39).

Physical activity is an important well-known factor, especially in relation to depression. People who are physically inactive are at an increased risk of developing depression, while those who are physically active have are more protected from developing depression. In line with our findings, an inverse association between physical activity and depression has been reported in earlier studies. However, inconsistent with previous studies, we did not find an association between anxiety and physical activity. There is strong evidence from animal studies that exercise and regular activity positively impacts the pathophysiological processes of anxiety.

Numerous studies and meta-analyses showed an inverse association between exercise and anxiety in clinical settings. Similar to the complex nature of anxiety disorders, no single mechanism sufficiently accounts for the anxiolytic nature of exercise. Physical activity positively impacts a number of biological, as well as psychological mechanisms (40). Exercise is known to be an effective therapeutic strategy for depression and anxiety (41, 42).

In the present study, a higher risk for depression and anxiety was shown among smokers compared to non-smokers and ex-smokers. The results of a meta-analysis of longitudinal studies (2016) revealed a bidirectional relationship between smoking and the occurrence of mental disorders. The bidirectional association between smoking and depression or anxiety may be explained with occasional smoking initially used to alleviate symptoms, but in fact exacerbating symptoms over time. (43). Several hypotheses have been proposed to explain the high rate of smoking among people with depression and anxiety. The theory of self-medication claims that people smoke to reduce symptoms of depression and anxiety, and thus suggests that anxiety and depression may lead to smoking (44, 45). Alternatively, it has been suggested that smoking may lead to depression or anxiety through its effect on neuro-circuitry, which increases susceptibility to environmental stressors. Animal models provide evidence that prolonged nicotine exposure deregulates the hypothalamic–pituitary–adrenal system, resulting in the hyper-secretion of cortisol and alterations in the activity of the associated monoamine neurotransmitter system, whose function is to regulate reactions to stressors, an effect that appears to normalize after nicotine withdrawal (37).

This study has some limitations. First, the cross-sectional design of the current study did not allow us to infer the cause-effect associations. For example, it is not clear whether mental health problems precede the adherence to smoking, or whether the smoking causes depression and anxiety. Second, the observed gender-specific association requires more research to determine the potential modifying effect of sex. It is not clear at this stage whether the different associations are true or attributable to methodological limitations. However, it is possible that some differences in lifestyle factors between men and women, such as smoking habits or physical activity, may explain the differences in risk between the sexes. Although the confounding effects of such variables have been considered in our multivariable fitted logistic model, the residual effects of such confounders or unknown or unmeasured confounders may influence the results. Finally, our study relied on the use of self-reported data.

The strengths of this study include the novelty of the topic in Iran and Middle Eastern countries as well as the use of validated questionnaires to evaluate mental health problems and physical activity. In addition, the large sample size of the study population with a wide variation in demographic variables may make our results generalizable to other populations.

CONCLUSION

In conclusion, this study provided new data in a rarely studied region and reinforced the available evidence regarding the relationship between lifestyle factors and two common mental health problems. Considering the adverse health outcomes associated with mental illness, as well as the large burden on individuals and society and the socioeconomic impact, there is a need to expand our knowledge in this context. We must develop new interventional strategies to reduce the incidence of psychiatric disorders and consequently their adverse health outcomes.

CONFLICT OF INTEREST

The authors have no conflicts of interest associated with the material presented in this paper.

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LABORATORY TESTS IN DIAGNOSIS OF MASTOCYTOSIS: LITERATURE REVIEW AND CASE REPORT

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LABORATORIJSKI TESTOVI U DIJAGNOZI MASTOCITOZE: PREGLED LITERATURE I PRIKAZ SLUČAJA

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ABSTRACT

Mastocytosis is a heterogeneous group of disorders characterized by abnormal growth and accumulation of mast cells (MCs) in the skin and/or other organ systems. Mastocytosis is a rare disease. The annual incidence is 5-10 cases per 1 million people. However, the majority of cases stay undiagnosed due to the lack of specific tests and a wide variety of clinical features of the disease. In mastocytosis, somatic mutations of KIT gene lead to autocrine dysregulation and constitutive c-KIT activation in the absence of its ligand SCF. Clinical symptoms of the disease are determined by MC mediator release and/or infiltration of tissues by MCs. According to the World Health Organisation classification updated in 2016 mastocytosis is divided to cutaneous mastocytosis (CM), indolent systemic mastocytosis (ISM), smoldering systemic mastocytosis (SSM), SM with an associated hematologic (non-MC-lineage) neoplasm (SMAHN), aggressive SM (ASM), MC leukemia (MCL) and MC sarcoma (MCS). The CM and ISM prognosis is excellent with (almost) normal life expectancy, unlike aggressive forms (ASM and MCL) with poor prognosis. In this paper the key aspects of clinical features and diagnostic criteria of mastocytosis are discussed. We present a case report of a patient with mastocytosis in the skin following psoralen plus ultraviolet A (PUVA) therapy with good response.

Keywords: *mastocytosis, cutaneous mastocytosis, systemic mastocytosis, case report, D816V mutation, KIT, tryptase*

SAŽETAK

Mastocitoza je heterogena grupa poremećaja koju karakteriše abnormalni rast i akumulacija mastocita (MC) u koži i/ili drugim sistemima organa. Mastocitoza je retka bolest. Godišnja incidencija je 5-10 slučajeva na milion ljudi. Međutim, većina slučajeva ostaje nedijagnostikovana zbog nedostatka specifičnih testova i širokog spektra kliničkih karakteristika bolesti. Kod mastocitoze, somatske mutacije KIT gena dovode do autokrine disregulacije i konstitutivne aktivacije c-KIT u odsustvu SCF liganda. Klinički simptomi bolesti su određeni oslobađanjem posrednika MC i/ili infiltracijom tkiva uz pomoć MC. Prema ažuriranoj klasifikaciji Svetske zdravstvene organizacije iz 2016. godine mastocitoza je podeljena na kožnu mastocitozu (CM), indolentnu sistemsku mastocitozu (ISM), tinjajuću sistemsku mastrocitozu (SSM), SM sa povezanom hematološkom (non-MC-lineage) neoplazmom (SMAHN), agresivnu SM (ASM), leukemiju MS (MCL) i MC sarkom (MCS). Prognoze CM i ISM su odlične sa (skoro) normalnim očekivanim životnim vekom, za razliku od agresivnih oblika (ASM i MCL) sa lošom prognozom. U ovom radu se razmatraju ključni aspekti kliničkih karakteristika i dijagnostičkih kriterijuma mastocitoze. Dat je prikaz slučaja pacijenta sa mastocitozom kože nakon terapije psoralenom plus ultraljubičasta A svetlost (PUVA) sa dobrim odgovorom.

Ključne reči: *mastocitoza, kožna mastocitoza, sistemska mastocitoza, prikaz slučaja, mutacija D816V, KIT, triptaza*

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INTRODUCTION

The term 'mastocytosis' denotes a heterogeneous group of disorders characterized by abnormal growth and accumulation of MCs in the skin and/or other organ systems (1).

This unique disease, characterized by symmetrical spread with pigmented maculopapular lesions, was for the first time described in 1869. In 1878 a term, "urticarial pigmentosa" (UP) was introduced. In 1879, Paul Ehrlich was the first to discover MCs. Later, in 1887 Paul Gerson Unna discovered that skin lesions contained focal accumulations of MCs. It was first assumed that pathologic accumulation of MCs is restricted to skin, but in 1949 a clinical case of systemic mastocytosis was described: autopsy revealed accumulation of MCs in visceral organs. In 1953, term "mastocytosis" became generally recognized (1-3).

At the 'Year 2000 Working Conference on Mastocytosis' clinical, histologic, immunologic and biochemical markers of mastocytosis were defined and criteria for the diagnosis and differential diagnosis were developed. According to WHO classification in 2001 and 2008, mastocytosis was regarded as a subgroup of myeloproliferative neoplasms. In the current revised WHO classification (2016) mastocytosis is an independent nosological entity, which is divided according to the affected organ system into cutaneous mastocytosis (CM), systemic mastocytosis (SM) and localized MC tumors (1, 4, 5) (Table 1).

Diagnostic criteria for MC according to WHO

CM is diagnosed based on a typical clinical presentation, histological evaluation of skin biopsy (typical dense multifocal MC infiltrates in derma) with no signs of SM.

Diagnosis of SM requires the presence of the following criteria: major criterion: dense multifocal MC infiltrates in a histologic and immunohistochemical evaluation of a biopsy sample of bone marrow or different extra-cutaneous organs (aggregation of > 15 MCs). Minor criteria confirm a neoplastic nature of the pathologic process: 1. Cytomorphologic examination of bone marrow and/or other extra-cutaneous organs reveals > 25% of atypical MCs, 2. Presence of activating point mutation KIT D816V*, 3. MCs expressing CD2 and/or CD25*, 4. Sustained elevation of serum tryptase > 20 ng/ml (not valid for SM-AHNMD).* Not applicable for skin.

Table 1. Mastocytosis classification

Types and subtypes	Diagnostic criteria and significant clinical signs
I. Skin involvement.	
Mastocytosis in the skin (MIS)	CM criteria are fulfilled, SM not excluded because of insufficient diagnostics (preliminary diagnosis)
Cutaneous mastocytosis (CM) 1) maculopapular cutaneous MC (MPCM)/ Urticaria pigmentosa (UP) 2) diffuse CM 3) skin mastocytoma	CM criteria are fulfilled, SM criteria not fulfilled (1-2 minor SM criteria may be present)
	Diagnosed predominantly in adults
	Diagnosed predominantly in children
	Diagnosed predominantly in children
II. Systemic mastocytosis (SM)	SM criteria fulfilled: 1 major + 1 minor criterion or 3 minor criteria Skin lesions +/-
1) Indolent SM (ISM)	No C-finding(s), < 2 B-findings, Skin lesions ++/-
2) Smoldering SM (SSM)	No C-finding(s) , ≥ 2 B-findings, Skin lesions +/-
3) SM with an associated clonal hematologic non-mast cell lineage disease (SM-AHN)	SM criteria fulfilled FAB/REAL/WHO criteria fulfilled
4) Aggressive SM (ASM) - classic -transforming into MCL (ASM-t)	≥1 C-finding, Skin lesions -/+ <5% of atypical MC in bone marrow smears 5-19% of atypical MC in bone marrow smears
5) Mast cell leukemia (MCL) - phase with no circulating MC (aleukemic) - phase with circulating MC (leukemic)	≥1 C-finding, ≥20% of atypical MC in bone marrow smears Skin lesions --/+ MC <10% in blood smears short or absent MC >10% in blood smears
III. Localized mast cell tumors	
1) Mast cell sarcoma (MCS)	Focal malignant tumor, SM criteria not fulfilled
2) Extracutaneous mastocytoma*	Focal benign tumor, SM criteria not fulfilled

* Extracutaneous mastocytoma is excluded, as no cases are registered during the last 20 years.

Epidemiology of MC

Mastocytosis is a rare disease. Yearly incidence is 5-10 new cases per 1 million people. Some researchers assume that real incidence is much higher, but patients with minimal symptoms often do not seek medical advice. Although mastocytosis is a sporadic somatic disease, several cases of familial mastocytosis with dominant inheritance are registered (1, 8).

Depending on the age of initial presentation, all cases can be divided into pediatric mastocytosis (65%) and adult mastocytosis (35%) (9). This classification has an important prognostic value (10). CM is more prevalent in children; in most cases all symptoms disappear during puberty (1). According to recent research, in children with small monomorphic maculopapular lesions, the disease usually undergoes progression, whereas heterogenous lesions of different size (usually large lesions) disappear without treatment before adolescence.

In adults, mastocytosis is usually characterized by lifelong systemic involvement with no spontaneous remissions (11).

Clinical manifestations and prognosis of MC

The most prevalent type among cutaneous forms of mastocytosis is maculopapular CM, which presents as intensely pigmented reddish-brown macules and/or papules (12, 13). Some authors state that the color of lesions depends on the proliferation of epidermal melanocytes, which express c-KIT during the regulation of melanogenesis, similar to MCs. Several studies demonstrated that KIT-mutations lead to focal albinism in humans.

In case of mastocytoma, a single elastic node sized 2-5 cm with smooth or creased surface is visualized. Occasionally, 3-4 nodes can be present predominantly on the neck and forearms. This subtype of mastocytosis is found only in newborns and is characterized by spontaneous regression (14).

Diffuse SM is characterized by skin erythema and thickening up to erythroderma (15).

Clinical presentation of mastocytosis is associated with uncontrolled proliferation of MCs, which depends on the degree of MCs differentiation and the production of a variety of mediators, including histamine, leukotrienes, proteases, heparin and others (1, 16).

In patients with mastocytosis symptoms, associated with MC mediator excretion, are present, including periodic flushing (erythema, appearing spontaneously or as a reaction to a number of triggers), itching, urticarial dermographism, positive Darier's symptom (spots get reddish and swollen when rubbing) (17). Other symptoms, associated with MC mediators include: anaphylactic reactions, recurrent syncopes, occasional hypotension, gastrointestinal distress such as diarrhea, abdominal pain, gastric ulcers and neurological and psychiatric disorders (5, 14).

Excessive proliferation of MCs in SM leads to visceromegaly, which can cause organ failure (Table 2). Besides, more aggressive forms of SM are associated with less skin involvement (18, 19).

MC can substitute other bone marrow cell lineages, which can manifest as hypo-/aplastic anemia, neutropenia, thrombocytopenia, which can lead to recurrent infections and hemorrhages (spontaneous petechia and/or bruising) (20, 21).

MCS is very rare and is characterized by local destructive (sarcoma-like) growth. Tumor is comprised of MCs with low differentiation. Several cases of atypical tumor locations are described, including larynx, colon with subsequent generalization of process with visceral organ and haemopoietic tissue involvement. Thus, late phase MCS may be similar to ASM or MCL (22).

Prognosis in patients with CM, ISM and SSM is usually favorable, in contrast to ASM, MCL or MCS where prognosis is usually unfavorable (1).

Table 2. Clinically significant findings in SM

B-findings (B, borderline 'benign')	1) dense multifocal infiltrates in bone marrow, with MCs >30% and/or total serum tryptase level > 200 ng/ml 2) dysmyelopoiesis: hypercellular bone marrow with loss of fat cells and signs of myelodysplasia or myeloproliferation of non-mast cell lineages, with insufficient criteria for SM-AHN. Blood tests are normal or demonstrate non-significant consistent deviation of measures. 3) Visceromegaly: hepatomegaly without liver function impairment and/or lymphadenopathy on palpation or visualization (lymph nodes >2 cm) and/or splenomegaly without hypersplenism.
C-findings (C, critical)	1) abnormal myelopoiesis in bone marrow with cytopenia; blood samples reveal: absolute neutrophil count <1* 10^9/l, Hb <100 g/l, platelet count <100*10^9/l, without non-MC hemopoietic malignization. Visceropaty: 2) hepatomegaly with liver function impairment, ascites and/or portal hypertension 3) splenomegaly with hypersplenism 4) malabsorption with hypoalbuminemia and weight loss as a result of MC infiltration of mucous layer of digestive tract (23) 5) Significant osteolysis focuses and/or severe osteoporosis with spontaneous pathologic fractures, osteosclerosis

Laboratory tests for MC

Histologic and immunohistologic evaluation.

Diagnosis of mastocytosis is traditionally based on the detection of dense multifocal infiltrates consisting of atypical MCs in skin, bone marrow and visceral organs.

The main criterion for the diagnostics of SM is the detection of dense multifocal infiltrates usually presented along blood vessels in a bone marrow biopsy sample. The number of MCs in aggregate should be >15. Cytomorphologic evaluation of bone marrow aspirate may reveal a secondary criterion of SM: >25% of atypical MCs (fusiform cells). In this case, a diagnosis of SM is verified (1, 18, 24).

Focal aggregates of normal MCs in bone marrow may be detected in case of reactive MC hyperplasia during parasitic infections, in case of tumors, aplastic anemia, immunocytoma or some chronic inflammatory conditions.

Apart from dense infiltrates, diffuse MC infiltration of bone marrow may be seen (mixed infiltration). In that case two variants may be distinguished:

1. disseminated MCs which do not change normal structure of bone marrow; this variant is mainly seen in ISM;
2. disseminated MCs which change normal structure of bone marrow, signs of myelodysplasia and myeloproliferation are present. This variant is mainly seen in ASM and MCL.

Another important histologic sign of SM is fibrosis and osteosclerosis of bone marrow, which are typically present in ISM and never seen in MCL (1).

Cytomorphologic evaluation of bone marrow smears

In most patients with SM, the proportion of MCs in bone marrow smears (among all cells) is < 5%. This information has diagnostic value, as it makes possible to exclude MCL, in which the proportion of MCs is ≥ 20%.

Other haemopoietic lineages in bone marrow smears should be evaluated to exclude SM-AHN (1, 16).

Cytomorphologic evaluation can reveal the following cell types (1, 25):

1) Non-metachromatic blasts - signs of maturation are absent, cytoplasmatic content is low, nuclear chromatin is thin, nucleoli are seen.

2) Metachromatic blasts - high nucleoplasmic ratio, nuclear chromatin is thin, several metachromatic granules (at that stage of maturation MCs and basophils are indistinguishable).

MC - lineage:

3) Typical tissue MCs - round or oval cells, small or medium-sized, with centrally located round or oval nucleus, without nucleoli, cytoplasm contains a lot of metachromatic granules, less commonly cytoplasm is hypo-/degranulated, low nucleoplasmic ratio.

4) Atypical MCs type I are characterized by the presence of two or three of the following signs: (1) fusiform cells, (2) eccentric or centrally located oval nucleus, (3) hypogranulated cytoplasm with focal accumulation of granules, without signs of degranulation.

5) Atypical MCs type II - cells of different forms with bi/multilobular nucleus, of mature morphology (condensed chromatin, low nucleoplasmic ratio) or immature morphology (high nucleoplasmic ratio and thin nuclear chromatin). Nucleoli may be present, cytoplasm is usually hypogranulated, without signs of degranulation.

Pathomorphologic classification of SM (1):

1) Malignant SM: metachromatic blasts + atypical MCs of type II >20% of all MCs in bone marrow smears.

2) Benign SM: metachromatic blasts in bone marrow smears + atypical cells of type II <10% of all MCs. Other MCs may have typical tissue morphology or may be presented by atypical type I MCs.

Laboratory findings

Elevation of serum tryptase, namely of alpha-tryptase, which is secreted by MCs in different organs. In CM patients, tryptase levels are usually within normal limits or slightly elevated (26). In SM, tryptase levels are elevated (> 20 ng/ml). Total tryptase levels in SM correlate with the load of neoplastic MCs. At the same time, the elevation of this enzyme is seen in different myeloid tumors. In fact, serum beta-tryptase level may temporary rise in case of allergic reaction (1, 18).

Detection of abnormal expression of CD2 and/or CD25 by MCs using flow cytometry

Cytoplasmic membrane phenotype of MCs is different from basophils and other myeloid cells. In SM, MCs express CD2 and/or CD25, which are normally expressed by T-lymphocytes and NK-cells and absent on normal MCs (1).

Detection of activating somatic point mutations of c-KIT

KIT receptor (CD117), coded by c-kit oncogene, is a tyrosine kinase with five extracellular Ig-like domains (27). The first three domains are able to bind with growth factor (stem cell factor - SCF), which activates an intracellular signaling cascade via c-KIT, promoting mitotic activity and proliferation of myeloid progenitors of MCs (28, 29).

Somatic point mutation in c-KIT (Asp-816→Val or D816V) activates ligand-independent growth of neoplastic MCs (30). This mutation is found in more than 90% of adult patients with SM and in children with monomorphic CM (21, 31, 32). C-KIT Gly-839→Lys mutation is registered in children with polymorphic CM (33, 34).

KIT D816V mutation is found in most patients with ISM (>90%), who are characterized by favorable prognosis (35, 36). This leads to a conclusion that there are other non-D816V KIT mutations, contributing to the pathogenesis and determining aggressive course of the disease (32, 37). In fact, several other somatic mutations in TET2, SRSF2, ASXL1, CBL, RUNX1 and RAS in patients with SM-AHN, ASM, MCL were detected (5, 24).

Non-KIT mutations in SM-AHN are found not only in MCs, but also in other myeloid cells, CD34-progenitor cells and sometimes in monocytes and B-lymphocytes (1, 38, 39).

However, KIT mutations are found in other neoplasms, including gastrointestinal stromal tumors, acute myeloblastosis, lymphomas and seminomas (1, 2, 20).

In addition to the abovementioned methods, a thorough evaluation for the determination of B- and C-findings is required.

Treatment of MC

At present, there are no standard treatment algorithms, as mastocytosis has a variable clinical presentation and unpredictable clinical course. Treatment is based on the clinical form and presentation. In case of MC mediator-related symptoms, antihistamines, mast cell membrane stabilizers (sodium cromolyne, ketotifen), leukotriene-receptor antagonists, corticosteroids and aspirin are recommended (40-46). In patients with risk of anaphylactic shock, these drugs are recommended for chronic administration. If immune therapy is ineffective, one should consider experimental treatment with recombinant humanized monoclonal IgG1κ antibodies (Omalizumab) (47). In osteoporosis, bisphosphonates are prescribed (in case of contraindications), in resistant cases therapy with RANKL inhibitors may be considered. Skin lesions demonstrate positive, but temporary response to psoralen plus ultraviolet A (PUVA)-therapy, local corticosteroids (48). In SSM, ASM “immunomodulating” anticancer agents are used, including interferon-alpha and systemic corticosteroids. In MCS, surgical treatment with subsequent radiotherapy and/or chemotherapy is performed (49).

Patient with SM-AHN demand separate treatment plans for SM and AHN. In some cases imatinib – a specific inhibitor of c-Kit receptor activity provides positive effect (40-44). In case of rapid disease progression, experimental chemotherapy or bone marrow transplantation should be considered.

Clinical case of MC

Patient A, 20 years old, is sick since she was 14, when she first developed urticaria pigmentosa lesions on thighs. Gradually, new skin lesions on face, trunk, upper and lower extremities developed. During the examination, generalized symmetrical polymorphic skin lesions – round reddish-brown macules and papules up to 5 mm in diameter with smooth surface were found (Figure 1). She was Darier's symptom positive (Figure 2). Mucous lining is not affected. An insignificant enlargement of regional neck and submandibular lymph nodes is present. Subjective complaints: itching after an exposure to different irritating stimuli (cold, heat, rubbing). Clinical, biochemical and immunochemical blood test are normal. Clinical signs of nerve system, musculoskeletal system or digestive system involvement are absent. Abdominal ultrasound did not reveal any signs of organomegaly. Pathohistological examination of skin biopsy sample revealed: non-uniform epidermal atrophy with preserved cellular and basal membrane stratification. Basal layer contains elevated number of cells with melanin granules – keratinocytes and melanocytes. Sclerosis of dermal layer with moderate lymphohistiocytic infiltration of capillaries in superficial dermal layers. Toluidine blue staining reveals individual MCs with metachromatic granules of lilac color. Conclusion: MIS (Figure 4). High-sensitive ASOqPCR for KIT D816V mutation (45) in peripheral blood cells is positive; mutant fraction is 29%. Serum tryptase activity IgE (ImmunoCAP) 30.10 ng/ml. Based on the clinical and laboratory findings, the patient was diagnosed with MIS. A thorough evaluation of bone marrow to exclude systemic process is planned. Treatment: desloratadine in a standard dose, topical corticosteroids, 20 sessions of PUVA-therapy. Treatment was well tolerated, no side-effects were registered. Treatment resulted in process stabilization, no new lesions are present. Subjective complains are absent (Figure 3).

Figure 1. Disseminated skin rash on legs: (A) front, (B) back

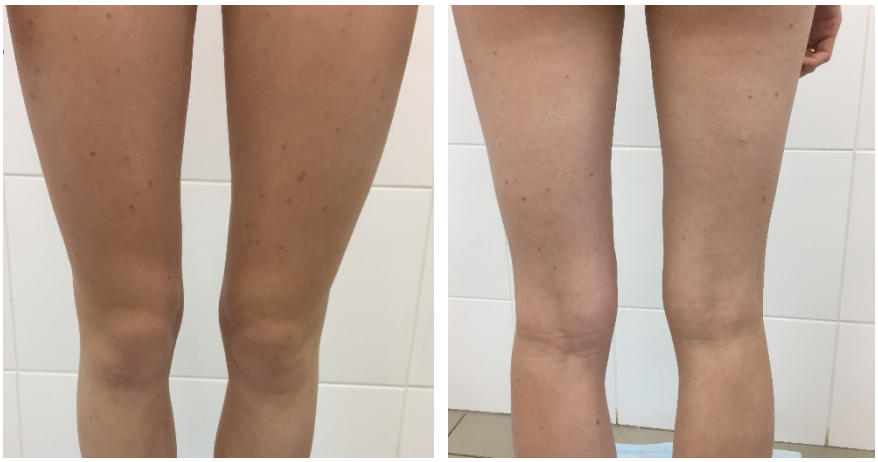


Figure 2. Darier's symptom

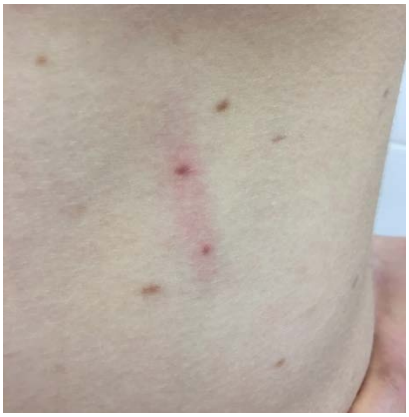


Figure 3. Disseminated skin rash on the back:
before (A) and after the treatment (B)

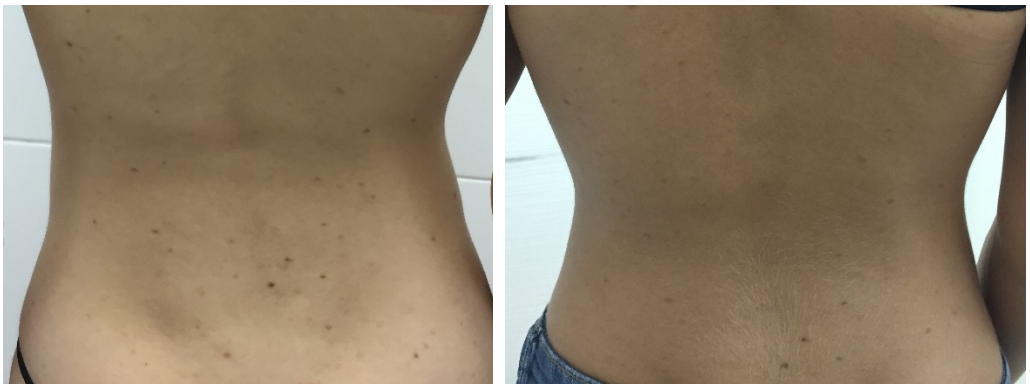
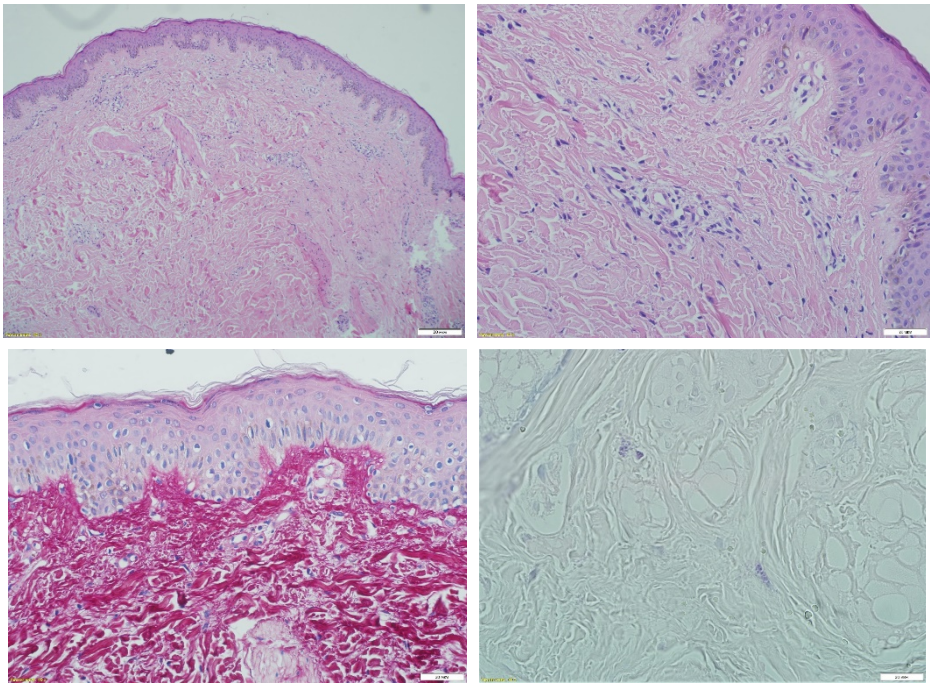


Figure 4. Skin biopsy. Hematoxylin and eosin stain (A, B), van Gieson's stain (C):
increased number of cells with melanin granules in basal layer of epidermis,
areas of sclerosis in derma, lymphohistiocytic infiltration around vessels.
Toluidine blue staining (G): Isolated MCs with metachromatic granules



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EPIDURAL ABSCESS AND SIGMOID SINUS THROMBOSIS AS INTRACRANIAL COMPLICATIONS OF THE MIDDLE EAR CHOLESTEATOMA

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EPIDURALNI APSCES I TROMBOZA SIGMOIDNOG SINUSA KAO INTRAKRANIJALNE KOMPLIKACIJE HOLESTEATOMA SREDNJEG UHA

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ABSTRACT

The otogenic intracranial complications are rare manifestations in modern era of antibiotics. An early antibiotic therapy often covers typical clinical signs and symptoms for each complication. A sigmoid sinus thrombosis is often associated with other intracranial complications, as in this case, an epidural abscess. We are presenting a case of 12-year-old girl with the sigmoid sinus thrombosis and epidural abscess as complications of chronic infection to the middle ear with cholesteatoma. In the active phase of chronic inflammation of the middle ear she was treated with the antibiotic therapy that covered early symptoms of intracranial complication development. A humid attic perforation of the tympanic membrane with protrusion of choleastoma and evident signs of bony wall destruction to the external auditory canal was noticed by performing routine otomicroscopy and otoendoscopic examination. Assuming intracranial complication, magnetic resonance imaging (MRI) of the endocranium was undertaken. The MRI showed inflammatory changes of both middle ears with intracranial complications: the right sigmoid sinus thrombosis and epidural abscess of the same side. During the surgery we have noticed an extensive middle ear cholesteatoma with significant destruction of the bony tissue and purulent collection between sigmoid sinus changed with granulation and respective dural segment of the posterior cranial cavity.

Timely diagnosis, multidisciplinary approach with an adequate choice of the antibiotic therapy and surgical technique have a crucial prognostic significance.

Key words: S*igmoid sinus thrombosis, epidural abscess, middle ear inflammation, cholesteatoma*

SAŽETAK

Otogene intrakranijalne komplikacije u današnjoj eri antibiotika su rijetka pojava. Rana antibiotska terapija često maskira tipične kliničke znakove i simptome za svaku komplikaciju. Tromboza sigmoidnog sinusa često je udružena s drugim intrakranijalnim komplikacijama, kao što je to u ovom slučaju epiduralni apsces. U radu je prikazan slučaj 12-godišnje pacijentkinje s trombozom sigmoidnog sinusa i epiduralnim apscesom kao komplikacijama hronične upale srednjeg uha s holesteatomom. U aktivnoj fazi hroničnog upalnog procesa srednjeg uha, pacijentkinja je tretirana antibiotskom terapijom koja je maskirala rane simptome razvoja intrakranijalnih komplikacija. Otomikroskopskim i otoendoskopskim pregledom, uočena je vlažna atik perforacija membrane timpani kroz koju je prominirao holesteatom s znakovima destrukcije koštanog zida spoljašnjeg slušnog hodnika. Zbog sumnje na intrakranijalnu komplikaciju, sproveden je NMR pregled endokranijuma, na kojem su očitane zapaljenske promjene oba srednja uha s intrakranijalnim komplikacijama: trombozom desnog sigmodnog sinusa i epiduralnim apscesom s iste strane. Intraoperativno je uočen ekstenzivni holesteatom desnog srednjeg uha s izraženom destrukcijom koštanog tkiva i prisustvom gnojne kolekcije između granulomatozno izmjenjenog sigmoidnog sinusa i pripadajućeg segmenta dure zadnje lobanjske jame.

Na vrijeme postavljena dijagnoza, multidisciplinarni pristup s adekvatnim izborom antibiotske terapije i hirurške tehnike, imaju presudan prognostički značaj.

Ključne riječi: *tromboza sigmoidnog sinusa, epiduralni apsces, srednje uho, upala, holesteatom*

ABBREVIATIONS

SST - Sigmoid Sinus Thrombosis;
LST - Lateral Sinus thrombosis;
EA - Epidural Abscess;
COM - Chronic Otitis Media;
MRI - Magnetic Resonance Imaging

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INTRODUCTION

A chronic middle ear inflammation (*Chronic Otitis Media - COM*) is characterized by insidious and asymptomatic onset, slow and long duration as well as with potentially large, destructive effects, especially when cholesteatoma is present. COM has more aggressive and persistent characteristics in childhood than in adult period of life. The main characteristic of choleasteatoma is a progressive growth with erosion of nearby bone structures due to the pressure effect and activation of the osteoclasts. Cholesteatomas can be classified as either congenital, that occur in 2-4% of cases or acquired. An annual incidence of acquired cholesteatomas is 3 in 100.000 in childhood and 9.2 in 100.000 in adult population, predominantly in males (1-3).

Intracranial complications due to otogenic infections are present even in the era of modern antibiotics (4). If they are not adequately and timely managed, they can be fatal. Ludman (5) has classified otogenic complications into intracranial (*extradural and subdural abscess, sigmoid sinus thrombosis, otitic hydrocephalus, meningitis and cerebral abscess*) and intratemporal (*facial paralysis, labyrinthitis*). Treatment includes antibiotic therapy, neurosurgical procedure when needed and otosurgical management in the temporal bone. COM, cholesteatoma and brain abscess are diagnosed mainly in adults, while COM and epidural abscess (*EA*) are more common in children (6).

Meningitis is the most common intracranial complication, associated with brain abscess and lateral sinus thrombosis (*LST*). Otogenic meningitis is most commonly followed by brain abscess, which aggravates timely diagnostics for each complication. Development of otogenic intracranial complications is significantly reduced with introduction of an antibiotic therapy, improved diagnostic methods and surgical management of COM. An early antibiotic treatment often covers specific clinical signs and symptoms for each complication (4, 7).

LST occurs when mastoid infection involves nearby lateral and sigmoid sinuses. Because of that, perisinus EA is common accompanying finding. LST treatment has been a subject of certain controversye. Most of the authors agree that minimal intervention includes intravenous application of antibiotics and mastoidectomy. Beside that, a recanalization of the blood vessel in order to develop collateral circulation, usually normalize pressure of the cerebrospsinal fluid after evacuation of the surrounding infection. EA abscess is an inflammatory process located within the potential space between the skull and lateral surface of the dura. A purulent collection is rare, more commonly a dural granulomatous tissue is in direct contact with suppuration from the temporal bone. EA treatment includes intravenous administration of antibiotics and surgical drainage. Cortical mastoidectomy with sufficient removal of the bone tissue enables direct inspection of dura and posterior cranial cavity. The most certain method of an early diagnostics of EA and sigmoid sinus thrombosis (*SST*) is magnetic resonance imaging (*MRI*) (8).

The case report presents a 12-year-old girl with SST and EA as a complication of the COM with cholesteatoma.

CASE REPORT

A 12-year-old girl, hospitalized at the Ear, Throat and Nose Department for a the headache, a right otalgia and otorrhea, postauricular pain, fever and vertigo. Previously, she underwent diagnostic evaluation and treatment in local general hospital and at the departments for paediatric and infective diseases. A redness, mild oedema and palpatory pain sensitivity over the right mastoid process was noticed during the clinical examination at the admission at the Ear, Throat and Nose Department.

After microaspiration of the purulent discharge from the right external auditory canal, a humid attic perforation of the tympanic membrane, with protrusion of cholesteatoma, was noticed by performing routine otomicroscopy and otoendoscopic examination (Figure 1). A dry attic perforation of the tympanic membrane was noticed on the left side. Signs of the posterior and upper wall bone destruction to the both external auditory canals were also noticed.

Both orthostatic and dynamostatic tests were positive: instability in walking and standing, staggered, especially during the tandem walk. Pure tone audiometry showed a right moderate, and left mild conductive hearing loss. Laboratory findings: C-reactive protein level was 0.5 mg/dL, white blood cells 8.5 x 10^9/L , neutrophill granulocytes 77.3 %, procalcitonin 0.05 ng/mL. Findings of EEG, lumbar puncture and examination of the eye fundus were normal. MRI of the endocranium was indicated and performed. MRI showed inflammatory changes of both middle ears with endocranial complications: the right SST and EA of the same side (Figure 2).

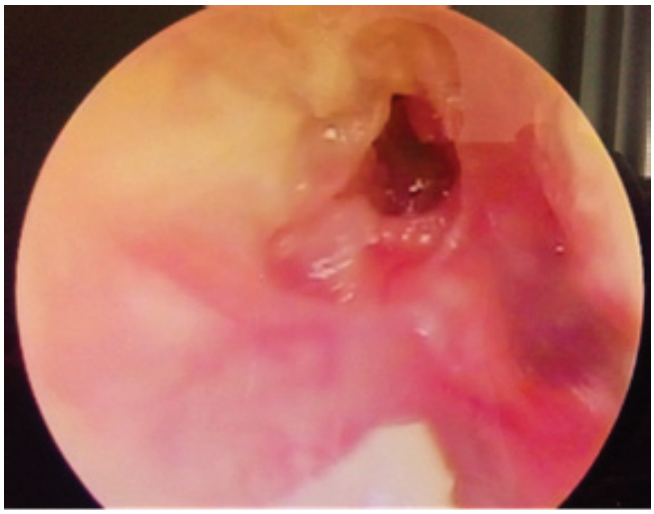

Figure 1. Otoendoscopic image: The humid attic perforation of the tympanic membrane, with protrusion of cholesteatoma.

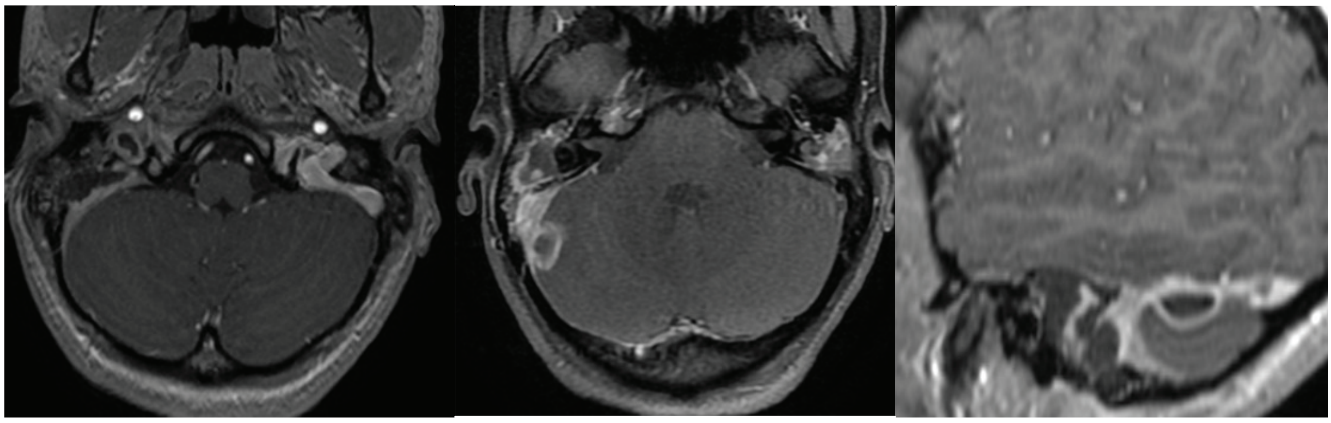

Figure 2. Initial MR images (T1W+C, axial and sagittal view): at the convexity of the right cerebellar hemisphere epidural empyema; the right sigmoid sinus is without normal opacification and dilated due to content of thrombus, with an erosion of the anterior wall and in continuity with right mastoid and middle ear that are filled with granulation tissue.

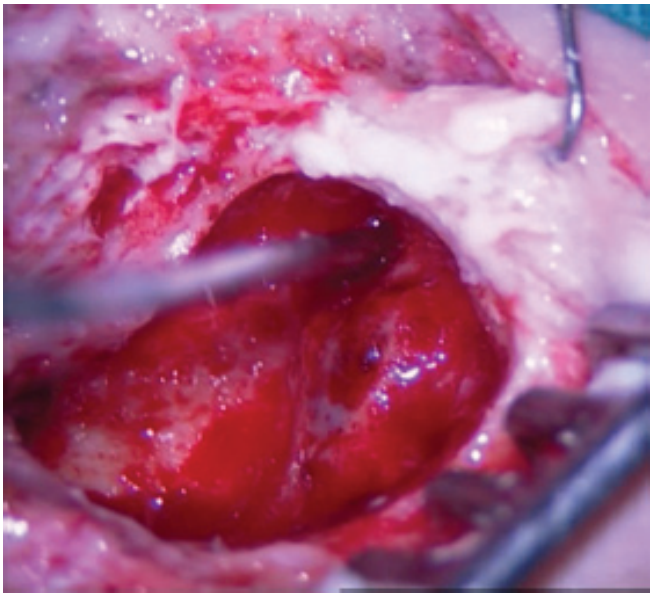

Figure 3. Intraoperative otomicroscopic image: The sigmoid sinus and posterior cranial cavity dura were covered with granulomatous and abscess collection in the epidural space.

A neurosurgeon was consulted and declared that there were no indications for neurosurgical treatment. A triple systemic antibiotic therapy was introduced (third generation cephalosporines, metronidazole and glycopeptide antibiotic), as well as antiedema therapy (osmotic diuretic), analgesics and antipyretic therapy. An otosurgical treatment was indicated under general anaesthesia.

During the surgery we have noticed an extensive cholesteatoma in the area of the mastoid process, which completely filled antrum and aditus ad antrum and destroyed mastoid cells. The bony plate of the sigmoid sinus and belonging segment of the bony wall of the posterior cranial cavity were destroyed. The sigmoid sinus and posterior cranial cavity dura were covered with granulomatous and abscess collection in the epidural space (Figure 3). During the trepanation a huge purulent collection was spontaneously drainaged from the epidural abscess, followed by liquorrhea which stopped spontaneously. A smear from the abscess was sent for microbiological analysis. After a consultation with a neurosurgeon, a lavage was performed with 3 % hydrogen peroxide solution, isotonic saline solution, povidone iodine solution and antibiotics from aminoglycoside class.

After elevation of the tympanomeatal lobe and removal of the posterior bony wall of the auditory canal, we noticed presence of cholesteatoma in the area of attic, oval fossa and retrotympanon as well as dehiscence of the attic tegmen and bony canal of the tympanal part of the facial nerve. An ossicular chain was disarticulated in the area of incudostapedal joint, while the suprastapedial structures were preserved. The mucosa of the tympanal cavity was hypertrophic, and the Eustachian tube orifice was not occluded. The fragments of the cholesteatoma were sent to the pathohistological analysis (Figure 4). The surgery was finished with open technique of tympanoplasty (*tympa-*

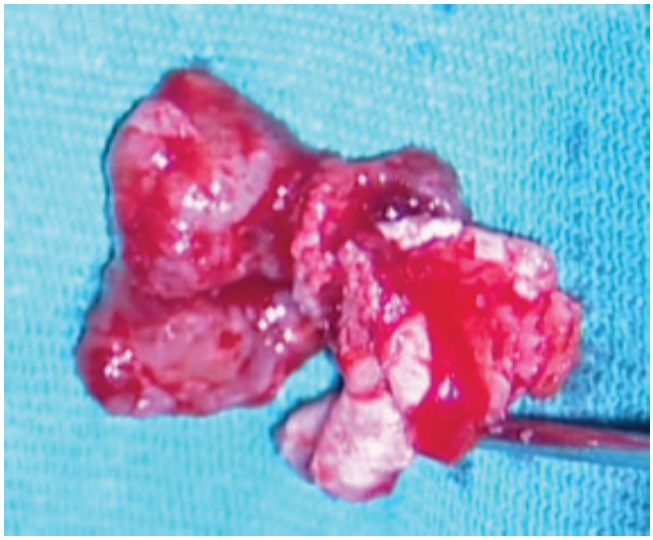

Figure 4. Intraoperative otomicroscopic image: The removed cholesteatoma.

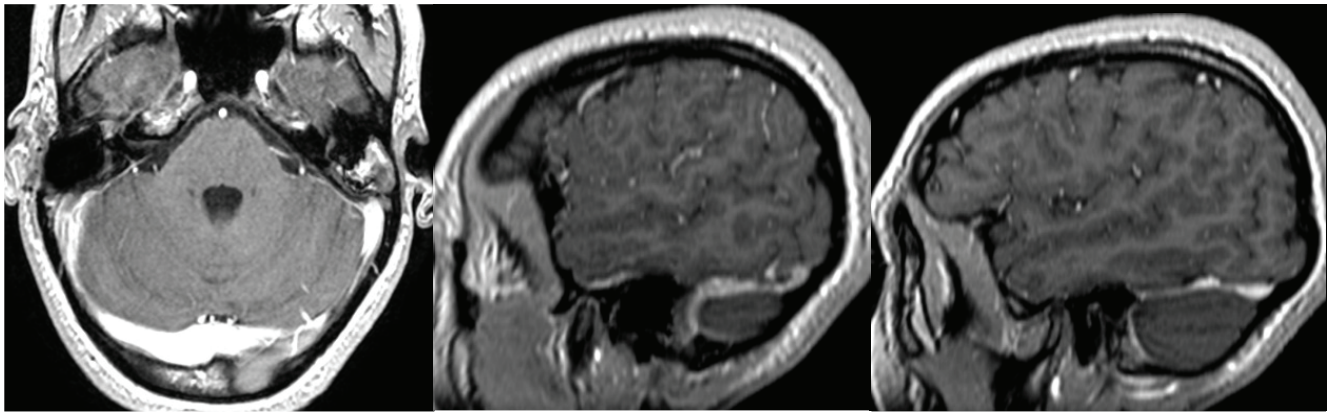

Figure 5. On the control MR exam (T1W+C, axial and sagittal view) it is visible a complete resolution of the epidural empyema and partial recanalization of the right sigmoid sinus.

nomastoidectomy). The operative and early postoperative period passed correctly.

The microbiological analysis of the smear taken from the abscess isolated *Staphylococcus epidermidis*, while the pathohistological analysis showed *Cholesteatoma*. On the ninth postoperative day a control MRI of the endocranium was performed: the abscess in the posterior cranial cavity was not found, while the sigmoid sinus showed signs of partial recanalization (Figure 5) . The patient was discharged on the tenth postoperative day in good general condition and with good postoperative finding. She came after seven days for a control examination, and a postoperative finding was good , as well as her general condition. Surgery of the left ear was indicated and she was operated after four months.

DISCUSSION

Most of the intracranial complications are caused by COM and cholesteatoma (95,8%). They usually occurred in the third decade of lifetime, predominately with male patients. Intracranial and extracranial complications of COM with paediatric populations are relatively rare in developed countries (9).

LST as a complication of otogenic infections can still present a serious threat that requires a prompt management. It is often associated with other intracranial complications, as in this case, with EA. The cholesteatoma manifests more aggressive characteristics in children, with different pattern of spreading and larger incidence of relapses. In patients with otogenic complications the most common intraoperative findings are granulations and cholesteatoma, which was the case with our patient as well. Spreading of the infection through the posterior wall of mastoid process into the posterior segment of the cranial cavity due to the cholesteatoma can cause, a perisinus abscess can be formed. The abscess presses the bone, creating necrosis of the anterior part of the sinus and intima as well as accumulation of fibrin, red blood cells and platelets, which results in thrombus formation on its wall. A thrombus can penetrate towards bulbus of the inferor vena cava, submucous tissue and even cause an embolism (9-14).

Headache, vomiting and neck stiffness associated with otorrhea and hearing loss are dominating clinical findings of LST. But symptoms may be atypical, hardly recognizable and almost undetectable due to the antibiotic application. The most common symptoms of the patients with LST, as was in this case, are characterized by a permanent and/or significant temperature rise associated with otorrhea, postauricular edema and otalgia (7).

The case report is also interesting for its silent and clinically undetected development of the EA. In the active phase of the COM, the patient was given an antibiotic therapy that most probably covered early symptoms of the intracranial infection. These facts lead to the conclusion that an otologist always has to bear this in mind and to check if in the active phase of COM an intracranial infection exists. In general, the focal neurological signs are absent. Although EA can be manifested by weak fever, headache and otalgia, a patient is usually asymptomatic and without focal neurological signs (8, 15).

The microbiological analysis of the smear taken from the abscess cavity isolated *Staphylococcus epidermidis*. However, in most of the cases of brain abscess whose etiology is of COM, a gram-negative bacteria were isolated (6).

Radiological examinations are useful in diagnostics, but they have a limited role in identification of tegmen dehiscence, mastoid erosion or meningoencephalocele. In order to increase the probability of correct diagnosis in cases suspected otogenic meningitis and bone defects, it is useful to perform a computed tomography (*CT*) scan with any possible reconstruction, while MRI can explain intracranial phlogistic processes. EA can often be overseen in CT scan, if a huge volume of pus is not present. It is for this reason that, MRI is considered a method of choice. In the case of LST, a priority is given to the magnetic resonance angiography due to the precise visualization of the vascular flow (16, 17).

From the authors experience and the literature review, it is recommended that paediatric patients with otogenic thrombosis of the dural sinus and in the case of increased

intracranial pressure (*ICP*) be treated conservatively. A triple antibiotic therapy is administered empirically until the results of microbiological analysis arrive and it should be given when there is a suspicion of possible development of the otogenic complications. A systemic administration of broad-spectrum antibiotics during six weeks is usually sufficient treatment. Treatment of LST is usually with anticoagulants not recommended. A risk of intracerebral haemorrhage, together with possibility of embolism, limits their usage. In the case of EA, if it is indicated, a neurosurgical intervention includes craniotomy and drainage. Otosurgical treatment includes mastoidectomy or tympanomastoidectomy. In the presented case, tympanomastoidectomy successfully eradicated pathological process in the middle ear.

CONCLUSION

A unique pathophysiology of each intracranial otogenic complication requires different diagnostic and therapeutic approaches. MRI is a method of choice in an early diagnostics of the otogenic intracranial complication. Timely diagnosis, multidisciplinary approach with an adequate choice of the antibiotic therapy and surgical technique have a crucial prognostic significance.

Authors declare that there is no conflict of interest.

Notice: A study derived from the scientific-research project: "*Significance of expression of the inflammatory mediators in middle ear cholesteatoma*", received a clearance from the Ministry of science and technology of the Republic of Srpska Government (No. 19/6-020/961-68/15) dated 12th of December 2016.

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CYSTIC LESIONS OF ANTERIOR MEDIASTINUM: CASE REPORT

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CISTIČNE LEZIJE PREDNJEG MEDIJASTINUMA: PRIKAZ SLUČAJA

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ABSTRACT

Cystic lesions of the mediastinum are uncommon, comprising 12% to 18% of all primary mediastinal tumors and unless they attain a large size and cause compressive symptoms, these tumors are generally asymptomatic and are discovered incidentally upon radiologic investigation of some other condition.

We present in this paper a case of cystic lesions of the mediastinum in a 70-year-old male patient who underwent a surgery for mediastinal mass removal. Histopathology report had shown it was the case of mature cystic teratoma of anterior mediastinum.

Based on a review of the literature, as well as our experience, we conclude that best treatment for cystic lesions of anterior mediastinum is complete surgical resection if possible.

Keywords: *cystic lesions of the mediastinum, teratomas, complete surgical resection*

SAŽETAK

Cistične lezije medijastinuma su retke, i čine oko 12% do 18% svih primarnih tumora medijastinuma, a ukoliko ne dostignu odredjenu veličinu kada prouzrokuju kompresivne simtome, ovi tumori su asimtomatski i otkrivaju se kao usputni nalaz tokom radioloških ispitivanja drugih stanja.

U ovom radu prezentujemo slučaj cistične lezije medijastinuma u pacijenta starog 70 godina, kod kojeg je uradjena hirurška intervencija i uklanjanje iste u potpunosti. Rezultati histopatologije su pokazali da je u pitanju cistični teratom prednjeg medijatinuma.

Na osnovu literature, kao i na osnovu našeg iskustva, zaključili smo da je najbolji tretman za cistične lezije medijastinuma kompletna hirurška resekcija, ukoliko je to moguće.

Ključne reči: *cistične lezije medijastinuma, teratomi, kompletna hirurška resekcija*

INTRODUCTION

Germ cell tumors are uncommon neoplasms that usually arise in the gonads. The most common extragonadal site is anterior mediastinum. It is estimated that only 1–3 % of all germ cell tumors arise in the mediastinum (1).

Mediastinal germ cell tumors are classified into three catagories: benign germ cell tumors, seminomas, and non-seminomatous germ cell tumors, also called malignant teratomas. Benign mediastinal teratomas accounts for 60% of all germ cell tumors and they are divided into three groups as epidermoid cysts, dermoid cysts, and teratomas (2). These tumors can characteristically be cystic or solid or a combination of the two, contain multiple germ cell layers, and are composed of tissue foreign to the organ or anatomic site in which they arise (3).

Cystic lesions of the mediastinum are uncommon, comprising 12% to 18% of all primary mediastinal tumors and unless they attain a large size and cause compressive symptoms, these tumors are generally asymptomatic and are discovered incidentally upon radiologic investigation of some other condition (4, 5).

We present in this paper a case of cystic lesions of the mediastinum in a 70-year-old male patient. During the diagnostic search of dispnea and back pain, CT scan of chest was performed. It showed a large heterogeneous soft-tissue dense mass in the anterior part of the mediastinum. Patient underwent a surgery for removal of the detected mass. After performed surgery, a microscopy shown a case of mature cystic teratoma.

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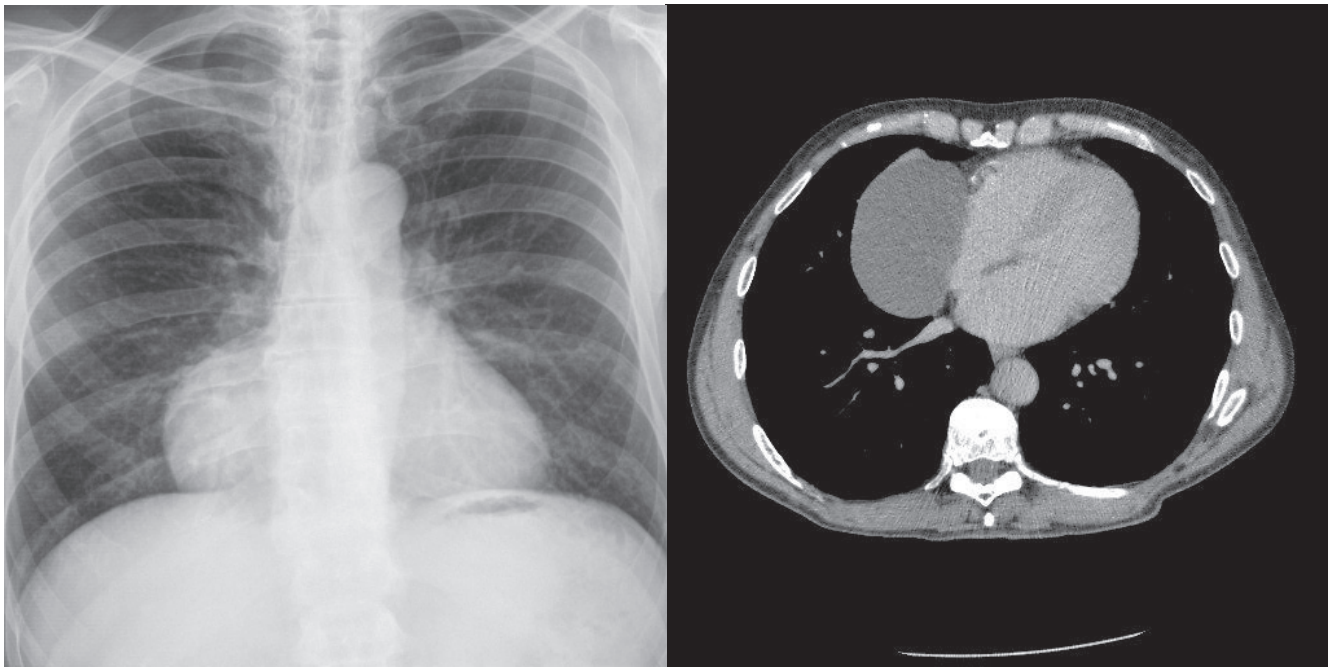

Figure 1. Left: Preoperative chest X-ray; Right: Preoperative CT chest scan of the patient

REPORT OF A CASE

A 70-year-old male presented with a mass in the anterior mediastinum. The mass was incidentally detected on a chest X-ray during the evaluation of persistent dyspnea and back pain lasting for previous three months. During his examinations, he was approached in a multidisciplinary way, and the imaging diagnosctic followed. CT scan of chest showed a large heterogeneous soft-tissue dense mass of 96mm × 67cm × 85mm in the anterior mediastinum extending to right hemithorax (Fig. 1).

Based on our experience with mediastinal masses, and reviewing complete medical history, the patient was planned for complete resection. He underwent a right posterolateral thoracotomy through the 5th intercostal space. Intraoperatively a large cystic mass present in anterior mediastinum (Fig. 2).

With careful blunt and sharp dissection, the mass was dissected all around and was resected completely without injuring any vital structures. Apical thoracostomy tube was placed and wound was closed in layers. The patient recovered uneventfully. Serial postoperative X-rays showed a complete right lung expansion (Fig. 3). The patient was discharged from the hospital one week after the operation. Histopathology report described a mature teratoma with cystic structures.

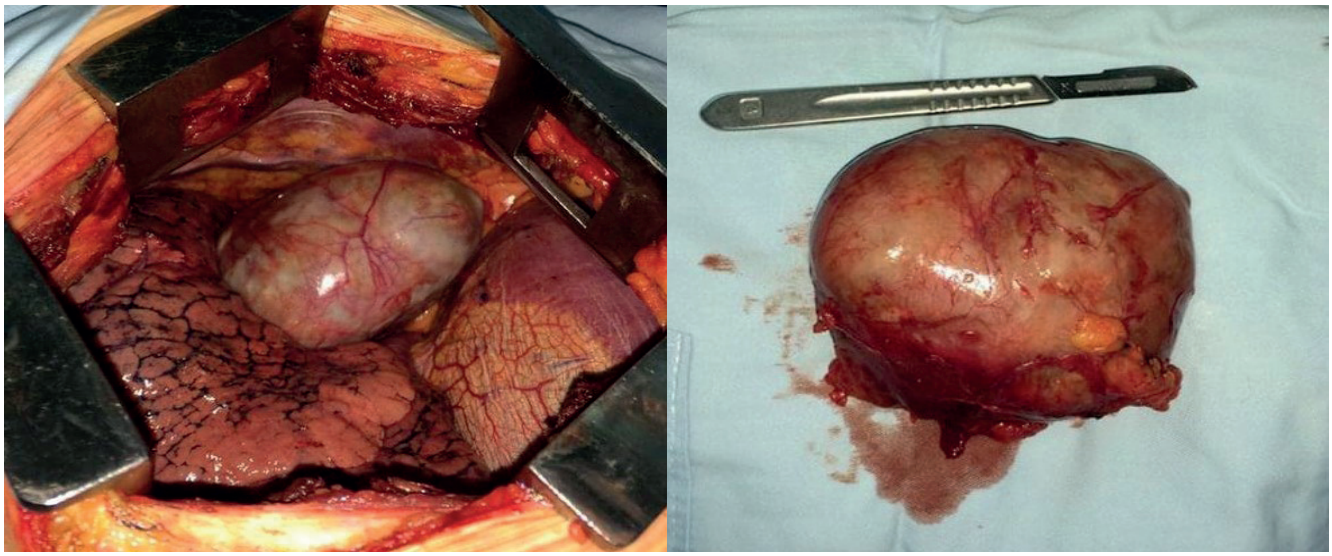

Figure 2. Left: Intraoperative finding of the mediastinal mass; Right: Extirpated mediastinal mass with surgical knife for size comparisement

DISCUSSION

Cystic lesions of the mediastinum are uncommon, comprising 12% to 18% of all primary mediastinal tumors and unless they attain a large size and cause compressive symptoms, these tumors are generally asymptomatic and are discovered incidentally upon radiologic investigation of some other condition (4, 5). Mediastinal cysts can be classified on the basis of their anatomical location and histomorphology. Thus, the cysts may be found either in the superior, anterior, middle or posterior mediastinum. Histologically, they may be classified into foregut cysts, cystic teratomas, thymic cysts and a large miscellaneous group. Foregut cysts are further categorised on the basis of their anomalous embryonic origin into bronchogenic, oesophageal, gastric and undifferentiated cysts (6).

Despite this wide repertoire of location and morphology, the symptomatology is nearly identical. Chest pain is a common symptom and is thought to be result of irritation or inflammation of the parietal or mediastinal pleura. Other symptoms like cough, dyspnea and dysphagia are all considered manifestations of compression or irritation of major airways and oesophagus by the cysts. Severity would depend on the size of the lesion (7). In our case, patient had sympthoms of dispnea and back pain two months before the surgery.

Benign mediastinal teratoma accounts for 60% of all mediastinal germ cell tumors, which in turn account for 15–20% of all anterior mediastinal masses. These tumors have been described in patients with ages ranging from 7 months to 65 years, most occur in young adults, with an approximately equal incidence in males and females (8).

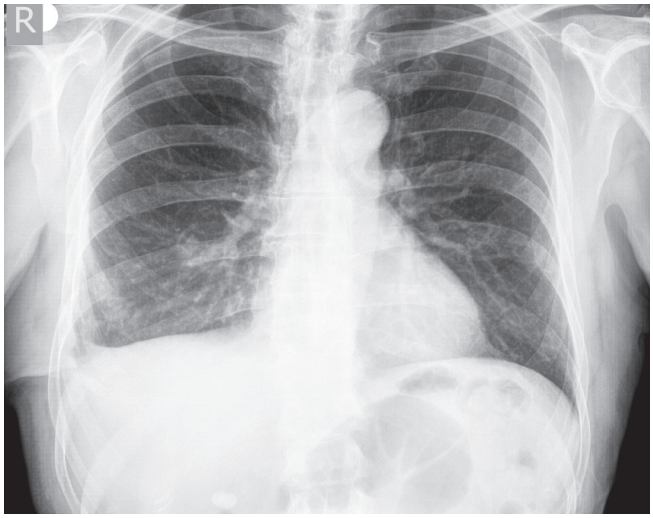

Figure 3. Postoperative chest X-ray

Mature teratomas are neoplasms derived from endodermal, mesodermal and ectodermal origin. Within the mediastinum these lesions are most commonly seen in the anterior mediastinum. Given their origin, these lesions are usually filled with sebaceous material, but may also contain macroscopic fat, bone, hair or other tissues. A small percentage may present as a primarily cystic lesion (9).

These tumors are slow growing and in recent years, 50 to 60% of patients have been asymptomatic at the time of diagnosis by routine chest radiography or other imaging diagnostical procedures. In our report that was the case. At radiography, cystic teratomas usually appear as a sharply marginated, round or lobulated anterior mediastinal mass that extends to one side of the midline. At CT scan, these

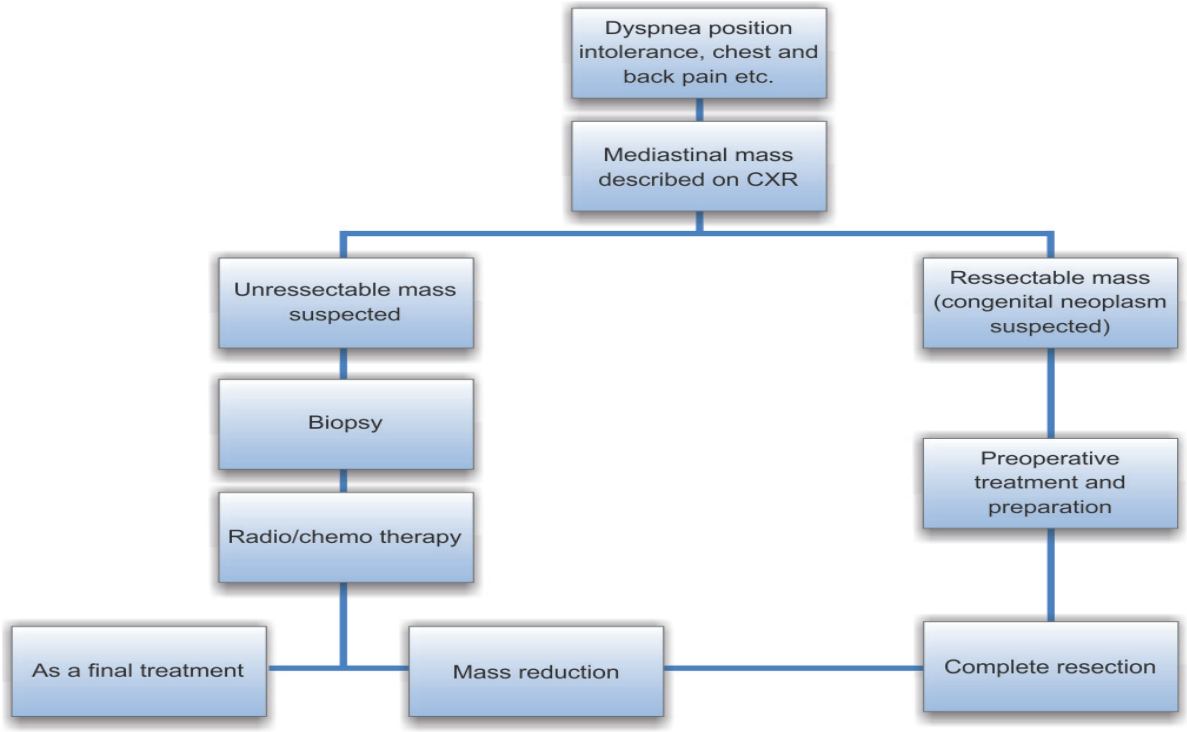

Figure 4. Short algorithm for diagnosis and treatment of mediastinal mass

tumors are heterogeneous, well-defined masses with walls of variable thickness that may enhance. They may contain all four tissue types, including soft tissue, fluid, fat, and calcium, but fluid-containing cystic components are usually prominent (10).

Benign lesions have no sex predilection; however, malignant ones are more common in males. Mediastinal teratomas rarely produce symptoms except when they attain large size or may rupture into the lung and bronchial tree, pleural space, pericardial space, or great vessels which can lead to the life threathinig complications (11, 12, 13).

Despite the value of various noninvasive diagnostic studies, definitive diagnosis is established only by surgical excision and tissue biopsy (Fig. 4). Transtracheal and percutaneous cystic aspirations have been proposed as alternatives to operation, but these methods are not widely accepted because of possible cystic recurrence, which carries a substantial morbidity rate or risk of complications related to the diagnostic procedure (14, 15).

Many of the patients who do not undergo surgery at diagnosis develop symptoms related to growth of the cyst, which means that an operation then involves a higher morbidity and mortality rate, together with a risk of malignancy and development of complications (16).

There are those who recommend conservative treatment for mediastinal cysts, on the ground that it avoids surgical morbidity and mortality. To the contrary, the prognosis after complete excision is excellent, and the morbidity and mortality rates associated with surgery are low as it was shown in our case (17).

CONCLUSION

Based on a review of the literature, as well as our experience, we conclude that best treatment for cystic lesions of anterior mediastinum is complete surgical resection if possible. Surgical procedure, if performed well, has low mortality and morbidity rates, with excellent prognosis for long term survival rate and complete treatment.

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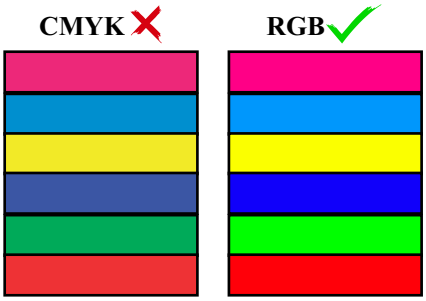

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