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Table Of Contents

BCL-2 FAMILY OVEREXPRESSION AND CHEMORESISTANCE IN ACUTE MYELOID LEUKEMIA

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PREKOMERNA EKSPRESIJA I HEMOREZISTENTNOST BCL-2 FAMILIJE U AKUTNOJ MIJELOIDNOJ LEUKEMIJI

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ABSTRACT

The family of Bcl-2 proteins is one of the most responsible for apoptosis pathway, that is a critical process to the maintenance of tissue homeostasis. Bcl-2 is an essential apoptotic regulator belonging to a family of functionally and structurally related proteins known as the Bcl-2 family. Some members of this family act as anti-apoptotic regulators, whereas others act in pro-apoptotic function. The relationship between the pro and anti-apoptotic proteins can regulate whether cells begin the apoptosis or remain its life cycle. Increasing of Bcl-2 expression has been found in some hematologic diseases, such as Acute Myeloid Leukemia (AML) and their effects on responsiveness to anticancer therapy have been recently described. Thus, this review aims to discuss apoptosis and the role of the Bcl-2 family of proteins in chemoresistance when overexpressed in patients committed with Acute Myeloid Leukemia submitted to chemotherapy treatment.

Keywords: *Acute Myeloid Leukemia, Apoptosis, Bcl-2 family, Chemotherapy resistance*

SAŽETAK

Familija Bcl-2 proteina je jedna najznačajnijih za odvijanje apoptoze, procesa od ključnog značaja za održavanje tkivne homeostaze. Bcl-2 je osnovni regulator apoptoze koji spada u porodicu funkcionalno i strukturno povezanih proteina poznatih kao Bcl-2 familija. Neki članovi

ove familije deluju kao anti-apoptotički regulatori, dok ostali pospešuju apoptozu. Odnos između

pro i anti-apoptotskih proteina određuje da li ćelije započinju apoptozu ili nastavljaju životni ciklus. Povećanje ekspresije Bcl-2 postoji u nekim hematološkim bolestima, kao što je akutna mieloidna leukemija (AML) i nedavno su opisani efekti povećanja ekpresije Bcl-2 na antitumorsku terapiju. Usled toga, ovaj revijski rad ima za cilj da razmotri apoptozu i ulogu hemorezistencije Bcl-2 familije proteina kod pacijenata sa akutnom mijeloidnom leukemijom koji se leče hemoterapijom i kod kojih postoji preterana ekspresija Bcl-2.

Ključne reči: *Akutna mieloidna leukemija, apoptoza, Bcl-2 porodica, otpornost na hemoterapiju*

INTRODUCTION

The mechanism of cell death is a decisive process to the maintenance of tissues homeostasis. Furthermore, its regulation leads to cell death through intracellular mechanism control (1-3). There are some natural mechanisms of cell death, which are most common seen in the literature such as autophagy, apoptosis, necrosis, and the last one added to this list was the necroptosis, which induce cells to die and disseminate especially carcinogen cells (2,4). These are mechanisms that occur inside the tissues in order to maintain the cell balance into the organisms (5). Apoptosis, was classified by Clarke in 1990 as the programmed cell death (PCD) type I, being an essential, critical and important process characterized by some alterations either biochemical or morphological to the cell structure leading to pack up the cell to be removed via phagocytosis and is triggered to remove diseased, damaged or aged cells (6-10). Apoptosis either can be activated by many oncogenes and the bcl-2 is one of them that play an essential function to support the cell lifespan. In fact, bcl-2 was identified as an important oncogene that drives some ability to activate and execute the apoptosis pathway (11). Moreover, it has been demonstrated that high levels of the Bcl-2 proteins especially the anti-apoptotic members leads to inhibition of apoptosis. The capacity to impairing the cell death confer to these proteins an interesting target for potential drugs used to treat cancers. However, the Bcl-2 family also

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have been associated to chemoresistance of many anticancer drugs what put it line highlighted for significance related to prognostic and treatment of many cancer such as Leukemia (12) especially Acute Myeloid Leukemia (AML). Bcl-2 has been frequently associated to reduction of the susceptibility of the current chemotherapies especially due to its ability to impair the apoptosis process (13,14evasion of programmed cell death (apoptosisevasion of programmed cell death (apoptosis). Here we suggest revising the role of bcl-2 family and its influence to trigger the chemoresistance of many current drugs used to treat patients committed with AML.

APOPTOSIS

Kerr and colleagues in 1972 suggested apoptosis as a definition for some pattern related to the morphology of the cell death during the embryonic phase after some observation of how cells were eliminated and also in adult healthy tissue turnover atrophy establishment after withdrawing some essential hormone (15). Apoptosis is a process that excludes undesirable cells with its progression morphological modifications can arise into the cell (16, 17). This process is complex and in human organisms have a close comparison with another organism known as *Caenorhabditis elegans* (18). Its regulation however, is performed by molecular components such as the B-cell lymphoma 2 family (Bcl-2 family) that has been in line highlighted due to their relevant role along of the cell death process [6]. Bcl-2 members are regulatory molecules for apoptosis process. Some components work to induce cells to die, acting as pro-apoptotic members such as Bad, Bak, Bax, Bid, Bim, Bmf, PUMA, and NOXA, whereas other act as anti-apoptotic components impairing the death process, which include the Bcl-2, Bcl-w, Bcl-XL, A1 and Mcl-1 (19).

The mechanisms of apoptosis are divided into two main pathways. The first is regulated by death receptor located on the cell surface known as extrinsic pathway, and the second has its focus on modification of the mitochondrial membrane permeability, known as intrinsic pathway (20). The death mechanisms for cancer cells, especially apoptosis and necrosis are not a simple issue, however (21-23).

Carcinogens cells have a high rate of proliferation, however these cells do not have their lives time longer than normal healthy cells; in fact, their lifespan is reduced (24). This was described on a study performed by Alex Carrel in 1925 who stated, "Malignant cells are sick cells which live shorter". Fisher later confirmed this results in 1937, showing that cells with malignant characteristics are sick and have a short lifetime, indeed (24). Fisher also demonstrated in his study that normal and carcinogens cells behave different when dead. Normal cells remains among the living cells in an inert state for long time, whereas cancer cells immediately are hydrolyzed and demise (25). Cancers are likely to develop in different organs such as into the bone marrow that is likely to arise some types known as leukemia, and one of the most well characterized leukemia is the Acute Myeloid Leukemia (AML) that is a severe and common complication among the individuals (26). AML is the most common manifestation of leukemia, being a genetic disorder identified by alterations of precursor or hematopoietic stem cells, resulting in blockage of their differentiation, as a consequence accelerated proliferation premature myeloid cells into the bone marrow, which leads to infiltration to other organs (27-30). Regarding the different therapeutic target for AML, it has been identified that the Bcl-2 proteins are associated with resistance to chemotherapy and cell survive especially the anti-apoptotic members when overexpressed. Therefore, focus on anti-apoptotic proteins and blockage their function might be an efficient alternative to induce apoptosis and activating pro-apoptotic members of Bcl-2 proteins resulting in the elimination of carcinogens cells (24, 31, 32, 33).

THE BCL-2 FAMILY OF PROTEINS

The Bcl-2 proteins are large components of the BCL family, which have an important function modulating process involving cellular death, either by the physiological or pathological pathway. In addition, it can be controlled by the abundance of modification via posttranslational mechanisms (34,35). These proteins family was firstly identified as a translocation problem in the chromosome 18q region 21 (18q21) and in the heavy chain of the gene from immunoglobulin located at the region 14q32, which result in decontrol in transcription of Bcl-2 proteins, this is characterized as a translocation t(14;18), mainly in follicular B-cell lymphomas (36-38). In addition, Bcl-2 family has a crucial role to regulate the apoptosis process in the mitochondria, which regulates the mitochondrial outer membrane (MOM), where the Bcl-2 members are located in (39,40,41). Based on its homology, this family has different subtypes, which are divided in three according to their functions, structure and domains (40,42). The division includes the members that prevent apoptosis or the anti-apoptotic members, whereas the others components stimulate apoptosis, pro-apoptotic proteins, which includes the Bcl-2 only proteins or BH3 pro-apoptotic only proteins (Fig. 1) (43, 44).

BCL-2 FAMILY AND APOPTOSIS REGULATION

The apoptosis regulation via Bcl-2 has been debated for many author along the years, some of them stipulate that Bak and Bax are sequestered by pro-survival proteins, whereas other argued that the main role of these proteins was insulated the BH3 only proteins members (45-50). An experimental study provided that the apoptosis mechanism is inhibited by pro-survival proteins, hence BH3-only proteins are sequestered while Bak and Bax are activated (51).

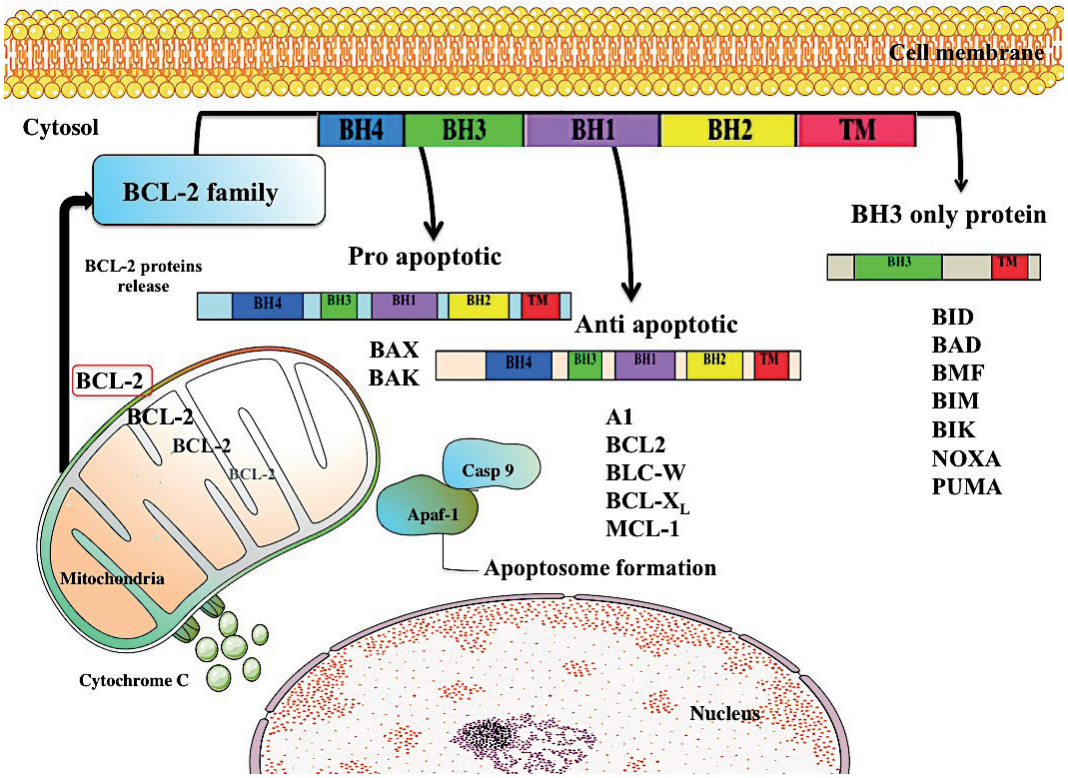

Figure 1. The Bcl-2 family members. The Bcl-2 family proteins are members that share domains and are divided into pro and anti-apoptotic proteins, their domains are known as Bcl-2 homology (BH domains). The figure shows the anti-apoptotic members, which have the function of neutralization, the death process into the cell, and includes A1, Bcl-2, Bcl-w, Bcl-xL and Mcl-1 molecules share BH homology domains from 1 to 4. On the other hand, the pro-apoptotic members are divided in two different groups known as effector and the BH3 only proteins. The members of the effector group are BAX and BAK and share homology domains from 1 to 4, however, the BH3-only proteins members have one BH domain and binding anti-apoptotic proteins including molecules such as BID, BAD, BMF, BIK, NOXA and PUMA.

Another important discovery is related to mitochondria scenery, which argues that the mitochondrial outer membrane (MOM) is extremely important to Bcl-2 family members interaction. In addition, these studies have suggested that such intercommunication among the members play an important role influencing the affinity among each member (47,48,50). Moreover, another character essential for apoptosis control is the tumor suppressor gene p53. It is crucial for apoptosis process due to its capacity to mediate the response to genotoxic stress (52). The apoptosis dependent of p53 activity was firstly observed in thymocytes of irradiated mouse according to Clarke and colleagues and Lowe and collaborators (53). The p53 is an extremely important tumour suppressor gene, mutation as well as inactivation on this gene is crucial for development and spreading of tumours. Indeed, most of the tumor types in humans are related to its mutation. However, when associated to haematological tumor types this arise in 11.1% only for notified cases. These data are according to the International Agency for Research on Cancer (IARC) version R15, that afford information and tools for analyses and studies of mutations related to many human cancers types giving support to investigate their impact in the clinical field (54,55). Furthermore, oncogenes for example adenovirus E1A combined with irradiation is likely to promote the activation p53 resulting in apoptosis, in most of the case for "transcription-dependent effects" (53,56,57). The p53 controls apoptosis through independent and dependent pathways and in addition, some stress signals have been associated with its activity, resulting in accumulation of it inside the cell either in the cytoplasm and nucleus (43, 57, 58).

BCL-2 FAMILY MAKES DECISION IN APOPTOSIS PROCESS

The members of Bcl-2 family regulate whether the cells remain alive or conduct them to apoptosis via some modifications of the mitochondrial membrane and consequent caspases activation. This process result from reciprocal action between anti and pro-apoptotic proteins, as a consequence of this intercommunication cell death process, is conducted to occur in order to maintain the homeostasis of the organism (43, 59, 60). The bcl-2 family has two different functions, being either anti-apoptotic, which avoid mitochondrial outer membrane permeabilization (MOMP) and pro-apoptotic activities or in other words promoting cell death via MOMP process. Furthermore, sharing of homology domains known as Bcl homology or also called BH regions is responsible for controlling the communication among the Bcl molecules

(43, 61, 62). Despite of anti and pro-apoptotic proteins being found in different cellular area, the anti-apoptotic have especial space into the mitochondrial membrane and endoplasmic reticulum, whereas the pro-apoptotic components are located into the cytoskeleton or cytosol (57,63).

PRO-APOPTOTIC MEMBERS

The pro-apoptotic members are the main proteins responsible for MOMP occurrence. These members' activities resulting in caspase activation and consequent release of cytochrome c from mitochondria through pores formation. The two principal members of this group are Bak and Bax (57, 70, 64). This group is also known as effectors proteins and has three BH domains. Bak and Bax molecules are antagonist killer, and associated to X protein respectively and separated promoting MOMP occurrence (57, 64).

Some research performed with mice that had an inadequate quantity of Bak and Bax proteins showed that these proteins are fundamental for apoptosis process in several cell types, especially in lymphocytes, due to their deficiency, which resulting in long life for cells, especially for hematopoietic cell. In addition, studies also showed that only Bax is enough to induce apoptosis process in most of the cases, however, it is extremely necessary to occur a signal that leads to its connection with the activator molecule (39, 66, 61).

ANTI-APOPTOTIC MEMBERS

The anti-apoptotic components are in contrast with the pro-apoptotic members, responsible for maintenance of MOM integrity preventing the mitochondrial pathway and consequent cell death, remaining the cell life over its life phases (57, 65). This group is basically compounded by different members such as A1 or Bfl-1 (or either the BCL-2 proteins associated to A1 gene), Bcl-B, Bcl-2, Bcl-XL, Bcl-w and Mcl-1 (or related to Myeloid Cell Leukemia) which interact with the BH3 only proteins that belongs to the pro-apoptotic group (66) (Fig. 1) (49). The function of Bcl-2 and Bcl-XL regulate the apoptosis process by binding to pro-apoptotic proteins Bax and Bak inhibiting them in BH3 region as a result of prevention of MOMP (table 1) (57, 44, 62, 66, 67).

Table 1. BCL-2 family members, locations and functions.

Protein	Location	Mechanism of action	Action	References
Bcl-2	Outer mitochondrial membrane, nuclear envelope, endoplasmic reticulum membrane	Preservation of the mitochondrial membrane integrity resulting in apoptosis inhibition	Antiapoptotic	57, 65
Bcl-xL	Transmembrane molecule in the mitochondria	Inhibition of caspase activation cascade by release of cytochrome c from mitochondrial pore formation	Antiapoptotic	57, 69, 64
Bcl-w	Completely on the mitochondria	Cytotoxic conditions reduce cell apoptosis leads to cell survive	Antiapoptotic	67
Mcl-1	Nucleus and mitochondria	Death promoter Bcl-2 associated, half-life reduced, interaction with NOXA, Bak1 and Bcl-2	Antiapoptotic	68, 70
Bax	Cytosol	Caspase cascade activation, release of apoptotic factor such as cytochrome c	Proapoptotic	57, 69, 64
Bak	Membrane of the integral mitochondrial proteins	Induces conformational changes resulting in larger aggregates during apoptosis process	Proapoptotic	57, 60, 65
Bid	Membrane and cytosol	Activate directly Bax protein and induces apoptosis	Proapoptotic	70, 71
Bim	Free Bim in the mitochondria	Bcl-2 or Bcl-xL is binding by free Bim resulting in inactivation of their anti-apoptotic activity, leads to apoptosis through deprivation of cytokines, microtubules perturbation and flux of calcium ions.	Proapoptotic	71, 72
Bad	Free Bad into the mitochondria	Heterodimer with Bcl-2 and Bcl-xL is formed by desphosphorylation of Bad, Inactivation of Bcl-2 and Bcl-xL allow Bak and Bax resulting in apoptosis mechanism	Proapoptotic	69

The Bcl-2 family of proteins is divided into antiapoptotic and proapoptotic members. Each one of them is located in different cell compartment and has different functions when activated. Their mechanism of action is specific, acting in some situation when is required to perform their specific role. Most of them are target to many drugs used in some pathologies such as cancers from different types.

According to Billard (2015), all anti-apoptotic members are able to bind to Bax however; only Mcl-1 and Bcl-XL might interact with Bak through the BH3 domain (fig. 2) (68). In order to develop their function precisely, the anti-apoptotic members require the BH4 domain (69). Despite Bcl-2 functions being located into the mitochondria, some studies have documented their functions either in the endoplasmic reticulum (ER) and nuclear cells membrane. Moreover, its activities have been demonstrated especially for Bcl-2 members through findings of sequence cytochrome b5 or also known as b5-Bcl-2 in the ER. It was showed that this sequence impairing apoptosis promoted by ER agents or expressed by Bax members (70).

BH3 ONLY PROTEINS

In mammalian cells, there are several molecules that compound the Bcl-2 family (fig. 2) (49, 71). The members of this family prevent cells from some cytotoxic damage such as UV and gamma irradiation, cytokine deprivation and drugs for chemotherapy purposes. Members of this family such as Bcl-XL and Bcl-w are the most important molecules that have the cited function. Some other members are identified as the binding proteins and are divided into two distinct groups, one of them compound by a trio of molecules to illustrate Bak, Bax, and Bok, which have a closer similarity to Bcl-2 members either in sequence or structure. The other group has three segments well conserved BH1, BH2, and BH3 or known Bcl-2 homology that form "hydrophobic groove" with anti or pro-apoptotic molecules, These molecules have this description due to their unique BH3 domain (59).

The main members of this subclass include the subtypes Bad, Bid, Bim, Bmf, Hrk, Noxa and Puma (table 1) (64,70,74). The BH3 only protein regulation is controlled by several and distinct mechanisms such as DNA damage, Cytokine deprivation, Tyrosine Kinase inhibitors, proteasome inhibitors, and cytotoxic inducement such as anticancer drugs (75, 76). Doerflinger and colleagues defined them as "the sentinels of cellular stress" because when activated, result in apoptosis initiator process (77-79). Their location has been recently discovered into the MOM, especially for the subtypes Bim, Bmf, Bid, Puma, and Noxa. Despite their location have not been prioritized in studies, it is necessary to understand their mechanisms, once this location is crucial to Bax activate Bim, tBid and Puma, and confer the role onto apoptosis process (79).

The activation process is performed by transcriptional and post-transcriptional mechanisms; however, the post-translational signal configures the most important, and once activated the response to death occur translocation in the mitochondria (64). The BH3 only proteins displace the Bak and Bax when apoptosis process initiates, especially Bid, Bim, and Puma are able to bind more efficiently and displace them, which can be bounded previously by anti-apoptotic members (80,81). In addition, BH3 members are divided into two main groups: The activators and sensitizers. The activators members include Bid, Bim, and Puma, due to their capacity to activate direct Bak and Bax, some studies also relate Noxa as an activator as well. On the other hand, the sensitizers' members include Bad and Bik and act releasing the activator, which was connected with anti-apoptotic molecules (81).

LEUKEMIA

Leukemia is defined as a neoplastic and malignant proliferation of the blood cells and the bone marrow. This abnormality affects especially the white blood cell (WBC's). As a consequence of the high proliferation rate, it leads to accumulation and interruption of the WBC's normal function and their production resulting in interruption of the other lineages such as erythrocytes and platelets synthesize characterizing anemia and thrombocytopenia (82,83). According to Hamerschlak, leukemia is divided into different subtypes, four major types are known as Acute Lymphoblastic Leukemia (ALL), and Chronic Lymphocytic Leukemia (CLL), Acute Myeloid Leukemia (AML), Chronic Myeloid Leukemia (CML) (83, 85).

ACUTE MYELOID LEUKEMIA

Acute Myeloid Leukemia (AML) is an extremely malignant hematological disorder proliferation and results from a clonal, undifferentiated and immature blood cells. AML is a high aggressive disorder that leads to apoptosis resistance and allows their growth and transformation of a hematopoietic precursor known as myeloblasts (84-85,86). Bone marrow and tissues infiltration characterize AML, and the myeloid precursors have abnormal synthesis resulting in their appearance in the blood stream. This abnormality leads to insufficiency of hematopoiesis process (86).

AML diagnosis is based on the presence of more than 30% of blasts cells in the bone marrow according to the French American British system (FAB) (34). On the other hand, according to World Health Organization (WHO), the diagnosis is based on 20% of nucleated cells knows as myeloblasts, however, associated with some molecular and genetics abnormalities located into the chromosomes especially (87,88,89). These myeloblasts were related to express high levels of bcl-2 proteins which have increased the chemotherapy resistance (90, 54).

BCL-2 PROTEINS' ROLE IN CHEMOTHERAPY RESISTANCE

According to Tzifi and colleagues, the apoptosis is regulated by caspases, that can be either an initiator such as caspase 9 that activate the effectors caspases 3 and 7 by the cleavage of an internal residues recognized as Asp in their substrates separating small and large unites, and triggered

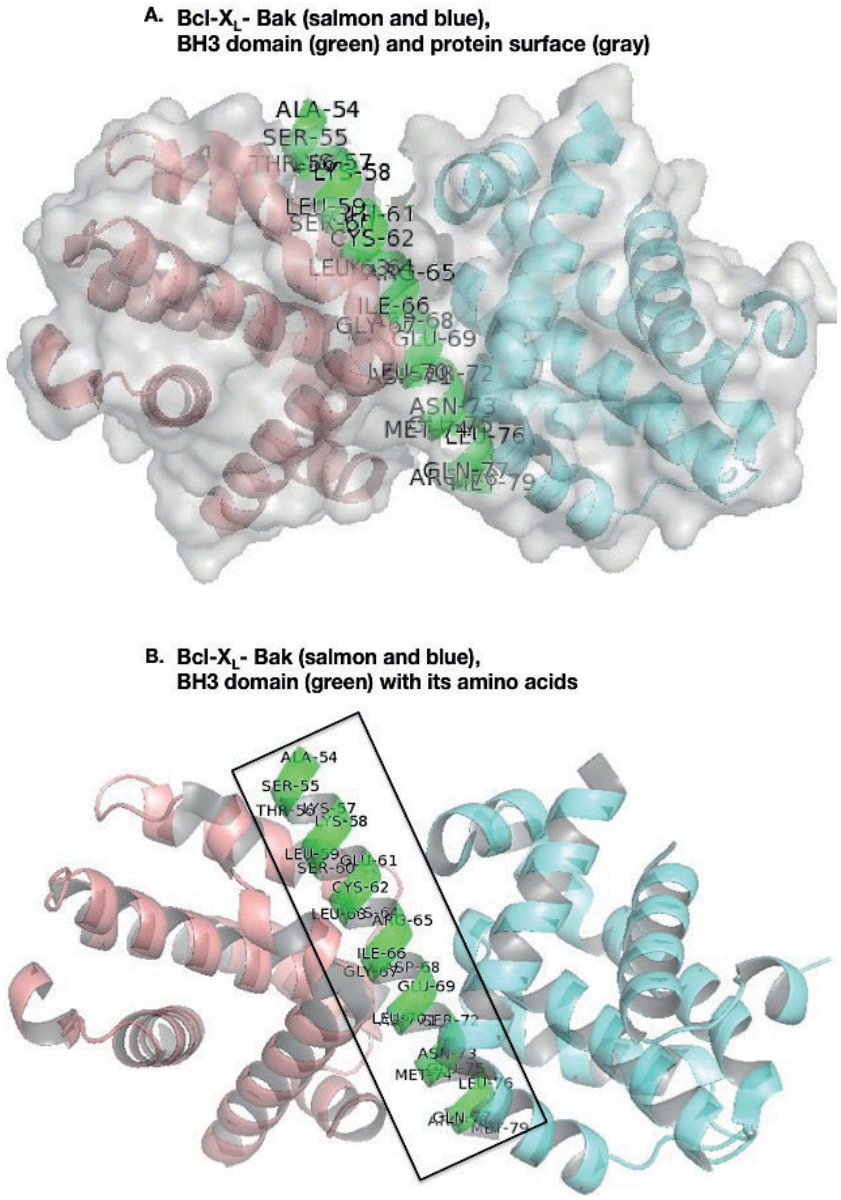

Figure 2.
Structure of the Bcl-X$_L$ interacting with Bak through the BH3 domain. It can be observed their structures connecting to each other through the BH3 domain (green). The binding groove is formed by some amino acids that give rise to different conformational structure and its organizational form. It is evidenced the structural interaction between the Bcl-XL and Bak. In (**A**) is demonstrated the complex formed by Bcl-X$_L$- Bak showed in salmon and blue, and the BH3 domain in green color, the protein surface is seen in gray. In (**B**) Bcl-XL-Bak (salmon and blue) and the BH3 (green) with the amino acids presence (black square) and (**C**) evidences the BH3 domain with its amino acids and (**D**) shows the BH3 amino acids sequences. Protein Data Bank (PDB) [69] entry for the displayed structure: Bcl-XL–Bak BH3 is 3PL7.

by the Bcl-2 proteins (82,91). These molecules are related to cell survivor, although their activity do not drive any function with the cell proliferation, and it is indeed that the tumor genesis process is linked to the cell survivor and deregulation of Bcl-2 proteins (46). Mcl-1 and Bcl-XL genes are highly expressed in humans diagnosed with AML and multiple myeloma according to recent studies (82).

In addition, it was observed that overexpression Bcl-2 family is related with resistance to cancer treatment (92). Indeed, it has been very common and frequently identified the apoptosis resistance in cancer therapies. Furthermore, when aberrant apoptosis pathway is identified, it is linked to chemotherapy and /or radiotherapy resistance, whereby most of the patients have been attended for many decades (92, 93). Yip and Reed in their study in 2008, argued that overexpression of both Bcl-2 especially the anti-apoptotic members have been associated with chemoresistance, which impair the apoptosis process through some stimuli such as oxidative stress, hypoxia, and deprivation of growth factor (70).

More and Letai also stated that most of the cancer cells are habituated to Bcl-2 proteins presence and these oncogenes play an important role inducing their survival and the pro-apoptotic Bcl-2 member's overexpression to oncogenic stimulus in tumor cells do not influence enough to overcome the overexpression of Bcl-2 anti-apoptotic proteins signalization into the cancer cells (62,94-97). Wei and Teh in 2012, argued that increasing of pro-survival members is related to resistance of many cancer therapies as confirmed by Danial, (2007) and Weyhenmeyer and colleagues (2012). In fact, these studies bring us evidences that hematologic disorders especially AML with overexpression of Bcl-2 members have more resistance to chemotherapy (29).

Another members of the Bcl-2 family are the BH3 only proteins, which play an essential and crucial role in chemotherapy resistance. These members modify their capacity to ligate Bcl-2 targets. In addition, Bim neutralizes potentially all apoptotic members, on the other hand, Bad has its function limited to other members such as "Bcl-2, Bcl-x, Bcl-w and Noxa that can bind only to Mcl-1 and A1" (97). Therefore, this role of function leads to predict to the responsiveness of cancer therapy such as chemotherapy to mitochondrial apoptosis both in cancer and normal cells. However, increasing the activity of BH3 only proteins is likely to result in direct activation of Bax and Bak resulting in cell apoptosis (97).

Bcl-2 proteins should occupy the binding site, however, cells that present high levels of BH3 occupied by Bcl-2 proteins are "highly primed" to induce death and measured by its affinity to BH3 members. On the other hand, BH3 member in excess and unoccupied by a Bcl-2 result in "low BH3 priming" as a result of it leads to high cell resistance to mitochondrial depolarization conducted by BH3 only proteins and result in effect of cytotoxic therapeutically methods (97, 98). If a comparison were performed with other pro-survival members activities in AML, BH3 members dislike no previous knowledge of prevailing levels of pro-apoptotic or anti-apoptotic members of Bcl-2 family. This, however, is extremely relevant in cases where Bcl-2 increasing result in stabilization and accumulation of Bim leading to lower level of unoccupied Bcl-2 than expected (98). As Vo and colleagues (2012) stated in their study, "the dominant pro-survival factor in human AML was Bcl-2 compared to Mcl-1 in hematopoietic stem cells with normal conditions" and that the target of the Bcl-2 members is likely to allow the final destruction of carcinogens cells in AML, while the toxicity of normal hematopoietic stem cells would be protected by Mcl-1. In order to overcome these findings some molecules have been designed to target some members of the Bcl-2 family. These compounds, binding to the hydrophobic site of the Bcl-2 anti-apoptotic member and perform its function like BH3-only proteins to induce the apoptosis process (99). Several studies have demonstrated some evidences that apoptosis has an important role in responsiveness to chemotherapy. In addition, was also observed that AML CD 34+ is more resistant to apoptosis than CD34- and this is also correlated to higher expression of bcl-2, Mcl-1, bcl-XL and low levels bax expression. This leads us to conclude that the AML cases that express CD34+ indicate the resistance to apoptosis (90). Some synthetic or even natural molecules have been described as inhibitors of the Bcl-2 family and are know especially as BH3 mimetic molecules (70). These BH3 mimetic components such as ABT-737 have the ability to bind to some blc-2 family molecules mainly to bcl-2, bcl-XL and bcl-w, however not to Mcl-1. This ligation leads to disruption of their synergy with pro-apoptotic members Bak and Bax intensifying the apoptosis, this ligation occurs with high affinity among these proteins (100, 101Bcl-xL and Mcl-1, resulting in resistance to apoptosis and association with poor prognosis. Docetaxel, an antimitotic drug that is the first-line treatment strategy for CRPC, is known to provide a small survival benefit. However, docetaxel chemotherapy alone is not enough to counteract the high levels of Bcl-2/Bcl-xL/Mcl-1 present in CRPC. ABT-737 is a small molecule that binds to Bcl-2/Bcl-xL (but not Mcl-1). ABT-737 is also known as a BAD mimetic that has shown efficacy in some types of the tumor including leukemia. After its ligation and inhibition of the cited molecules it can result in apoptosis of carcinogens cells no affecting the adjacent normal cells. However, in some tumor the resistance mechanism has been associated with overexpression of the Mcl-1 members that is an anti-apoptotic protein that the ABT-737 does not target to (70). As has been suggested by Vo in 2012, if pro-apoptotic members being targeted in AML treatment it might allow a small molecule of the BH3 only proteins with selectivity upon Bcl-2 such as ABT-737, it will target therapeutically, efficient, and safely than most of the drugs and chemotherapies currently available and used for AML treatment (98, 102).

CONCLUSION

The high expression of the bcl-2 have been related to chemotherapy resistance reported in many populations around the world, what can be inferred is that due to their overexpression the induction of apoptosis is affected. Indeed the development of a therapeutic method, which targets the BH3 molecules, might have high and precise activity to treat AML in most of the cases and designing molecules to target especially the members of Bcl-2 family might be an alternative, once they have been found increased in many types of tumor. One of the alternatives to achieve the solution for this problem is decrease these proteins levels via the new emergent technologies. Moreover, another criteria that should have been considered is the patient safety, once receiving the drug this must have a total security regarding its side effect and therapeutic efficiency, when compared to several other drugs and therapies existent and that have been currently released from the pharmaceutical companies worldwide.

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CONFLICT OF INTEREST

Author declare no conflict of interest.

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EPILEPSY DETECTION USING DWT BASED HURST EXPONENT AND SVM, K-NN CLASSIFIERS

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OTKRIVANJE EPILEPSIJE UPOTREBOM DTW BAZIRANE HURSTOVE EKSPONENCIJE I SVM, K-NN KLASIFIKATORA

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ABSTRACT

Epilepsy is a typical neurological issue which influence the focal sensory system and can make individuals have seizure. It can be surveyed by electroencephalogram (EEG). A wavelet based HURST EXPONENT strategy is displayed for the analysis of epilepsy. This strategy deals with the nonlinear analysis of EEG signals. Discrete wavelet transform is used to disintegrate the original EEG signal into specific subbands. The hurst exponent of different sub-bands is employed and then fed into two classifiers, namely SVM and KNN. The highest classification accuracy obtained in the presented work is 99% for healthy subject data versus epileptic data is obtained by SVM. However, the corresponding accuracy between normal subject data and epileptic data using SVM is obtained as 99% and 93% for the eyes open and eyes shut conditions, respectively. The detailed analysis of the methodology and results has been discussed in the paper.

Keywords: *Hurst exponent (HE), Support vector machine (SVM), Discrete wavelet transform (DWT), K- nearest neighbor (KNN)*

SAŽETAK

Epilepsija je tipični neurološki pormećaj pod uticajem fokalnog sanzornog sistema koji može da izyzove epi-napade kod obolelih. Može da se otkrije analizom elektroencefalograma (EEG). Razvijena je strategija analize epilepsije pomoću HURSTOVE EKSPONENCIJE zasnovane na malim talasima, koja se bavi nelinearnom analizom EEG zapisa. Koristi se diskretna transformacija malih talasa kako bi se originalni EEG zapis dezintegrisao na specifične podgrupe. Primenom Hurstove eksponcije na različite podgrupe svrstvaju se u odgovarajuće klasifikatore, pre svega SVM i KNN. Najveća tačnost klasifikacije je postignuta u navedenom radu i iznosi 99% za zdrave osobe u poređenju sa obolelim od epilepsije primenom SVM. Međutim, odgovarajuća preciznost za razlikovanje zdravih osoba od obolelih od epilepsije upotrebom SVM iznosi 99% i 93% u zavisnosti od toga da li su oči otvorene ili zatvorene. Detaljna analiza primenjenih metoda i dobijenih rezultata je prikazana u radu.

Ključne reči: *Hurstova eksponencija (HE), podrška vektor mašina (SWD), diskretna transformacija malih talasa (DWT), K-najbliži komšija (KNN)*

INTRODUCTION

Epilepsy is a standout amongst the most predominant neurological issues in people. It is described as repeating seizures in which strange electrical action in the mind causes the loss of awareness or an entire body shaking. Patients are unaware of the event of seizure because of the irregular way of such seizures which may expand the danger of physical damage. Surveys demonstrate that 4-5% of the aggregate total population has been experiencing epilepsy.

Electroencephalogram is one of the most important tools for examination of epilepsy. Electroencephalogram is the recorded portrayal of electrical action delivered by terminating of neuron inside the mind along the scalp. For recording of EEG, terminals will be glued at some key focuses on the patient's head. Anodes get the signs and will be recorded in a recording gadget through wires which are associated with terminals.

As total visual examination of EEG signal is exceptionally troublesome, automatic detection is preferred. Fourier transform has been mostly utilized as a part of feature extraction of EEG signals. However as EEG signal is a non stationary signal, Fourier examination does not give precise outcomes. Most effective tool is wavelet transform. Better information can be extracted from the individual frequency sub signals rather than extracting it directly

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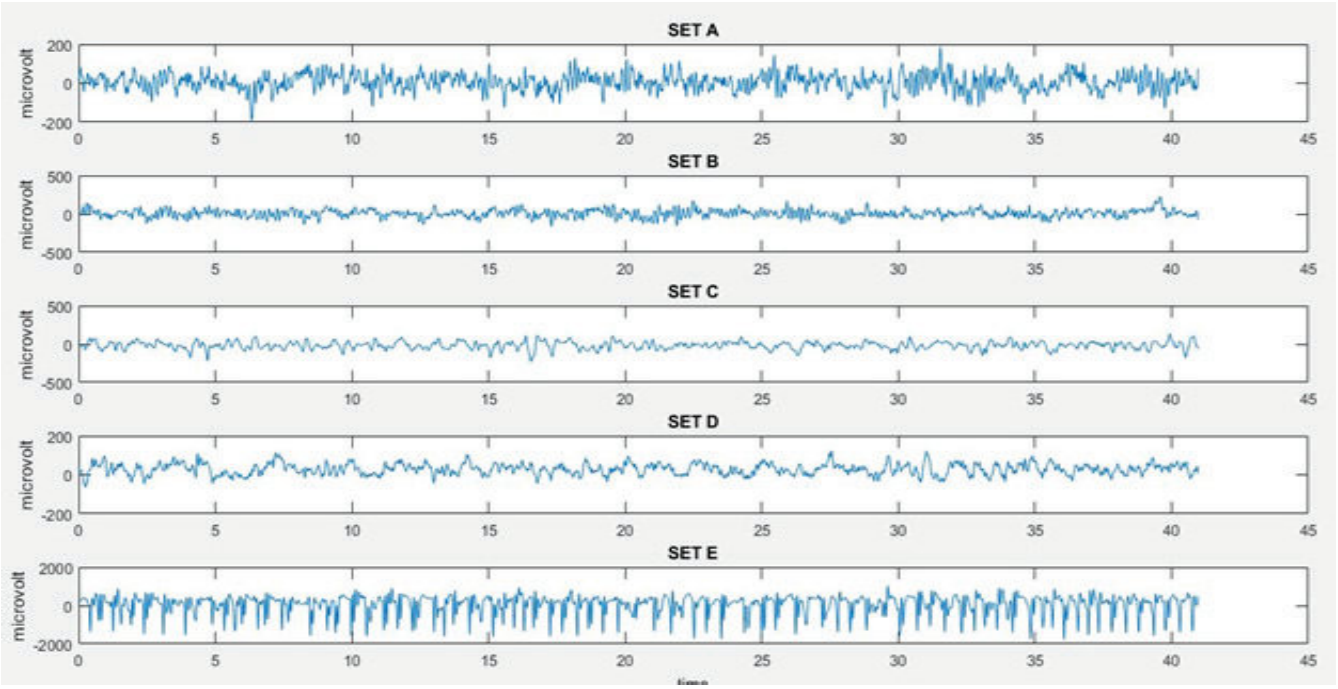

Fig. 1 Sample Plots of A-E Data Sets.

from the EEG signal. Also by using wavelet transform, it becomes easier to get the time and frequency information of a signal together, which makes it practical to obtain the transient components in EEG. Research works in recent studies can be categorized as time domain, frequency domain, time-frequency domain and nonlinear methods of analysis. Since the EEG signals are complex, nonlinear and non-stationary in general, time-frequency domain and non linear analysis methods are most broadly utilized in epilepsy detection. Nonlinear components, for example, time lag (TL), hurst exponent (HE), implanting dimension (ED), relationship dimension (CD) and biggest Lyapunov exponent (LLE) are extracted from the EEG and their each sub-band to describe and distinguish the epileptic seizure.

Nonlinear investigation is another prominent research strategy which could better reflect the attributes of the EEG signals. The utilization of Hurst exponent (HE) has been demonstrated to accomplish great accuracy in recognizing seizures. Also, entropies like estimated entropy (ApEn) and test entropy (SampEn) are broadly connected to uncover the shrouded complexities existing in the EEG time arrangement. In this paper, the HE is introduced to characterize the EEG signals in terms of the stronger relative consistency and less dependence on data length.

DATA

The complete database used in this study consists of five sets denoted as A-E, each containing 100 samples. These datasets are explained in Table 1. The signals were recorded with the 128-channel amplifier system and digitized at 173.61 samples per second using 12 bit resolution. Sets C and D is an interictal data and is recorded when the patient in pre-ictal state. Set E, which is called ictal data, contains signals recorded during the epileptic seizure. Fig. 1 depicts the sample of EEG signals for each of the five sets.

METHODS

In this study, DWT is used to decompose the original EEG signal into six sub-band signals using fifth level decomposition. Complexity of this EEG signal is simplified by extracting Hurst Exponent values from each sub-band signal. For classification, SVM and KNN classifiers are used. The block diagram of the proposed approach is shown in figure below fig 2.

Discrete Wavelet Transform

Since EEG signal is a non-stationary signal, Fourier Transform cannot be used as it can better analyse stationary signals but not non-stationary signals. It provides the signal which is localized only in frequency domain. Also, it

Fig 2 Block diagram of proposed approach

does not provide multi-resolution analysis. Moreover, window size is not available. These drawbacks are overcome in Wavelet transform. Wavelet change is an exceptionally helpful approach used on a signal for time–frequency representation since it utilizes the variable size of windows. Long time and short time windows are utilized to get a low and high frequency resolution data respectively. In this way, WT gives specific frequency data and time data at low frequencies and high frequencies, individually. Continuous wavelet transform (CWT) and discrete wavelet transform (DWT) are the two types of a wavelet transform. If x (t) is the input raw signal then CWT of a signal is

$$SENSITIVITY\ (SEN) = \frac{true\ positives}{true\ positives + false\ negatives} \times 100\%$$

where a is the scaling parameter and b is the shifting parameter. Now it is a difficult task to find the wavelet coefficient at each scale so the scaling and shifting parameters are converted to powers of two. DWT is defined as

$$DWT(j,k) = \frac{1}{\sqrt{2^j}} \int_{-\infty}^{\infty} x(t) \Psi \left(\frac{t - 2^j k}{2^j} \right) dt$$

First of all, input signal is made to pass through a low pass and high pass filter and output from this is referred as approximation coeffiecient (A1) and detailed coefficient (D1) of first level. Similarly, all the coefficients of five levels are extracted as D1, D2, D3, D4, D5, A5. At each level of decomposition, filtering is used to double the frequency resolution and down-sampling is used to half the time resolution. While analyzing a signal, the most important parameter to be considered is the number of decomposition levels, which in this case is chosen to be five. Since above 40 hz, the frequency components may not contain useful information so only fifth level decomposition is done.

Hurst Exponent

At the surface of brain, the response is created in the form of wavelets. A desired feature is extracted in the form of wavelets which is called as feature extraction. Hurst exponent is extracted from these wavelets only.

Hurst type (HE) is an established parameter which is utilized as a part of this case for nonlinear analysis. In a time series, this parameter is used to quantify the correlation of points.

- HE < 0.5 indicates that the sequences are long range anti-correlations and anti-persistent
- HE > 0.5 indicates the sequences with long range correlations.

The presence or absence of long-range dependence and its degree in a time series is assessed through HE. During interruption of seizures, HE is very much useful in identifying deviations from the normal pattern of brain activity. Commonly, HE can be estimated using rescaled range analysis (R/S) of which the equation is defined below

$$HE = \frac{log(R/S)}{\log T}$$

Where R is the difference between the maximum and minimum of deviation and S represents the standard deviation of the time series. T denotes the duration of the sample data (11).

Support vector machine (SVM)

For binary classification tasks in machine learning and for high dimensional feature vectors SVM is a very well known tool due to its accuracy and capability to deal with a large number of predictors. For multi-class classification process, SVM gives much better results. SVM constructs $M(M-1)/2$ binary sub-classifiers, where M is the number of classes. To separate a pair of classes, each binary sub-classifier is trained and a prediction is made by the majority voting technique.

The SVMs tend to find an optimal hyper-plane in high dimensional feature space in order to maximize the distance between this hyper-plane and the nearest data point of each class. SVM classifiers gives much better results as compared to other classifiers. Standard optimization software can be used to find optimum parameters. General quadratic programming software will often fail for large sample sizes and to solve the optimization special-purpose optimizers need to be used. (25–29).

K-nearest neighbor (KNN)

It is a simple machine learning algorithm. A majority vote of neighbors is used to classify an object, with the object being assigned to the class most common amongst its *k*- nearest neighbors. *K* is a positive integer whose value is typically small. *k* = 1 implies that the object is assigned to the class of its nearest neighbor. To avoid tied votes in binary classification problems, k should be an odd number.

Assume each sample of our dataset has n attributes and an n dimensional vector: x = (x1, x2. . . xn) is formed by combining these attributes. These attributes are independent variables. The problem that we have to solve is that we have a new sample where x=u. We want to find the class of this sample. In the event, if we knew the function f, we could compute v= f (u) to classify this sample. But in this case we don't know about f.

The thought in k Nearest Neighbor strategies is to distinguish k samples in the training set whose autonomous factors x are like u, and to utilize these k tests to characterize this new specimen into a class. On the off chance that all we are set up to accept is that f is a smooth function, a sensible thought is to search for samples in training data that are close to it and after that to figure out from the estimations of y for these samples .When we discuss neighbors we are inferring that there is a separation that we can compute

between samples in view of the autonomous factors. For the minute we will concern ourselves to the most prominent measure of separation: Euclidean separation (7).

We observe that the sort of separation utilized has little impact on accuracy. We actualized the k-neares neighbor calculation on dimensionally lessened information. As we probably am aware dimensional diminishment lessens the connection between elements. We attribute the dimensional lessening to be the explanation behind unaffected characterization precision with separation sort.

Statistical parameters

To evaluate the performance of the classifier, two different parameters are employed, namely sensitivity and accuracy. Mathematically, these parameters are defined as,

$$SENSITIVITY\ (SEN) = \frac{true\ positives}{true\ positives + false\ negatives} \times 100\%$$

$$ACCURACY\ (A) = \frac{correct\ cllasified\ patterns}{total\ patterns} \times 100\%$$

RESULTS

DWT technique was used to decompose all the 500 epochs of normal, interictal and epileptic (ictal) EEG data sets. The sub-bands were divided as follows: A5 (0–2.70 Hz), D1 (43.4–86.8 Hz), D2 (21.7–43.4 Hz), D3 (10.85–21.7 Hz), D4 (5.43–10.85 Hz) and D5 (2.70–5.43 Hz). Fig 3 represents decomposition of a sample dataset A into different sub-bands.

The approximation and detail coefficients of all sub bands of the entire 500 EEG epochs were used to calculate HE values. For five data sets A-E the value of HE for entire 500 epochs are plotted in figure 4,5 and 6. Data set A and B have higher HE value as compared to data set E, this proves that the data set E is in more ordered form than the data sets A and B. Similarly the data sets C and D tends to have higher HE values than the data set E and lesser than data sets A and B. Hence we can conclude that data sets C and D are more ordered than the data sets A and B and are less regular than E.

Tabulation of average HE values for wavelet coefficients of the six sub bands (D1 to D5 and A5) of data sets A,B,C,D and E is given in table 2. We can come to a conclusion that epileptic EEG i.e. set E is more regular or less complex as compared to normal i.e. set A and B and data sets representing interictal periods i.e. sets C and D. We also observed that the complexity of data sets C and A is almost similar, when recorded for normal subjects. Conversely , the complexity of data set E is lower than the complexity of data set D, when recorded from epileptic patient throughout the ictal period.

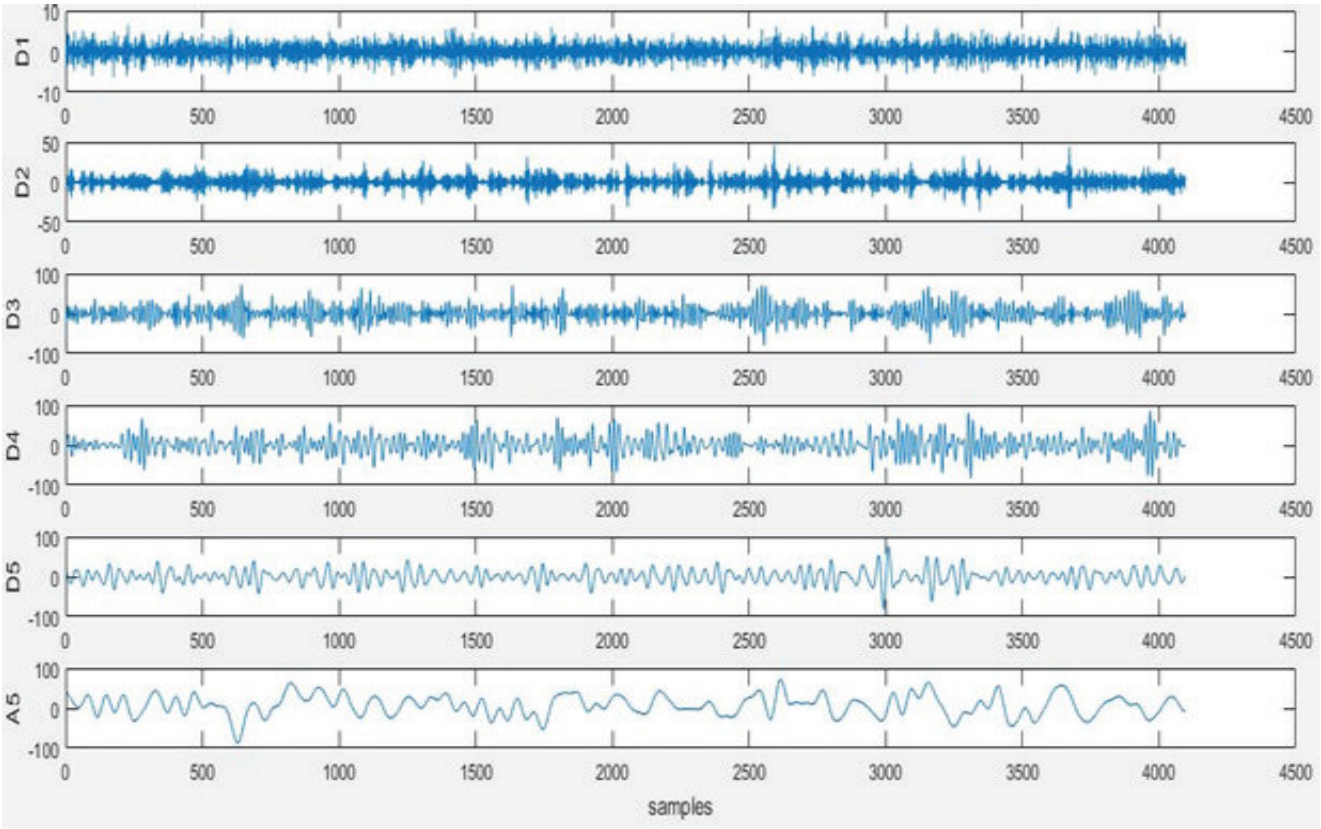

Fig 3 Decomposition of Set A into sub-bands

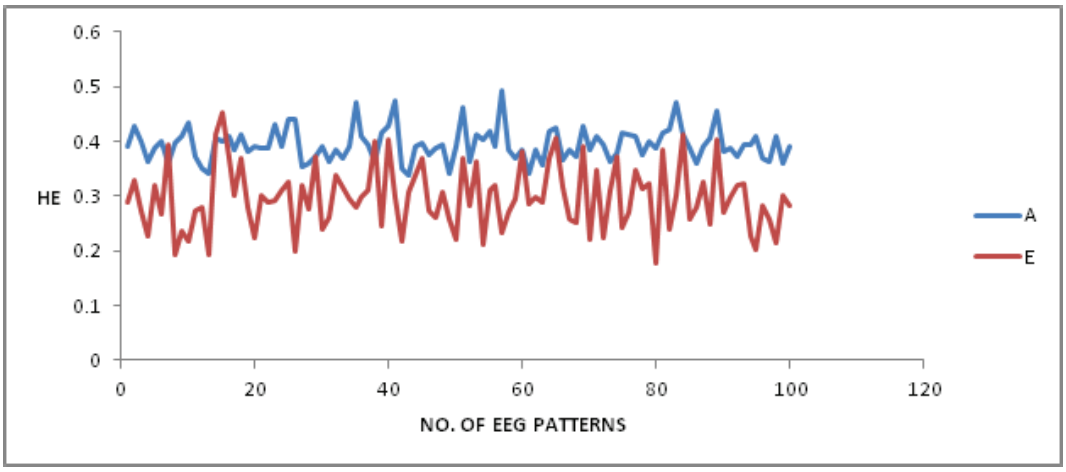

Fig 4 DWT based HE values of sub-band (D1) for data sets (A and E)

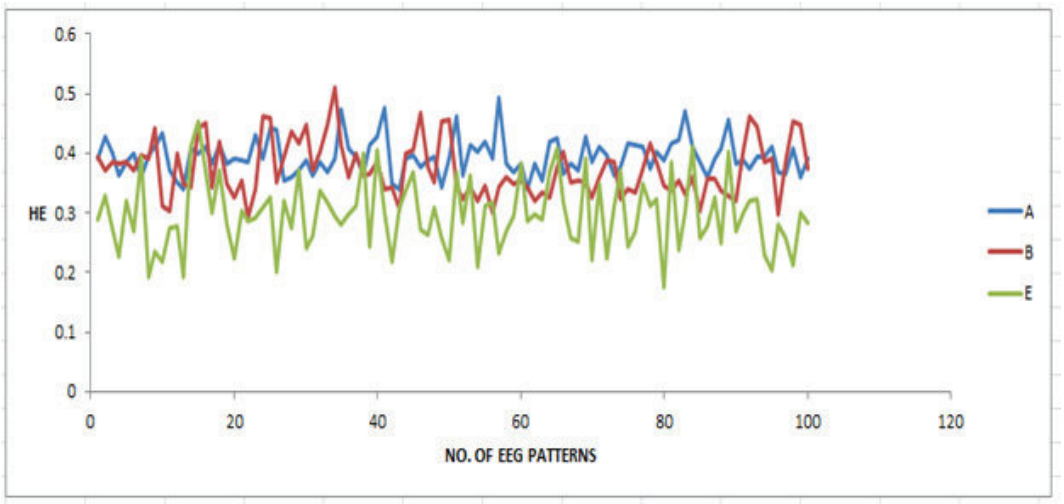

Fig 5 DWT based HE values of sub-band (D1) for data sets (A,B and E)

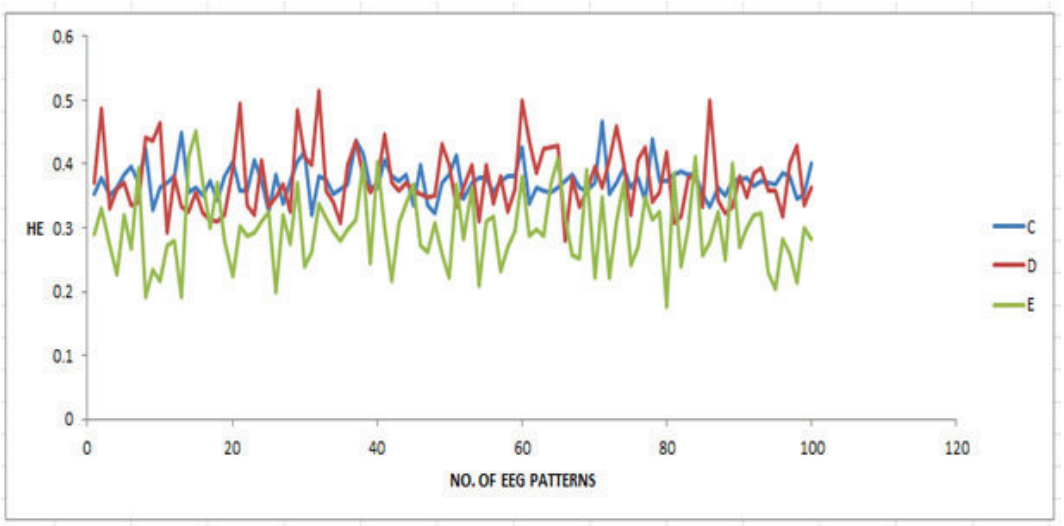

Fig 6 DWT based HE values of sub-band (D1) for data sets (C,D and E)

A significabt difference was found between the DWT based HE values of different sub bands of all the data sets , this fact is very much evident from data in table 1. The differences in these values is used to formulate feature vector of an entire band (0-86.8 Hz) of EEG signal. The SVM and KNN classifiers thus uses these six features as an input in or-

Table 1: Statistical parameters of SVM and k-NN

	SVM			KNN		
Sets	Accuracy (%)	Sensitivity (%)	Elapsed Time (sec)	Accuracy (%)	Sensitivity (%)	Elapsed Time (Sec)
A-E	99.00	100.00	0.0184	98.00	98.00	1.2885
B-E	95.00	95.91	0.0208	95.00	94.00	0.7638
C-E	99.00	100.00	0.0018	98.00	96.00	0.7695
D-E	93.00	97.77	0.0022	91.00	94.00	0.7587
BCD-E	91.50	85.10	0.0038	95.00	90.00	0.7883
AB-E	94.67	88.89	0.0007	95.33	92.00	0.9628
AC-E	97.33	94.23	0.0007	98.00	96.00	0.8158
AD-E	93.33	90.00	0.0006	93.33	94.00	0.7489
BC-E	93.33	93.47	0.0008	96.00	92.00	0.7444
BD-E	89.33	88.63	0.0007	93.33	90.00	0.7616
CD-E	94.00	88.67	0.0008	94.00	94.00	0.7541
ABC-E	95.50	93.61	0.0008	96.50	92.00	0.7522
ACD-E	95.00	85.71	0.0008	95.00	94.00	0.7521
ABD-E	92.50	85.71	0.0008	94.50	90.00	0.7592
ABCD-E	93.20	81.13	0.0171	95.60	90.00	0.67422

der to further classify the EEG as healthy,interictal and ictal. The following cases have been taken up for consideration.

While processing class 0 is allotted value 1 and class 2 is allotted as 1 in the target vector , for all the cases.Implementation of SVM is done using MATLAB software version 7.8.0 (R2016a). The input feature vector thus obtained is divided randomly into training data set and testing data set. For training the SVM the training data set is used, whereas the accuracy and effectiveness of the trained SVM for given EEG signal is determined using the training set. Similar procedure is carried out for classification using KNN classifier.

The input data matrix which is prepared from the HE values has 100 rows and 6 columns in this work. In this matrix each row represents one observation and its column is one feature. In the same way, the feature vectors of sets B, C, D and E have 100 observations each. Present binary classifier task comprise of 200 observation of six features for case 1 to case 4, 300 observation with six features for case 5 to case 11, 400 observation with six features for case 12 to case 14 and 500 observation with six features for case 15. For testing of classifier 50% of input data set is used and remaining 50% is used for training.

Comparison with existing state-of-art work

The work done by various researchers in this field of epilepsy detectionis tabulated in Table 2. We have drawn here a comparison between the results obtained in other methods and our proposed approach. For making the results more realistic only the methods using same data sets and similar cases is incorporated.

For case 1 and case 3, the classification accuracy obtained from our work 99% (using SVM) is one of the best presented for these data sets. Tzallas et al.'s (38) work also represent this result by using the method of time-frequency analysis combined with ANN.

For case 2 and case 4, the accuracy obtained after classification process is 95%, 93% (using SVM) are the best presented. Nicolaou et al. (23) also looked upon these cases and reported their accuracy as 82.8%and 79.94% respectively. His method comprises using permutation entropy with SVM for the same data sets.

For case 12, the accuracy obtained after classification process is 95% (using both SVM and KNN). This particular case was also presented in Guo et al.'s (41) work , which were obtained by using the line length features centered on multi resolution decomposition combined with ANN.

Case 6 to Case 12 and case 15 is presented in our work for the first time and has given satisfactory results. As already mentioned the approach used by us involves using wavelet analysis (using DWT) for EEG signal decomposition combined with extracting HE (for all six sub bands) and using SVM and KNN classifiers for the classification process.

CONCLUSION

Manual detection of epileptic seizure is a very costly and time consuming process. In this work hurst exponent has been employed which simplifies automatic seizure detection process. Fifth leveldecomposition of EEG signals has been performed to decomposethe signal into different sub-bands by DWT to obtain the detail wavelet coefficients ($D1$–$D5$) and approximate wavelet coefficients ($A5$). The HE values are calculated from $D1$–$D5$ and $A5$ which provides the best detec tion rates. The 99% classification accuracy is obtained using SVM for cases 1 and 3 and 98% accuracy is obtained using KNN classifier. It can be concluded that using DWT based

Table 2: Comparison of classification accuracy obtained by proposed method and others existing methods

RESEARCHERS	YEAR	METHODS	CASES	ACCURACY (%)
Nigam and Graupe (35)	2004	Non linear preprocessing filter – diagnostic neural network	A-E	97.20
Srinivasan et al. (20)	2005	Time and frequency domain features – recurrent neural network	A-E	98.66
Kannathal et al. (36)	2005	Entropy measures – adaptive neuro-fuzzy inference system	A-E	92.22
Polat and Günes (37)	2007	Fast Fourier transform – decision tree	A-E	98.72
Subasi (34)	2007	Discrete wavelet transform – mixture of expert model	A-E	95.00
Guo et al. (19)	2009	Discrete wavelet transform – relative wavelet energy-MLPNNA	A-E	95.20
Ocak (18)	2009	Discrete wavelet transform – approximate entropy (ApEn)	A-E	99.60
Subasi et al (40)	2010	DWT – PCA, ICA, LDA and SVM	A-E	98.75 (PCA)
Guo et al. (41)	2010	Line length feature – ANN	A-E ACD-E ABCD-E	99.60 97.75 97.77
Guo et al (43)	2011	GP-based feature extraction – KNN classifier	A-E	99.20
Nicolaou et al. (23)	2012		A-E B-E C-E D-E	93.55 82.88 88.00 79.94
Akbarzadeh-T and Naghibi-Sistani	2013	Discrete wavelet transform – Hurst exponent and Lyapunov exponent	A-E B-E	96.90 96.50
Kaya et al.	2014	1-D local binary patterns + BayesNet	A-E	95.50
Riaz et al.	2015	Empirical mode decomposition based temporal and spectral features + SVM	A-E	93.00
Mingyang Li , Wanzhong Chen , Tao Zhang	2016	Double density discrete wavelet transform- Hurst exponent and fuzzy entropy	A-E	100
Present reporting		Discrete wavelet transform- Hurst exponent (SVM)	A-E B-E C-E D-E BCD-E AB-E AC-E AD-E BC-E BD-E CD-E ABC-E ACD-E ABD-E ABCD-E	99.00 95.00 99.00 93.00 91.50 94.67 97.33 93.33 93.33 89.33 94.00 95.50 95.00 92.50 93.20

Hurst exponent method using SVM clasiifier gives more satisfactory results as compared to other methods. The presented method may prove to be a useful tool in epilepsy detection.

DECLARATION

The authors declare no conflict of interest

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ANTIMICROBIAL ACTIVITY OF AQUEOUS EXTRACTS OF POTENTILLA REPTANS L. RHIZOME AND AERIAL PART

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ANTIMIKROBNA AKTIVNOST VODENIH EKSTRAKATA NADZEMNOG DELA I RIZOMA POTENTILLA REPTANS L.

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ABSTRACT

Potentilla reptans is a little studied plant of the genus Potentilla, the family Rosaceae. The aim of this study is to determine antimicrobial effects of aqueous extracts of P. reptans aerial part and rhizome against standardized bacterial strains.

The antimicrobial activity of aqueous extracts of P. reptans aerial part and rhizome was tested against one fungus, Candida albicans, and two standard bacterial strains, Staphylococcus aureus and Escherichia coli, using an agar diffusion method.

Both examined extracts showed a significant antimicrobial activity against Escherichia coli and Staphylococcus aureus at the concentrations of 10 to 150 mg/ml. The rhizome extract showed stronger antimicrobial effect against the tested strains of bacteria than the aerial part extract.

The obtained results represent preliminary results of antimicrobial activity of this plant and suggest that in future, the studies should examine antimicrobial activity against other bacterial strains and minimum inhibitory concentration.

Keywords: *Potentilla reptans; Antimicrobial effect; Agar diffusion method.*

SAŽETAK

Potentilla reptans je malo istraživana biljka roda Potentilla familije Rosaceae. Cilj ove studije je utvrđivanje antimikrobnih efekata vodenih ekstrakata herbe i rizoma P. reptans na standardizovanim bakterijskim sojevima.

Antimikrobna aktivnost vodenih ekstrakata herbe i rizoma Potentilla reptans je ispitivana na jednu gljivicu Candida albicans i dva standardna bakterijska soja Staphylococcus aureus i Escherichia coli korišćenjem agar-difuzione metode.

Oba ispitivana ekstrakta su pokazala da u koncentracijama od 10 – 150 mg/ml ispoljavaju značajnu antimikrobnu aktivnost prema Escherichia coli i Staphylococcus aureus. Ekstrakt rizoma je pokazao jači antimikrobni efekat nego herba kod oba ispitivana bakterijska soja.

Dobijeni rezultati predstavljaju preliminarne rezultate o antimikrobnom delovanju ove biljke i ukazuju da buduća istraživanja mogu da idu u smeru ispitivanja antimikrobnog delovanja na drugim bakterijskim sojevima, kao i utvrđuvanje minimalne inhibitorne koncentracije.

Ključne reči: *Potentilla reptans; Antimikrobno delovanje; agar difuziona metoda.*

ABBREVIATIONS

P.rep-a - Potentilla reptans aerial part **P.rep-r** – Potentilla reptans rhizome

INTRODUCTION

Potentilla reptans L. (*P. reptans*) is one of three hundred *Potentilla* species belonging to the genus *Potentilla*, the family Rosaceae. The genus *Potentilla* is mostly characterized by perennial, rarely biennial or annual herbaceous plants (1). *P. reptans* is a perennial herbaceous plant with an erect rhizome. The stem is herbaceous, thread-like, creeping, up to 100 cm long, and the leaves are palmately five or seven lobed. It is usually found near the shores, in wet and flood meadows (2). The rhizome and aerial part of this plant are used in traditional medicine in the treatment of rheumatism, scabies, diarrhea, viral infections and as a remedy for wound-healing detoxification or internally

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in jaundice and dysentery (1). Studies that have examined the pharmacological characteristics of *P. reptans* aerial part proved its antioxidant and anti-ulcer activities (3, 4). Anti-inflammatory effect of the rhizome and aerial part was evaluated and proven by experimental mouse ear edema model (5). The results proved the presence of following compounds (Chinic acid, Caffeic acid, Protocatechuic acid, Luteolin-7-O-glucoside, Quercetin-3-O-glucoside, Rutin, Quercetin, Kaempferol-3-O-glucoside, Apigenin-7-O-glucoside) in the aerial part of *P. reptans L.* and Catechin as a dominant compound in the rhizome of this plant, as well as the presence of Chinic acid, Gallic acid, Protocatechuic acid, Epicatechin, Quercetin (5).

A large number of *Potentilla* species showed moderate to high antimicrobial activity. Antimicrobial activity was demonstrated against *Streptococcus mutans* and *Streptococcus sobrinus,* while moderate antibacterial activity was observed against *Staphylococcus aureus* and *Bacillus substilis,* and there is no such activity or it is very weak against *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia* (1). A study showed antimicrobial activity of *P. reptans* aerial part and rhizome against gram-positive *Staphylococcus aureus* and *Bacillus subtilis* and gram-negative *Escherichia coli* and *Pseudomonas aeruginosa* bacterial strains (6).

The aim of our study is to justify the use of this plant as antimicrobial agent in traditional medicine.

METHODS

Plant material

The aerial parts of *P. reptans* were collected from May to August 2010, and rhizome in October of the same year. Plant material was dried for two weeks (in a windy, shady place). Before preparation of the extract, the material was kept at the temperature of 6-8^{0}C. Immediately prior to extraction, the plant material was powdered. The voucher specimens were deposited in herbarium of botanical garden of Department of Biology, Faculty of Natural Sciences, University of Belgrade, Serbia, no. BEOU 16405.

Preparation of dry extracts

Aerial part and rhizome extracts were obtained by the infusion method (7). For extraction, 20g of dried and powdered aerial part (*P.rep-a*), and rhizome (*P.rep-r*) and 200 ml of boiling distilled water were used. The resultant extract was filtered and evaporated using a rotary vacuum evaporator at 40^{0}C, (RV05 basic IKA, Germany).

Testing of antimicrobial activity

In order to determine possible antimicrobial activity, *P.rep-a* and *P.rep-r* aqueous extracts were tested in vitro against one fungus and two standard ATCC bacterial strains using the agar diffusion method (8). Standard bacterial strains used in the test were: *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922, as well as the fungus *Candida albicans* ATCC 10231.

The testing started at concentrations ranging from 10 μg/ml to 10 mg/ml, wherein the positive results were observed at concentrations above 10 mg/ml. The experiment was further carried out at concentrations: 10, 50, 75, 100 and 150 mg/ml which were obtained by diluting the appropriate amount of extract in distilled water.

Müeller Hinton agar medium was used for testing the sensitivity of bacteria to the obtained extracts (HiMedia Laboratories, India, LOT 0000099844), and Sabouraud agar plate was used for the fungus *Candida albicans* (Institute of Immunology and Virology "Torlak", Belgrade).

The concentration of bacterial broth was diluted (10^2 organisms/ml) and volume of 0,1 ml of each broth was applied to the surface of the agar plate. Then the appropriate strain substrate was poured into sterile 90 mm diameter petri dishes, so that the thickness of the solidified agar was 4 mm. The reservoirs of diameter 12 mm were made in agar and in each well was introduced appropriate concentration of extracts in volume – of 150 μl.

Thus prepared agar plates were incubated at 37^{0}C during before 24 h. Inhibition zone diameters of plant extracts and standard substances were determined. Inhibition zones were determined by measuring the diameters in millimeters (12 mm diameter of the reservoir was subtracted from the displayed values of the inhibition zone diameter), and in cases when the inhibition zone diameter was smaller or equal to 12 mm, the tested sample was considered to be inactive (8).

Statistics

Statistical analysis was performed using SPSS software. Inhibition zone values for each disk were shown in a scatter diagram, and linear regression lines were calculated using the least squares method. Diffusion zone values for each concentration of the extracts were plotted on scatter diagrams, and regression lines were calculated by the least squares method. The significance of the difference in the activity of the extracts against selected microorganisms was calculated using Mann – Whitney test ($p < 0,05$).

RESULTS

Agar diffusion test results showed that the aqueous extracts of *P. reptans* aerial parts and rhizome at the concentrations of 10 – 150 mg/ml had significant antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*. Maximum antimicrobial activity was displayed at the highest concentrations applied. Aerial part extract at the concentration of 150 mg/ml showed 95,24% of ceftriaxone activity (30 μg/disk) against *Escherichia coli,* and 68,75% against *Staphylococcus aureus*. Rhizome extract at the concentration of 150 mg/ml showed 133,3% activity of the standard against *Escherichia coli*, and 87,5% of ceftriaxone activity (30 μg/disk) against *Staphylococcus aureus*. The strain of *Candida albicans* showed moderate sensitivity to *P.reptans* rhizome and the highest applied concentration exhibited 50% activity in comparison to that of the refer-

ence substance - nystatin (25 µg/disk). The results of antimicrobial activity are shown in Table 1.

The mean value of the diameter of inhibition zones *P.rep-r* versus *Staphylococcus aureus* was 25.2 ± 1.16 mm for *P.rep-a* 15.2 ± 3.93 mm. While the mean value of the diameter of inhibition zones *P.rep-r* versus *Escherichia coli* was 23.4 ± 1.5 mm and for a *P.rep-a* 17.2 ± 0.86 mm. The differences in antimicrobial activity of the extracts against respective bacteria are displayed in Figures 1 and 2.

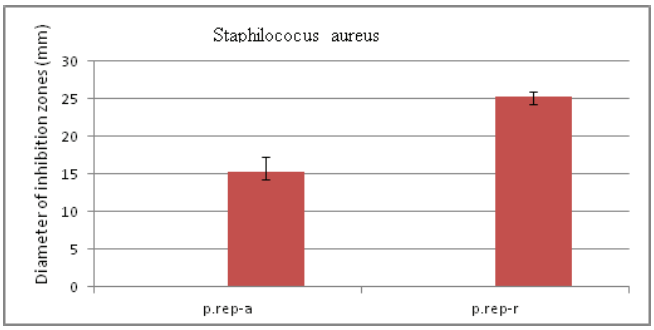

Fig. 1. The difference in activity of extracts of P. reptans aerial parts and rhizome against Staphilococus aureus p > 0,05

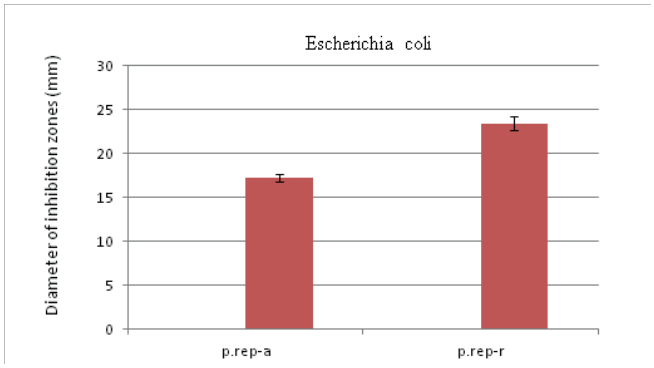

Fig. 2. The difference in activity of extracts of P. reptans aerial parts and rhizome against Escherichia coli p > 0,05

DISCUSSION

Many of the plants used in traditional medicine were studied in order to prove their antimicrobial activity and justify their use in the treatment of various diseases caused by variety of microorganisms. Antimicrobial activity of *P. reptans* rhizome and aerial parts in this study was tested on standardized strains of microorganisms. The extacts of solvents such as ethanol, methanol, acetone, chloroform etc, are richer in active compounds than aqueous solvents and this greatly influences the appearance of antimicrobial effects (9). The tested aqueous extracts were obtained in a manner that is most commonly used in traditional medicine.

The 75% ethanol extract of *P. reptans* rhizome at concentration of 8 µg/mL showed significant antimicrobial effect against S. aureus, as well as the 25% ethanol extract of the rhizome at concentration of 40 µg/mL. The 25% ethanol extract of *P. reptans* leaves showed antimicrobial activity against *B. subtilis* at concentration of 40 µg/mL. Decoctions and 25% ethanol extracts of *P. reptans* root showed the activity against *E. coli* at the concentration of 200 µg/mL (6). Our study results were in accordance with the results that had been shown before but the concentration of our extracts (10 µg/mL) did not show any effect. However, at higher concentrations *P. reptans* aerial part and rhizome showed a significant antimicrobial activity. This difference can be explained by the presence of tannin catechin in large quantities in the rhizome and probably its higher extraction with alcoholic solvents which were used in other study.

Table **1.** Antimicrobial activity of aqueous extracts of *P. reptans* rhizome and aerial parts

Diameter of inhibition zones (mm)				
Material		*Escherichia coli*	*Staphylococcus aureus*	*Candida albicans*
P.rep-a	10 mg/ml	15	0	0
	50 mg/ml	16	16	0
	75 mg/ml	17	18	0
	100 mg/ml	18	20	0
	150 mg/ml	20	22	0
P.rep-r	10 mg/ml	19	22	0
	50 mg/ml	22	23	0
	75 mg/ml	23	26	0
	100 mg/ml	25	27	16
	150 mg/ml	28	28	17
Ceftriaxone 30 µg/disk		21	32	-
Nystatin 100 IJ/disk		-	-	34

Methanol extract of *P. recta* applied at the concentration of 10 mg/ml caused the inhibition zone (15 mm) against *S. aureus* (10). In this study, aqueous extracts of *P. reptans* rhizome at the concentration of 10 mg/ml also induced the inhibition zone against this bacteria (22 mm), while the aerial part extract at this concentration did not show a significant activity against *S. aureus* and it showed the inhibition zone of 16 mm at the concentration of 50 mg/ml. The inhibition zones after application of 10 mg/ml of *P. reptans* aerial part and rhizome extracts against *E. coli* were 15 mm and 19 mm. These values were significantly larger than the results obtained after application of 10 mg/ml *P. recta* extract (12 mm) (10).

Aerial parts of nine *Potentilla species* (*P. argentea, P. fruticosa, P. recta, P. rupestris, P. erecta, P. anserina, P. nepalensis, P. thuringiaca, P.grandiflora*) showed moderate effect against gram-positive bacteria (*Staphylococcus aureus, Bacillus suptilis*) at minimum inhibitory concentration of 12,5-100 mg/ml, while these extracts did not have any effect against gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae*). Moderate antifungal effect against *Candida albicans* was indicated (11). In our study, the rhizome showed moderate effect against *Candida albicans* but *P. reptans* aerial part did not show any effect.

Catechin is a dominant flavonoid from *P. reptans* rhizome and it may be assumed that it is the main carrier of the extract

activity (5,12). The activity of tannins in the plants of *Potentilla genus* against gram-positive, gram-negative bacteria and fungi was studied before when antibacterial and antifungal activity was observed (13). The study performed on three sorts of triticum showed that catechin and its derivates were synthetised in larger quantities within infectious plants rather than healthy ones, and the level of catechin also decreased after the infection ended, so it was considered that the synthesis of catechin was a defence response against pathogen attack (14). As for antimicrobial activity, it was proved that catechin had antimicrobial activity but the mechanism of action has not been explained yet. There is a conjecture that hydroxyl group of catechine molecule as a result of dehydrogenation gets replaced with carboxyl group which can bond with phospolipids within cell membrane, what can induce damages of membrane and physiological functions (15). Microbiological effect of aqueous extracts of *P. reptans* can be induced not only by catechin but larger number of secondary metabolites of different chemical structures which are present in extracts at the same time (16).

Large number of Potentilla species showed moderate effect not only against gram-positive bacteria but also against fungus *C. albicans* while there was not any effect against gram-negative bacteria or the effect was very weak (11). Although it was showed that *Potentilla* plants had moderate antifungal activity, for example tested *Potentilla* species had MIC range of 25-100 mg/ml against *Candida albicans* (7), while *P. recta* at concentration of 10 mg/ml induced the inhibition zone of 21 mm, in our study none of the extracts at this concentration showed clear inhibition zone. Only at concentrations higher than 100 mg/ml the rhizome extract showed the inhibition zone of 16mm.

Antimicrobial activity of aqueous extracts of *P. reptans* aerial part and rhizome showed antimicrobial properties at concentrations of 10 to 150 mg/ml. Aqueous extract of *P. reptans* rhizome at concentrations of 100 to 150 mg/ml showed antifungal activity against *C. albicans* while the aerial part did not show any effect against this fungus. There was a significant difference in the activity of aerial part and rhizome extract against both bacterial strains. The rhizome showed statistically significantly stronger antimicrobial activity against examined bacterial strains than aerial part extract.

The results are in accordance with the results of other *Potentilla species* of this genus, as well as with the results of this species, significantly weaker though. Such weak results can be justified by the method of extract preparation. Obtained results confirmed the use of this plant in traditional medicine for the treatment of diarrhea and other conditions caused by various bacterial strains.

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REACTIONS OF PLATINUM(II) COMPLEXES WITH SULFUR- AND NITROGEN- CONTAINING BIOMOLECULES: SELECTIVE INTERMOLECULAR MIGRATION OF THE THIOETHER-BOUND PLATINUM(II) COMPLEX TO THE N7 NITROGEN ATOM OF GUANOSINE-5'-MONOPHOSPHATE

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REAKCIJE KOMPLEKSA PLATINE(II) SA SUMPOR- I AZOT-DONORSKIM BIOMOLEKULIMA: SELEKTIVNA INTERMOLEKULSKA MIGRACIJA PLATINA(II) KOMPLEKSA SA TIOETARSKOG SUMPORA NA N7 ATOM AZOTA 5`-GUANOZIN-MONOFOSFATA

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ABSTRACT

The interactions of platinum(II) complexes with nitrogen- and sulfur-containing biomolecules are responsible for aider antitumor activity due to attack on DNA or a variety of toxic side effects. Therefore, the monofunctional Pt(II) complexes, [Pt(Gly-Gly-N,N,O)I]+ (Gly-Gly is the dipeptide glycyl-glycine coordinated through the oxygen and two nitrogen atoms) and [Pt(Gly-L-Met-S,N,N)Cl] (Gly-L-Met is the dipeptide glycyl-L-methionine coordinated through the sulfur and two nitrogen atoms) have been used to study their interactions with S-methylglutathione (GS-Me) and guanosine-5'-monophosphate (5'-GMP). All reactions have been studied by 1H NMR spectroscopy and at room temperature in 50 mM phosphate buffer at pD 7.4. The investigation of the competitive binding of 5'-GMP and GS-Me to the Pt(II) complexes (1:1:1 molar ratio) has shown that in the initial stages of the reaction the corresponding Pt(II) complex only reacts with GS-Me, and second step of the reaction is very slow intermolecular displacement of the S-bound thioether ligand with N7 atom of the guanine base of 5'-GMP. The obtained results have been analyzed in relation to the antitumor activity and toxicity of Pt(II) complexes.

Keywords. *Platinum(II) complexes; S-methylglutathione (GS-Me); guanosine-5'-monophosphate (5'-GMP); Intermolecular migration.*

SAŽETAK

Interakcije kompleksa platine(II) sa biomolekulima koji sadrže sumpor i azot donorske atome su veoma značajne kako za antitumorsku aktivnost usled reakcija Pt(II) komplesa sa DNA tako i za toksično delovanje ovih kompleksa. U ovom radu su ispitivane reakcije monofunkcionalnih kompleksa platine(II), [Pt(Gly-Gly-N,N,O)I]+ (Gly-Gly je je tridentatno koordinovani glicil-glicin preko atoma kiseonika i dva azotova atoma) i [Pt(Gly-L-Met-S,N,N)Cl] (Gly-L-Met je tridentatno koordinovani glicil-L-metionin preko atoma sumpora i dva azotova atoma) sa 5`-guanozin-monofosfatom (5`-GMP) i S-metil-glutationom (GS-Me) primenom 1H NMR spektroskopije, na sobnoj temperaturi u 50 mM fosfatnom puferu na pD = 7,4. Konkurentne reakcije 5'-GMP i GS-Me sa Pt(II) kompleksom (molski odnos 1:1:1) pokazuju da u prvoj fazi Pt(II) kompleks reaguje samo sa GS-Me, dok u sledećem koraku dolazi do veoma spore intermolekulske migracije sa S-koordinovanog tioetarskog liganda na N7 atomom guanina iz 5`-GMP. Dobijeni rezultati ovih ispitivanja su diskutovani u smislu antitumorskog i toksičnog delovawa kompleksa platine.

Ključne reči: *Platina(II) kompleksi; S-metil-glutation (GS-Me); 5`-guanozin-monofosfat (5'-GMP); intermolekulska migracija.*

ABBREVIATIONS

DNA - deoxyribonucleic acid
D_2O - deuterium oxide
HCl - hydrochloric acid
$K_2[PtCl_4]$ - potassium-tetrachloridoplatinate(II)
KI - potassium iodide
$KHCO_3$ - potassium bicarbonate
KOH - potassium hydroxide
NMR - nuclear magnetic resonance

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INTRODUCTION

Due to the clinical success of cisplatin, carboplatin, and more recently oxaliplatin, platinum(II) complexes represents important class of inorganic compounds as well as their mechanism of action during their antitumor activity (1,2). These successes have provided an increasing interest in the interactions of Pt(II) complexes with nitrogen- and sulfur-containing biomolecules which has revealed new and interesting findings, both thermodynamic and kinetic. The interactions of these platinum(II) complexes with sulfur-containing biomolecules are responsible for a variety of biological effects, such as inactivation of platinum(II) antitumor complexes, development of cellular resistance to platinum drugs, and toxic side effects such as nephrotoxicity (3). Additionally, it was found that the thioether-containing amino acid methionine plays an important role in the metabolism of platinum anticancer drugs. Not only does interaction with sulfur-containing protein appear to be important in cell entry of platinum drugs, also targeting of nucleic acids processing with platination of DNA lesions play important roles in their antitumor activity (4-6).

Discovering that *S*-bound thioether ligand can be selectively displaced by one of the nitrogen atoms of the imidazole ring in the histidine side chain opens a chance for designing a new Pt(II) complex for selective covalent modification of proteins. The monofunctional $[PtCl(dien)]^+$ complex, with the dien (dien is 1,5-diamino-3-azapentane) acting as a non-removable tridentate ligand, has been shown to be a very useful model for the study of the kinetics and mechanism of the interactions of Pt(II) antitumor complexes with nitrogen- and sulfur-containing biomolecules. Results obtained by the NMR investigation of the competitive binding of the L-methionine (L-Met), *N*-acetylated dipeptide glycyl- L-methionine (Ac-Gly-L-Met) and guanosine-5'-monophosphate (5'-GMP) to the monofunctional $[PtCl(dien)]^+$ complex have shown that 5'-GMP selectively displaces Pt-S-Met bound (7, 8). Slow intramolecular displacement of a $[Pt(L)]^{2+}$ unit (L is dien, Gly-Gly, or Gly-Met) from the sulfur to the nitrogen atom of imidazole ring in *N*-acetylated dipeptide L-methionyl-L-histidine (Ac-L-Met-His) has been observed (9). In the reaction with $[PtCl(dien)]^+$ complex this migration reaction is strongly selective to the N1 atom of the imidazole ring, while with $[Pt(Gly\text{-}Gly\text{-}N,N,O)I]^+$ complex this migration reaction occurs to the N3 atom of imidazole ring of the histidine side chain. No migration reaction from the sulfur to the either N1 or N3 nitrogen atom of the imidazole was observed for the reaction of Ac-L-Met-His peptide with [Pt(Gly-L-Met-*S,N,N*)Cl] complex.

In this work we have sought to gain further insight into sulfur-nitrogen intermolecular migration. Thus, ^{1}H NMR spectroscopy is applied for investigation of the reaction of the monofunctional Pt(II) complexes, [Pt(Gly-L-Met-*S,N,N'*)Cl] and $[Pt(Gly\text{-}Gly\text{-}N,N,O)I]^-$, in which Gly-L-Met is glycyl-L-methionine coordinated through the sulfur and two nitrogen atoms and Gly-Gly is glycylglycine coordinated through two nitrogen and oxygen atoms, with *S*-methylglutathione (GS-Me) and guanosine 5'-monophosphate (5'-GMP).

EXPERIMENTAL

2.1. Synthesis of Platinum(II) Complex

The complex $K[Pt(Gly\text{-}Gly\text{-}N,N`,O)I]$ was prepared by a modification of a literature method (9, 10). To $K_2[PtCl_4]$ (0.2076 g, 5.00 x 10^{-4} mol) dissolved in 5 cm^3 of water was added 0.3320 g (2.00 x 10^{-4} mol) of KI and the mixture was heated at 60 °C for 5 min. Subsequently, an aqueous solution (5 cm^3) of the peptide glycyl-glycine (0.0660 g, 0.5 mM) was added to the obtained reaction mixture and the heating (60 °C) with stirring was continued for 30 min. During this time, the pH of the reaction mixture was controlled every 5 min and adjusted to about 6.5 with 1 M $KHCO_3$ solution. The obtained solution was concentrated to 5 cm^3 under vacuum and then left at room temperature over night. The obtained yellow crystals were filtered off, washed with ethanol and air dried. Yield 0.098 g (40%). Calculated for $K[Pt(Gly\text{-}Gly\text{-}N,N`,O)I] = C_4H_6N_2O_3IKPt$ (FW = 491.18): C, 9.78; N, 5.70; H, 1.23%; found: C, 9.63; N, 5.81; H, 1.34%. ^{1}H NMR (D_2O, 200 MHz); δ = 3.99 (s, 2H, CH_2) and δ = 3.62 (s, 2H, CH_2). ^{13}C NMR (D_2O, 200 MHz); δ= 51.72 (CH_2); δ = 53.37 (CH_2); δ= 182.55 (C=O) and δ= 194.00 (COO).

Complex $[Pt(Gly\text{-}L\text{-}Met\text{-}S,N,N)Cl]\cdot H_2O$ was prepared by a modification of the method of Freeman et. all (11). $K_2[PtCl_4]$ (207.6 mg, 0.5 mmol) was disolved in 3 ml of water and to this an aqueous solution of glycyl-L-methionine (103.2 mg, 0,5 mmol) was adeded. The pH of the solution was adjusted to c. 3.5 by the addition of 1 M KOH and mixture was stirred at 50 °C for 3 h. The yellow solution obtained was cooled to room temperature and then the pH was reduced to c. 2 by the additional of 1 M HCl. The solution was left overnight at room temperature; crystals of $[Pt(Gly\text{-}L\text{-}Met\text{-}S,N,N)Cl]\cdot H_2O$ were removed by filtration, washed with a smoll amount of ethanol, and air-dried. The yield was 136 mg (60%). The pure complex was obtained by recristallisation from a smol amount of water and cooling. Calculated for $[Pt(Gly\text{-}L\text{-}Met\text{-}S,N,N)Cl] \cdot H_2O = C_7H_{15}ClN_2O_4PtS$ (FW = 348.52): C, 18.5; H, 3.3; N, 6.1%; found: C, 18.4; H, 3.3; N, 6.1%. ^{1}H NMR (D_2O, 200 MHz); δ = 3.99 (s, 2H, CH_2), δ = 2.66 (s, 3H, CH_3). ^{13}C NMR (D_2O, 200 MHz); δ= 51.72 (CH_2); δ= 182.55 (C=O) and δ= 194.00 (COO).

2.2. pH measurements

All pH measurements were made at room temperature. The pH meter (Mettler Toledo Seven Compact S220-U) was calibrated with Mettler Toledo certified buffer solutions of pH 4.00 and 7.00. The results were converted into pD by the standard formula: pD = pH + 0.41 (12). However, in conceptual references to acidity

and basicity, the common symbol pH is used. Elemental microanalyses for carbon, hydrogen, and nitrogen were performed at the Faculty of Chemistry of the University of Belgrade.

2.3. ^{1}H NMR Measurements

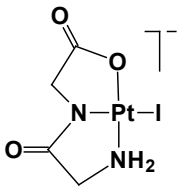
Reactions of peptides with Pt(II) complexes were followed by ^{1}H NMR spectroscopy using a Varian Gemini 2000 spectrometer (200 MHz for ^{1}H and 500 MHz for ^{13}C). Equimolar amounts of the Pt(II) complex and ligands (GS-Me and 5'-GMP) were mixed in an NMR tube. The final solution was 20 mM in each reactant. All reactions were carried out at 298 K and at pD 7.4 in 50 mM phosphate buffer solution prepared in D_2O solvent. The internal reference was TSP (sodium trimethylsilylpropane-3-sulfonate). The kinetic data for the reactions of Pt(II) complexes with thioether-containing ligand (see Table 1) were obtained from ^{1}H NMR measurements at 298 K. The values of the rate constants for these reactions were determined when the data from the early part of the reaction (up to 2 h) were fitted to a second-order process (13) by plotting $x/a_o(a_o-x)$ against t (a_o is the initial concentration of the thioether ligand and x is the concentration of the Pt(II) complex with *S*-bound thioether ligand at time t.

RESULTS AND DISCUSSION

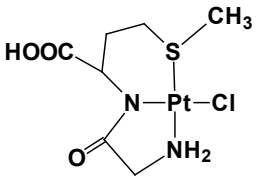
Intermolecular Migration of Pt(II) Complexes

Reactions of monofunctional Pt(II) complexes, [Pt(Gly-L-Met-*S,N,N'*)Cl] and [Pt(Gly-Gly-*N,N',O*)I]$^-$, in which Gly-L-Met is glycyl-L-methionine coordinated through the sulfur and two nitrogen atoms and Gly-Gly is glycylglycine coordinated through two nitrogen and oxygen atoms, with *S*-methylglutathione (GS-Me) and guanosine 5'-monophosphate (5'-GMP) have been studied by ^{1}H NMR spectroscopy (**Figure 1**). All reactions were carried out in equimolar amounts of the reactants in 50 mM phosphate buffer at pD 7.4 and at 25 °C. The formation of the products in these reactions was followed by ^{1}H NMR spectroscopic measurements of the chemical shifts of *S*-methyl protons in *S*-methylglutathione and those for H8 proton in guanosine-5'-monophosphate.

The platinum(II) complex was stable under the above mentioned experimental conditions and Gly-Gly and Gly-L-Met ligands stay tridentate coordinated to the Pt(II) during the reaction time. Release of the coordinated dipeptide from the Pt(II) was observed after 34 days. The detachment of the dipeptides from the Pt(II) was observed in the ^{1}H NMR spectrum by appearance of two new signals at 3.82 and 3.86 ppm due to methylene protons of the free Gly-Gly and at 2.11 and 3.82 ppm for *S*-CH3 protons and CH_2 protons of the free Gly-L-Met, respectively. Addition of the free dipeptide to the reaction mixture caused an increase of these two signals in both cases.



[Pt(Gly-Gly-*N,N',O*)I]$^-$



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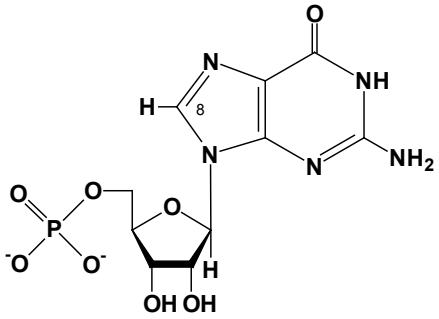

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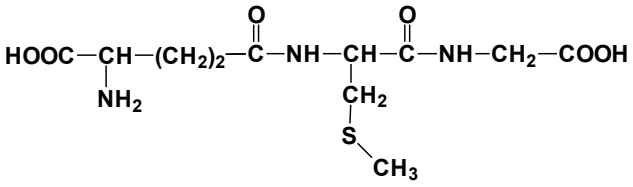

S-methylglutathione

(GS-Me)

Figure 1. The platinum(II) complexes, [Pt(Gly-Gly-*N,N',O*)I]$^-$ and [Pt(Gly-Met-*S,N,N*)Cl], *S*-methylglutathione (GS-Me) and guanosine 5'-monophosphate (5'-GMP) employed in this study.

In the initial stage of the reaction Pt(II) complexes forms a kinetically favored Pt(II)-GS-Me complex with unidentate coordination of GS-Me through the sulfur atom of the *S*-methylglutathione residue (**Figure 2** and **3**). The singlet for the *S*-methyl protons of the free *S*-methylglutathione at 2.11 ppm was shifted downfield and new resonance at 2.54 ppm for the *S*-bound *S*-methylglutathione (**Figure 1** and **4**) appeared in the spectrum. This Pt(II)-GS-Me-*S* complex is an intermediate kinetically favored product, which is at the same time thermodynamically labile. The platinum(II)-thioether bond can be cleaved in the presence of a strong nucleophile at physiological pH values. In the second step of the reaction intermolecular displacement of the *S*-bound thioether ligand with the N7 nitrogen atom of the guanine base of 5'-GMP has been observed (**Figure 2** and **3**). This migration reaction is very slow and strongly selective to the N7 nitrogen atom of the 5'-GMP. The monodentate binding of the platinum(II) to the N7 nitrogen atom of the 5'-GMP was registered from the simultaneous decline of the resonance at 8.17 ppm due

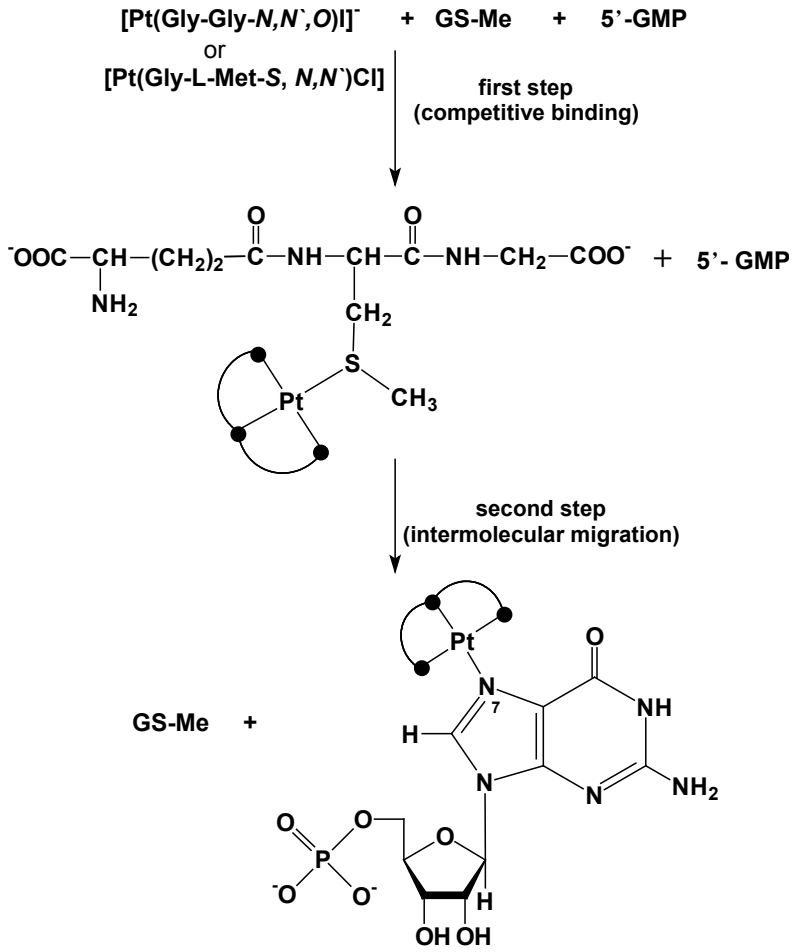

Figure 2. Competitive binding of the methionine sulfur atom in *S*-methylgluthathione (GS-Me) and guanosine *N7* atom in guanosine 5'-monophosphate (5'-GMP) in the reaction with [Pt(Gly-Gly-*N,N',O*)I]⁻ and [Pt(Gly-L-Met-*S,N,N*)Cl] complexes. The components were reacted in 1:1:1 molar ratio at pD 7.4 in 50 mM phosphate buffer and at 25 °C.

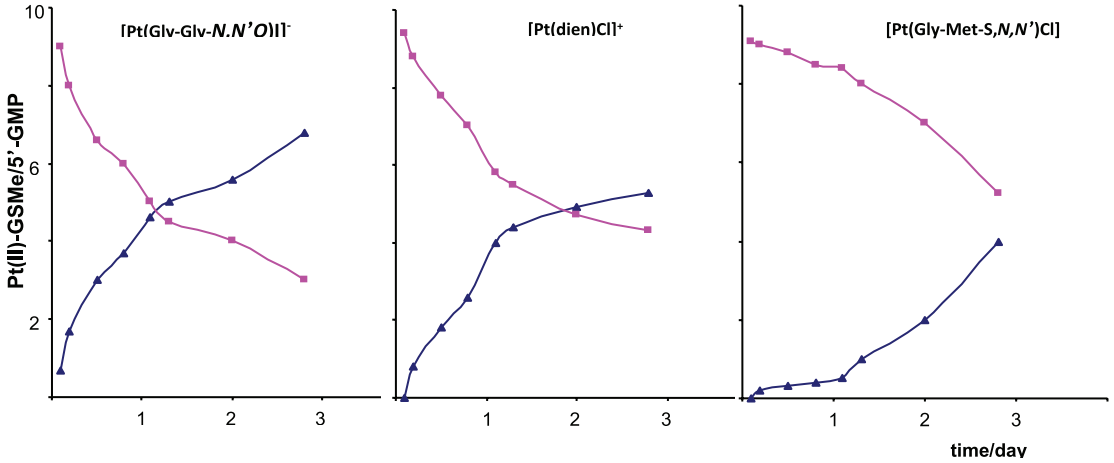

Figure 3. Time depedance of product formation in the reaction of [Pt(Gly-Gly-*N,N',O*)I]⁻ , [Pt(dien)Cl]⁺ and [Pt(Gly-L-Met-*S,N,N'*)Cl] complexes with GS-Me and 5'-GMP (1 : 1 : 1 mol ratio)at pD 7.4 in 50 mM phosphate buffer and at 25 °C: (■) Pt(peptide-***S***) complex; (▲) Pt(5'-GMP-*N7*) complex.

to the H8 proton of the free 5'-GMP and the growth of a resonance at 8.85 ppm, corresponding to the same protons of the 5'-GMP coordinated to platinum(II) (**Figure 4**). These chemical shifts are in accordance with those previously reported for the reactions of platinum(II) complexes with 5'-GMP (14).

The above-mentioned findings are in accordance to those previously reported for the selective intramolecular migration of $[Pt(dien)Cl]^+$ complex (dien is diethylenetriamine) from the methionine sulfur to the imidazole N1 atom of the *N*-acetylated L-methionyl-L-histidine (Ac-L-Met-His) (8), as well as to the N7 nitrogen atom of guanosine-5'-monophosphate (14). Also, it was found that this migration reaction is very slow and strongly selective to the N1 atom of the imidazole ring of the histidine side chain. On the other hand, no migration of the Pt(II) complex was observed in the reaction between [Pt(Gly-L-Met-*S,N,N'*)Cl] and Ac-L-Met-His dipeptide (8). It was explained by the fact that this complex, with a more sterically hindered Gly-L-Met ligand dipeptide, reacts more slowly with thioether-containing molecules than other two Pt(II) complexes and forms a more stable Pt(II)-sulfur bond (**Table 1**) (7, 8, 15) .

The present investigation shows that [Pt(Gly-Gly-*N,N',O*)I]$^-$ complex is more reactive with the methionine sulfur atom from the *S*-methylglutathione and undergo intramolecular migration to the N7 nitrogen atom in guanosine-5'-monophosphate than the $[Pt(dien)Cl]^+$ complex (**Table 1** and **Figure 3**). The reaction of [Pt(Gly-Gly-*N,N',O*)I]$^-$ with *S*-methylglutathione was much faster than with the corresponding [Pt(Gly-L-Met-*S,N,N'*)Cl] complex. Time dependence of the product formation in these reactions shows that, after 30 days of migration reaction, about 70% of [Pt(Gly-Gly-*N,N',O*)(5'-GMP-*N7*)] complex is present in the reaction mixture, while with [Pt(Gly-L-Met-*S,N,N'*)Cl], approximately 40% of the [Pt(Gly-L-Met-*S,N,N'*)(5'-GMP-*N7*)] complex was formed (**Table 1** and **Figure 3**).

The highest rate constant for [Pt(Gly-Gly-*N,N',O*)I]$^-$ in comparison with those of other platinum(II) complexes observed for reactions with sulfur-containing donors (**Table 1**) and the very rapid intramolecular migration of this complex in the reaction with guanosine-5'-monophosphate can be attributed to the *trans*-effect of the deprotonated peptide nitrogen, as well as to the electronegative oxygen atom in the coordination sphere of the investigated Pt(II) complex. These factors contribute to the weakness of the Pt-I or Pt-*S*-CH_3 bond and together with the large size of the iodido ligand could be considered for explanation of the fast reactivity of [Pt(Gly-Gly-*N,N',O*)I]$^-$ with the 5'-GMP.

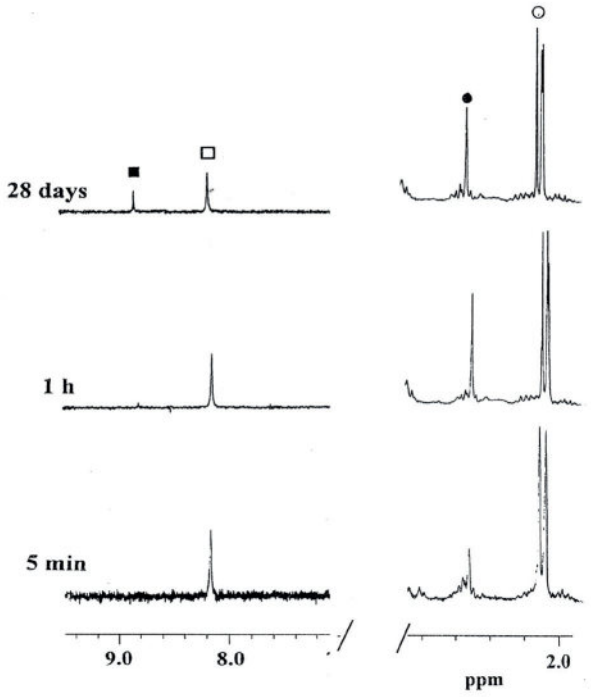

Figure 4. Part of the ^{1}H NMR spectra for the reaction of the [Pt(Gly-Gly-*N,N,O*)I]$^+$ complex with GS-Me and 5'-GMP (1 : 1 : 1 molar ratio) as a function of time. Chemical shifts are labeled for 5'-GMP bound (■) and free (□) as well as for GS-Me bound (●) and free (○).

CONCLUSION

The present study related the competitive binding of the sulfur- and nitrogen-contining biomolecules, *S*-methylglutathione and guanosine-5'-monophosphate, to the [Pt(Gly-Gly-*N,N',O*)I]$^-$ and [Pt(Gly-L-Met-*S,N,N'*)Cl] complexes shows that in the initial stages of the reaction the Pt(II) complex only reacts with *S*-methylglutathione forming kinetically favored product. In the second step

Table 1. Kinetic data for the reactions of Pt(II) complexes with thioether-containing ligands at room temperature, where k_2 is the second-order rate constant

Reactants [complex + ligand]	pD value	$10^3 k_2$/ $M^{-1}s^{-1}$	Ref.
$[Pt(dien)Cl]^+$ + L-Met	4.31	14	7
$[Pt(dien)Cl]^+$ + *S*-methylglutathione	5.41	33	15
$[Pt(dien)Cl]^+$ + Ac-L-Met-His	4.40	44	8
[Pt(Gly-Met-*S,N,N*)Cl] + L-Met	4.31	4.5	8
[Pt(Gly-Met-*S,N,N*)Cl] + S-methylglutathione	4.34	0.3	This work
[Pt(Gly-Met-*S,N,N*)Cl] + Ac-L-Met-His	4.40	8	9
[Pt(Gly-Gly-*N,N,O*)I]$^-$ + *S*-methylglutathione	4.21	40	This work
[Pt(Gly-Gly-*N,N,O*)I]$^-$ + Ac-L-Met-His	4.11	70	9

of the reaction very slow intermolecular displacement of the *S*-bound thioether ligand with the N7 atom of the guanine base of guanosine-5'-monophosphate is observed. Thiols, such as glutathione, are abundant sulfur containing ligands in cells and are responsible for inactivation of Pt(II) complexes, as well as numerous toxic side effects. The obtained results for intermolecular migration of the thioether-bound platinum(II) complex to the N7 nitrogen atom of guanosine-5'-monophosphate could support the hypothesis that Pt(II) initially bounded to a protein side chain may further react with nitrogen atom in DNA and therefore act as some kind of drug reservoir.

Acknowledgment

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INTERPLAY BETWEEN THE IMMUNOHISTOCHEMICAL EXPRESSION OF P53 AND THE PROLIFERATION INDEX IN THE KERATINOCYTE TUMORS OF THE SKIN

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UZAJAMNO DEJSTVO IZMEĐU IMUNOHISTOHEMIJSKE EKSPRESIJE P53 I INDEKSA PROLIFERACIJE U KERATINOCITNIM TUMORIMA KOŽE

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ABSTRACT

P53 is important for cell cycle regulation, and its overexpression is seen in malignant tumors. We examined correlation between p53 expression and cell proliferation, and its role in the pathogenesis of keratinocyte skin tumors. We used biopsies from patients with squamous cell carcinoma, actinic keratosis and keratoacanthoma. We examined cross-sections stained with HE and using anti-cytokeratin, anti-p53 and anti-Ki67 antibodies.

Expression of p53 is found in 87, 85% of SCC, in 83. 3% of AK and 13. 4% KA. The high index of p53 expression was higher in SCC and AK compared to KA. We also observed a positive correlation between the expression of p53 and localization of the tumors. The largest proportion of subjects with AK and SCC has a high index of p53 expression on photoexposed region. We also observed that p53 expression correlates with age whereby in AK p53 expression increases with age. The high index of proliferation is most frequent in SCC and KA. Also at AK we found a strong correlation between a moderate proliferation index and tumor localization in photoexposed region. Between the proliferation index and p53 expression we observed a significant positive correlation only in SCC.

Proliferation index and the expression of p53 are useful for the differentiation of precursor keratinocyte lesions and skin carcinoma. High p53 expression has been associated with the aging and significantly correlates with the exposure to UV radiation in SCC and AK. High expression of p53 in AK and SCC supports the importance of this oncoprotein in carcinogenesis of the skin.

Keywords: *Keratinocyte tumours, p53 expression, proliferative index, immunohistochemistry*

SAŽETAK

Protein 53 je značajan za regulaciju ćelijskog ciklusa i njegova pojačana ekspresija se viđa kod malignih tumora. Naš cilj je ispitivanje povezanosti ekspresije p53 sa proliferacijom ćelija i ispitivanje njegove uloge u patogenezi keratinocitnih tumora kože. Korišćen je biopsijski operativni materijal skvamocelularnog karcinoma (SCC), aktiničnih keratoza (AK) i keratoakantoma. Na presecima debljine 4µm , su primenjene rutinska HE i imunohistohemijska ABC metoda sa anti-cytokeratin, anti-p53 i anti- Ki67 antitelima.

Ekspresija p53 je nađena u 87, 85 SCC, u 83. 3% AK i u 13. 4% KA, pri čemu je visok indeks ekspresije p53 značajno češći u SCC i AK u odnosu na KA. Takođe je uočena pozitivna korelacija između ekspresije p53 i lokalizacije tumora. Statistički značajno najviše ispitanika sa SCC i AK ima visok indeks ekspresije p53 na fotoeksponiranoj reiji. Zapažena je znajana povzanost ekspresije p53 sa starošću ispitanika, pri čemu u AK sa godinama raste i ekspresija p53 . Visok indeks proliferacije je najčešći u SCC i KA , dok je u AK nađena jaka korelacija između umerenog indeksa proliferacije i lokalizacije tumora na fotoeksponiranoj regiji (Spearman's corelation p=0. 025). Izneđu proliferativnog indeksa i ekspresije p53 je zapažena značajna pozitivna koreleacija samo u SCC (p<0. 05 p=0. 028; Spearman's rho 0, 377)

Indeks proliferacije i ekspresija p53 su korisni za razlikovanje prekursorskih keratinocitnih lezija od karcinoma kože. Prekomerna ekspresija p53 je povezana sa procesom starenja i u značajnoj je korelaciji sa izlaganjem UV zračenju u SCC i AK. Visoka ekspresija p53 u AK i SCC podržava značaj ovog onkoproteina u karcinogenezi kože.

Ključne reči: *keratinocitni tumori, ekspresija p53, proliferativni indeks, imunohistohemija*

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ABBREVIATIONS

SCC - squamous cell carcinoma, **AK** - actinic keratosis
KA - keratoacanthoma

INTRODUCTION

Keratinocyte skin cancers are clinically and histopathologically heterogeneous group of lesions, caused by the proliferation of epidermal and adnexal keratinocyte. On the one side of the keratinocyte spectrum are a number of benign proliferation, whilst at the other end are malignant tumors which, not infrequently, have aggressive course with metastatic potential, such as squamous cell carcinoma (1, 2). Actinic keratosis also belongs to this keratinocyte spectrum (1). At the same time, keratinocyte tumors are the largest group of tumors of the skin, which is associated with these extreme fluctuations of climatic and meteorological factors, depletion of the ozone layer, ultraviolet and ionizing radiation (3, 4, 5). In addition to ultraviolet radiation, ionizing radiation and thermal damage to the chronic, frequent exogenous carcinogens are cigarette smoking, human papilloma virus, arsenic, vinyl chloride, polycyclic aromatic hydrocarbons, insecticides, herbicides, cadmium, etc. (6).

About 20% of keratinocyte skin cancers represents squamous cell carcinoma, which is the second most frequent of all cancers in the white population (1). In more than 90% of cases tumor is localized on the face, lower lip, neck, ear lobes and dorsal hand side, but can also have specific localisation on tongue, penis or vulva. Surrounding skin usually shows features of acantholytic damage (7). Clinically, it is most commonly presented as shallow ulcers often with ceratotonic crust and elevation of indure environment. Tumor sometimes begins as hyperkeratotic or verrucous focal point. Progression is followed by tumor enlargement, and when it reaches a few centimeters ulcers or necrosis might occurre. During enlargement tumor is spreading in softt tissue, cartilage, and bone. It can metastasize to lymph nodes and distant tissues and organs (7, 8).

Squamous cell carcinoma can arise *de novo* or from precursors such as actinic keratosis. A deregulation of the cell cycle is one of the most common changes during carcinogenesis (9). Cell unwinding cycle is highly organized and highly regulated process that involves a number of checkpoints, and ensures complete and accurate replication of DNA and cell components before the division. Carcinogenesis is the consequence of accumulation of disorders in the structure and function of genes regulating mechanisms of cell proliferation, DNA repair and apoptosis in the molecule. These are the oncogenes, tumor suppressor genes and genes controlling programmed cell death (10, 11, 12).

The most frequently mutated gene is TP53, located in humans on the short arm of chromosome 17 (17q13). Its importance is reflected in itsrole in suppressing cell transformation and tumorigenesis, Therefore it belongs to the class of typical suppressor gene. TP53 is organized in 11 egzogenic sequences encoding the synthesis of the p53 protein which plays an important role in cell cycle regulation and preserving stability of the genome (13, 14). Considering scarce and controversial results about the expression of p53 in the skin tumors of the keratinocyte histogenesis, in this study we investigated the association of p53 expression with proliferative index and clinicopathological characteristics of keratinocyte skin cancers.

MATERIALS AND METHODS

Patients and tissue samples

The study included 78 patients who underwent excision me-melanoma skin cancer in the period from January 1st to December 1st, 2008 in The centre for Plastic surgery in Clinical Center Kragujevac. Tissue samples were fixed in 10% buffered formalin solution, and after routine processing in autotehnicon, tumor tissues were embedded in paraffin and archived in The center of pathology in Kragujevac. Tissue sections (3-4μm) were stained with hematoxylin–eosin for histomorphological analysis of the tumor differentiation. For immunohistochemical staining we used paraffin embedded sections (3-4μm) and ABC method with anti-cytokeratin, anti Ki67- and anti p53- antibodies.

The study included 33 squamous cell carcinoma, 30 actinic keratosis and 15 keratoacanthoma. Patient clinicopathologic parameters are shown in Table 1. The study protocol was approved by the local Ethics Committee.

Immunohistochemistry

Paraffin sections were heated at 55°C to melt the paraffin, deparaffinized in xylene for 5 min three times and them rehydrated in a series of 100%, 96%, 70% and 50% alcohol. Antigen retrieval was performed by microwave heating for 20 minutes in 10 mM sodium citrate buffer (pH 6. 0). Endogenous peroxidase activity was blocked by 3% H_2O_2 in methanol for 20 min. Rabbit monoclonal Ki67 antibody (Abcam, Cambridge, UK; 1:100 dilution) and monoclonal mouse anti-human p53 (DAKO Denmark; 1:100 dilution) and monoclonal mouse anti-cytokeratin antibody (clone AE1/AE3; DAKO Glostrum, Denmark;1:200 dilution) were incubated at +4^0 C overnight. Immunostaining was performed by the

Table 1. Patient clinicopathological characteristics according to tumor histotypes

Tumor type (N)		Mean age ±SD (years)	Range MaleN(%)	Sex		Photo-exposed regions*	
				MaleN(%)	FemaleN(%)	Yes (N)	No(N)
SCC	(N=33)	73.39±10.43	41-88	19 (57.58)	14(42.42)	31	2
AK	(N=30)	67.37±7.93	56-77	15 (50.00)	15 (50.00)	28	2
KA	(N=15)	65.60±12.93	32-75	7 (46.67)	8(53.33)	8	7

*Photo-exposed regions are: scalp, face (cheek, forehead, chin, temple, nose, ear), surfaces of upper limbs, chest and upper back

Table 2. Comparison between patient's age and type of keratinocyte tumor.

Tumor type	SCC	KA	AK
SCC	/	p=0.006 *	p=0.002 *
KA	p=0.006 *	/	p=0.718
AK	p=0.002 *	p=0.718	/

** p<0.05 Mann-Whitney U test*

avidin-biotin peroxidase complex (ABC) method (Vectastain ABC-*Elite* kit, Vector Laboratories, Burlingame, CA).

Staining was visualized with 3, 3 diaminobenzidine tetrachloride (DAB). The slides were counterstained with Mayer hematoxylin and mounted in Canada balsam. Negative controls were done by replacing the primary antibody with phosphate buffered solution (PBS). The slides were examined by conventional light microscopy.

Quantification of immunohistochemical staining

In evaluating the expression of Ki67 and p53, only stained nuclei were taken into account, while for the evaluation of Ki67-positive cells per mm^2 by area the multipurpose test system M42 by Weibel was used. The objective micrometer (Reichert Wien 2mm/200) was used to determine the measuring area of 0. 016 mm^2

For testing Ki67 and p53, positive cells/mm^2 were counted successively by 10 "hot spots". The absolute value of the density of positive cells in the "hot spot" was determined stereometrically (15). The arithmetic mean of the obtained values of the "hot spots" represented the final number of Ki67 and p53 positive cells per mm^2 per case. The median was subsequently determined and the absolute values of the density of positive cells were divided into two groups: those with low expression level (value ≤ the median value) and those with high level of expression (values > the median value). These values represented the proliferative activity (proIDX). (1 + 2 + low or high index). It is also referred to an absolute value of a p53 in relation to deviation from the median obtained index of p53 expression (1 + 2 + or low: high). Cytokertine expression was not evaluated since cytokeratine antibody was used as a differencial marker for keratinocyte tumors.

Statistical analysis

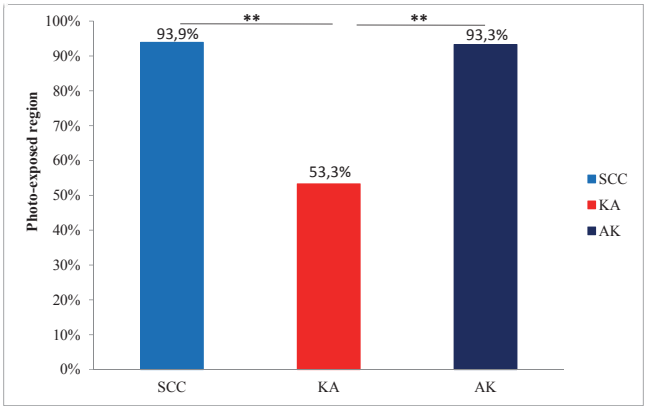
Data are presented as means ± SDor as proportions. Results were analyzed using the Chi-sqare test or Fisher's Exact test and, where appropriate, Mann-Whitney U test. Relationships between variables wereassessed using Spearman's Correlation test. Values of p< 0. 05 were considered as statisticallysignificant. Statistical analyses were performed using SPSS 22. 0. for Windows software (SPSS, Inc. , Chicago, IL).

RESULTS

Total number of patients with keratinocyte skin tumors was 78, and the average age of subjects was 69, 58 (range 32-88). Squamous cell carcinoma (SCC) was found in 33 patients and the average age was 73. 39 years. In the group with actinic keratoses (AK) 30 subjects were found, mean age 67, 37, while in the group with keratoacanthoma (KA) , 15 *to 70 years of age , there were the following results: SCC=3, AK= 9, KA=4. In the group of the patients from 71 to 80 years of age , distribution is: SCC=20, AK=13, KA=5, while in the age group from 81 to 90 years of age , distribution was the following SCC=6, AK=1, KA=1.* In the group of patients with SCC , 57. 58% of them were male and 42. 42% female, in patients with AK 50% were male and 50% female. In patients with KA 46. 67% of them were male and 53. 33% female. There were no significant correlations between the type of keratinocyte tumor and gender of patients, but we have noticed that in the SCC group, male subjects have been more affected (Table 1).

A statistically significant relation is noticed among the patient age and the patient type tumor carcinoma. Average age of the patients with SCC is statistically significantly higher than average patient age with AK (*Mann-Whitney U test;* p=0, 002) and also from average patient age range with the KA (*Mann-Whitney U test*; p=0, 006). There was no difference in average age between the patients with AK and the patients with KA (*Mann-Whitney U test*, p=0. 718). (Table 2).

Figure 1. Comparison between photo-exposed regions and different type of keratinocyte tumor.



***Chi-sqare test, p=0.001*
Values are expressed like percent of patients with tumor localized on photo-exposed region.

There was a statistically significant difference in the prevalence of patients with different tumor localization. In SCC and AK groups, tumor localization was significantly more often on photoexposed region (Chi-sqare test p = 0. 000), but there were no significant difference in the occurence of KA at photoexposed region and photo-non exposed (Chi-sqare test r = 0. 549). There is a significant difference in tumor occurrence in the region between photo-exposed SCC and KA (Chi-sqare test r = 0. 001) and between the AK and KA (*Chi-sqare test* p = 0. 001). SCC is significantly more likely to occur in the photo exposed region (93. 9% of cases) compared to KA (53. 3% of the cases). AK was also significantly higher (93. 3% cases) on photo-exposed region compared to KA (Chi-sqare test r = 0. 001). Between AK and SCC no significant differences in the incidence of the photo - exposed region was found. (Chi-sqare test r = 0. 942) (Fig. 1).

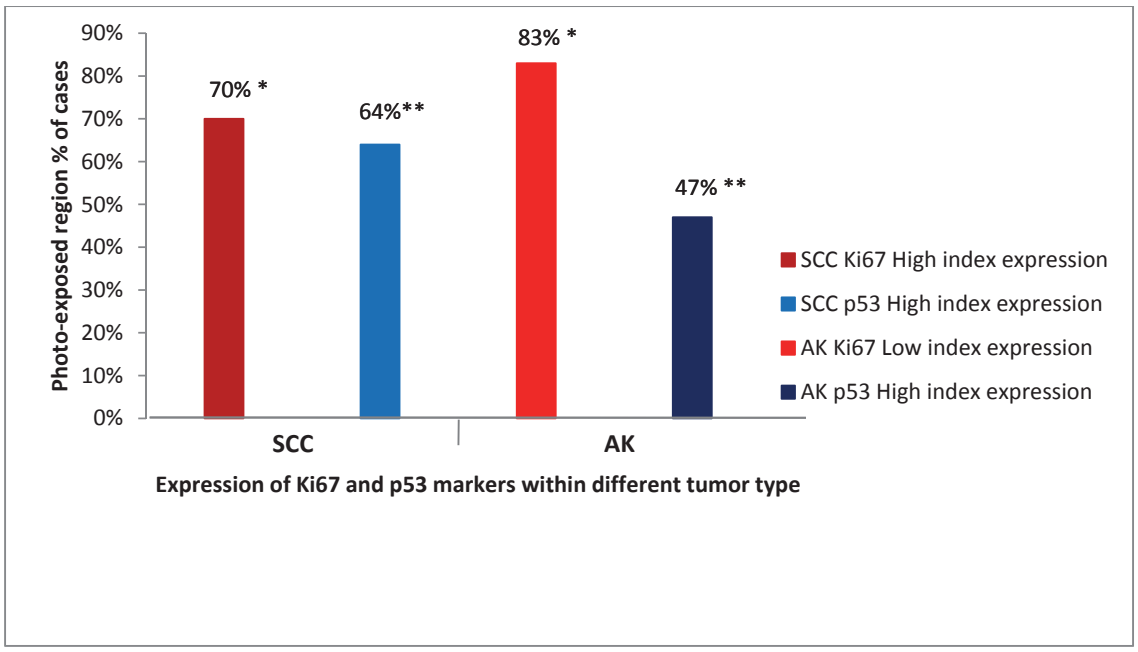
High proliferative index was found in 75. 8% of cases in the SCC group, 6. 7% in AK and 73. 3% in the KA. Percentage of patients with high proliferative index was highest in SCC and KA groups, and also there were no difference between these two groups. High proliferative index was significantly less frequent in AK in relation to the SCC (Chi-sqare test p = 0. 000) and KA (Chi-sqare test p = 0. 000). Low proliferation index was found in 24. 2% of cases in SCC group, in 90. 0% ofpatients in AK group and 26. 7% of the patients in KA group. Low proliferative index prevalence was significantly higher in AKcompared to SCC (*Chi-sqare* test; p = 0. 000) and KA (*Chi-sqare test* p = 0. 000) (Table 3).

There is significant correlation between the proliferation index (Ki67 expression) and the tumor of localized photo exposed region. The biggest number of patients with SCC (70%) has a high index of proliferation of the photo-exposed region, while in the AK patients there was significant corelation between the photo exposure region and the moderate proliferative index, that is encountered in 83% of cases (*Spearman's corelation* p = 0. 025). (Fig. 2). The connection between the high index of proliferation and tumor localization was not observed in the KA group.

A statistically significant number of men have a high proliferative index in comparison to women in SCC (*Chi-sqare test*, p = 0. 015) (Table 4).

Expression of p53 is found in 87. 8% SCC, in 83. 3% AK and 13. 4% KA . Hight index of p53 expression was found in 63. 6% of the SCC, in 50. 0% cases of AK, even in 6. 7% cases of KA. There were no significant difference in high expression of p53 between SCC and AK. High levels

Figure 2. Correlation between photo-exposed regions and ekspression of Ki67 and p53 markers within different tumor type.



**Spearman's correlation, p<0.05*
***Spearman's correlation, p<0.001*

Table 3. Values of p53 and Ki67 expression within different tumor types and intergroup analysis

		Type of tumor			Intergroup analysis		
		SCC	AK	KA	SCC-AK	SCC-KA	AK-KA
Expression of p53							
Level of expression	0	4 (12,1%)	5 (16,7%)	13 (86,7%)	-	p=0,000**	p=0,000**
	1+	8 (24,2%)	10 (33,3%)	1 (6,7%)	-	p=0,000**	p=0,003*
	2+	21 (63,6%)	15 (50%)	1 (6,7%)	-	p=0,000**	p=0,000**
Expression of Ki67							
Level of expression	0	0 (0,0%)	1 (3,3%)	0 (0,0%)	-	-	-
	1+	8 (24,2%)	27 (90,0%)	4 (26,7%)	p=0,000**	-	p=0,000**
	2+	25 (75,8%)	2 (6,7%)	11 (73,3%)	p=0,000**	-	p=0,000**

0: absent expression, 1+: low level expression, 2+: high level expression
**Chi-sqare test, p<0.05*
***Chi-sqare test, p<0.001*

of p53 expression were significantly more prevalent in AK and SCC compared to KA. (*Chi-sqare test*; p = 0. 000). Low level of expression was found in 24. 2% SCC, in 33. 3% AK and 6. 7% KA. The low level of expression is significantly more frequent in SCC compared to KA (*Chi-sqare test*, p = 0. 000) and significantly more frequent in AK compared to KA (*Chi-sqare test* p = 0. 003). The absence of expression of p53 was found in 12. 1% SCC, 16. 7%AK and 86. 7% KA (Table 3).

There is a correlation between p53 expression and tumor localization. We demonstrated a positive correlation between p53 expression and photo exposure of tumor regionin SCC and AK. Significantly more patients with SCC 64% and AK47% has a high index of p53 expression on

Table 4. Expression of p53 and Ki67 in SCC, AK and KA depending on the gender.

Ki 67 and p53 expression		**Type of tumor**						**Analysis according to the type of tumor**		
		SCC		**AK**		**KA**		**SCC M-F**	**AK M-F**	**KA M-F**
Gender		**M**	**F**	**M**	**F**	**M**	**F**			
	Ki67 expression									
Level of Ki67 expression	**0**	0 (0%)	0 (0%)	1 (3,3%)	0 (0%)	0 (0%)	0 (0%)	-	-	-
	1	3 (9,1%)	5 (15,2%)	12 (40%)	15 (50%)	2 (13,3%)	2 (13,3%)	-	-	-
	2	16 (48,5%)	9 (27,3%)	2 (6,7%)	0 (0%)	5 (33,3%)	6 (40%)	p=0,015	-	-
	p53 expression									
Level of p53 expression	**0**	2 (6,1%)	2 (6,1%)	2 (6,7%)	3 (10%)	5 (33,3%)	8 (53,3%)	-	-	p=0,031*
	1	6 (18,2%)	2 (6,1%)	6 (20%)	4 (13,3%)	1 (6,7%)	0 (0%)	p=0,014*	-	-
	2	11 (33,3%)	10 (30,3%)	7 (23,3%)	8 (26,7%)	1 (6,7%)	0 (0%)	-	-	-

M-males, F-females;
*Chi-sqare test, p<0.05

Table 5. Expresion of p53 by age, within different keratinocyte tumor.

Expression of p53		Type of tumor											
		SCC			AK				KA				
Age		<=60	61-70	71-80	81-90	<=60	61-70	71-80	81-90	<=60	61-70	71-80	81-90
Level of p53 expression	0	0 (0%)	1 (33%)	3 (15%)	0 (0%)	0 (0%)	2 (22%)	3 (23%)	0 (0%)	4 (80%)	4 (100%)	4 (80%)	1 (100%)
	1	0 (0%)	2 (67%)	4 (20%)	2 (33%)	3 (43%)	4 (44%)	3 (23%)	0 (0%)	1 (20%)	0 (0%)	0 (0%)	0 (0%)
	2	4 (100%)	0 (0%)	13 (65%)	4 (67%)	4 (57%)	3 (33%)	7 (54%)	1 (100%)	0 (0%)	0 (0%)	1 (20%)	0 (0%)

Intergroup analysis

Age		<=60			61-70			71-80			81-90		
		SCC-AK	SCC-KA	AK-KA	SCC-AK	SCC-KA	AK-KA	SCC-AK	SCC-KA	AK-KA	SCC-AK	SCC-KA	AK-KA
Level of p53 expression	0	-	p=0,000**	p=0,000**	-	p=0,001**	p=0,05*	-	-	-	-	p=0,000**	p=0,000**
	1	p=0,042*	-	p=0,009**	p=0,05*	p=0,001**	p=0,000**	-	p=0,000**	p=0,014*	p=0,000**	p=0,000**	-
	2	-	p=0,000**	p=0,000**	p=0,000**	-	p=0,000**	p=0,027*	p=0,000**	p=0,001**	p=0,000**	p=0,000**	p=0,000**

** Chi-sqare test, p≤0.05*
*** Chi-sqare test, p<0.001*

photo exposed tumor region(*Spearman's correlation*, p = 0. 000). (Figure 2). Correlation between the expression of this marker and localization was not observed in the KA. Significantly more women have absence of p53 expression in KA compared to men (*Chi-sqaretest*, p = 0. 031), while a statistically significant number of men have low index of p53 expression compared to women in SCC (*Chi-sqare test*, p = 0. 014) (Table 4).

In patients younger than 60 years of age, frequency of high index of p53 expression was significantly higher in the SCC compared to KA (*Chi-sqare test* , p = 0. 000). In this population there were no statistically significant difference in the prevalence of high index of p53 expression between the SCC and the AK, but the high index of p53 expression was significantly more frequent in the AK compared to KA (*Chi-sqare test*, p = 0. 000). The low index of p53 expression was significantly less frequent in SCC compared to AK, and also significantly more frequent in the AK compared to KA. In this group of patients the absence of p53 expression was significantly lower in the SCC and the AK compared to KA (Table 5).

In the group of respondents aged 61 to 70 years, a high index of p53expression was significantly more common in AK compared to SCC and KA, and statistical significance was not found between the SCC and KA. Low index of p53 expression were significantly more prevalent in the AK as compared to SCC and KA. Significantly more patients with SCC have a low index of p53 expression as compared

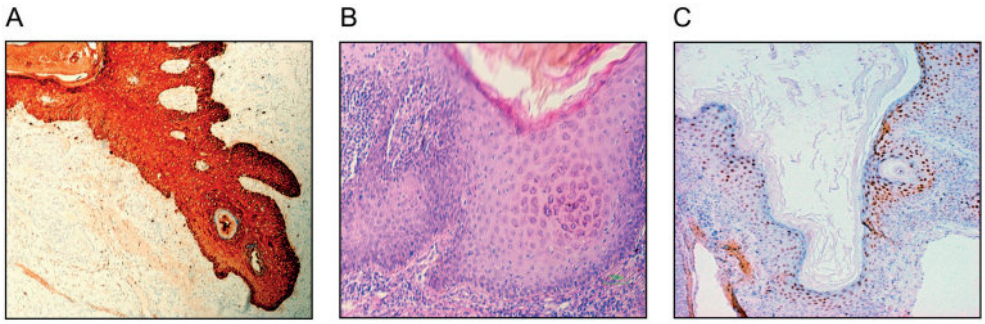


Figure 3: Keratoacathoma

A) Diffuse high expression of cytokeratin in whole epiderm with pronounced focal intensity in basal keratinocytes (AE1/AE3; magnification X 200),

B) Bottom of keratin plug, hiperplasion of granulose layer with increased mitotic activity (AB-PAS, magnification X 400),

C) Diffuse proliferative activity of epidermal keratinocites in the surroundings of keratin plug (Ki67, magnification X 200)

to KA. The absence of p53 expression was significantly lower in the SCC and the AK in relation to keratoacanthoma. (Table 5).

In subjects 71 to 80 years of high index of p53 expression were significantly more frequent in SCC compared to AK and KA, as well as AK in relation to KA. Low index of expression of this protein is significantly more common in SCC and the AK compared to KA. , Between AK and SCC there were no statistically significant differences in the low expression of p53. Also, in this population there was no significant difference in the absence of p53 expression between the different tumor types (Table 5).

In the group of subjects aged 81 to 90 years, high index of p53 expression was significantly more frequent in SCC compared to AK and KA, as well as in AK compared to KA. The low index of p53expression was significantly more frequentin SCC compared to AK and KA, while between the AK and KA there were no significant difference in the low p53 expression. The absence of p53 expression was significantly lower in patients with SCC and the AK compared to KA, while between the AK and the SCCno difference was found. (Table 5).

In patients younger than 60 years of age high index of expression of Ki67 was significantly more frequent in SCC and KA compared to AK, and statistically significant difference was not found between SCC and KA. The low index of expression of the p53 is significantly less frequent in SCCthan in AK, whereas statistical significance was not found between the SCC and KA. In this group of patients there is no statistical significance in the absence of the expression of Ki67 and tumor types.

In the group of respondents aged 61 to 70 years, a high index of expression of Ki67 was significantly more frequent in SCC and KA relative to AK, and statistical significance was not found between the SCC and KA. Low index of expression of Ki67 were significantly more frequent in AK in relation to the SCC and KA, while significantly more subjects with KA has a low index of expression of Ki67 in comparison to the SCC. The absence of expression of Ki67 was significantly less for SCC and KA in comparison to the AC, while between the SCC and KA there is no statistical significance.

In subjects aged from 71 to 80 years, high index of Ki67expression was significantly more frequent in SCC and KA in comparison to the AK, and between SCC and KA no difference was found. Low expressionindex of this protein is significantly more common in AK compared to SCC and KA, while between SCC and KA no difference was found. In this population subjects there was no significant difference in the absence of the Ki67expression between different tumor types.

In the group of subjects aged from 81 to 90 years, high index of expression of Ki67 was significantly more frequent in SCC compared to AK and KA, as well as the KA with respect to AK. The low index of expression of Ki67 is significantly more frequent in AK compared to SCC and KA, as in SCC compared to KA. In the study group, there were no significant difference in the absence of the Ki67expression in various tumor types.

Correlation analyzes of Ki67 and p53 expression in a keratinocyte tumors demonstrated a significant positive correlation between these two markers in the SCC (p<0. 05 p=0. 028; *Spearman's rho* 0, 377). Correlation between the expression of Ki67 and p53 was not found in the AK and KA.

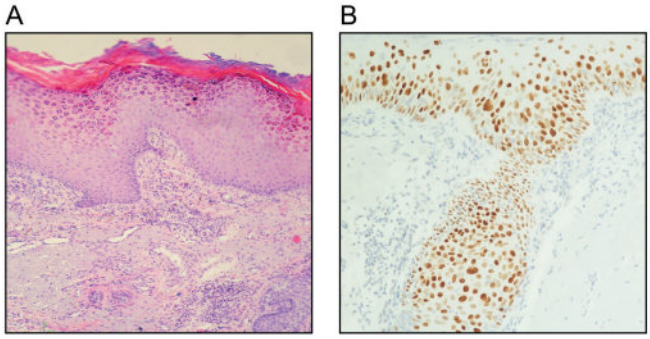


Figure 4: Solar keratosis

A) Acantosis and hiperplasion of granulose layer (HE, magnification X 200)

B) Diffuse nuclear p53 expression along whole epidermis (p53, magnification X 200)C) Diffuse p53 expression in moderate differentiated squamocelular carcinoma (p53, magnification X 200)

D) Moderate proliferative activity with nuclear and cellular atypia in moderate differentiated squamocelular carcinoma (Ki67, magnification X 200)

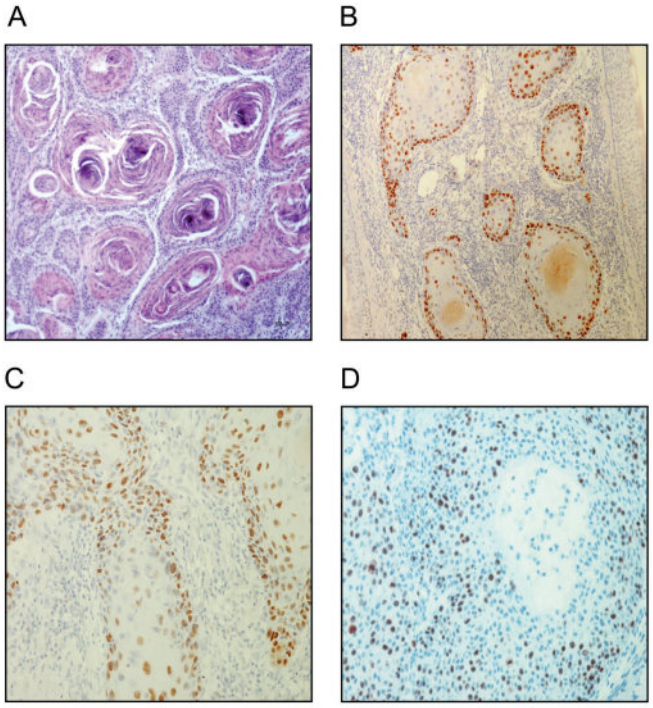


Figure 5: Squamocelular carcinoma

A) Well diferented type with epithelial cells distributed in bulbar shape and "cancer pearls" in the center (HE, magnification X 200)

B) Intensive nuclear p53 expression predominantly on periphery of "tumor islands", in well diferrentiated squamocelular carcinoma (p53, magnification X 200)

DISCUSSION

The development of squamous cell carcinoma is a multistage process in which a number of molecular disorders occurs that lead to neoplastic transformation of keratinocytes. The key events in keratinocyte carcinogenesis include activation of protooncogenes and inactivation of tumor suppressor genes. A classic example of a suppressor gene is a gene TP53 which encodes a multifunctional protein p53. Somatic mutations of this gene are the most frequent gene mutations in human cancers, and has been estimated to occur in 50-90% of malignant tumors, depending on the type and stage of the tumor (16, 17). From 1989. till today, in over 2700 published papers numerous evidences for more than 35000 TP53 mutations were described (18, 19). The mutated gene is changing the structure and function of the encoded protein, initiate the process of oncogenesis and lead to accumulation of p53 in tumor tissue (20, 21).

As expected, reports on the expression of p53 in keratinocyte tumors are quite heterogeneous. In our study, the expression of p53 was present in 87. 8% of SCC, 83. 3% of AK and 13. 4% of KA patients, which is consistent with previous reports of Karagece et al and Park et al (22, 23). However , Dornelas et al have recently demonstrated the expression of p53 in all cases (100%) tested in the SCC and the AK (24), while in the study of Khodaeiani et al, p53 was detected in 50. 20% of SCC, and was absent in KA patients (25). Almost more than two decades ago, Lee et al. demonstrated that p53 expression was present even at the 78. 8% of the tested KA and 75. 5% of the SCC (26). In our study, we showed significantly higher frequency of high index of p53 expression in the SCC and the AK in relation to KA. These findings are in favor of concept that the common factor, such as actinic damage may be responsible for both lesions (SCC and the AK). In the other hand, majority of SCC is a p53 pozitive, regardless of whether they are localized on the photo exposed parts of the body or not, which indicates that, in addition to actinic damage, in the keratinocyte carcinogenesis other pathogenetic mechanisms can also be involved (26, 27, 28)

A statistically significant majority of our patients with SCC and the AK has a high index of p53 expression in tumors that are localized to the photo exposed region. Numerous reports confirmed that keratinocyte tumors occurring as a result of exposure to ultraviolet radiation (3, 29, 30, 31). About 90% of UV radiation consists of UVA rays (320-380 nm in the solar spectrum), while the UVB rays (290-320 nm in the solar spectrum), make up about 1-10% of the UV radiation. However, when absorbed by the keratinocyte DNA, UVB rays cause genotoxicity and mutations of the p53 gene (24 29, 32). P53 induction is detected in the skin after 30 minutes after exposure to UVB radiation (33). The presence of p53 expression in a high percentage (83. 3%) in the AK, in this study, confirms the observation that the mutation of the p53 gene is early event in keratinocyte carcinogenesis (32) .

P53 belongs to transcriptional factors and when enabled, allows the expression of several genes, one of which is p21, which belongs to the group of inhibitors of cyclin - dependent kinases (CKIs), and arrest cells in the G1 phase of the cell cycle. Protein p53 can halt the progression of the cycle in the G2 phase and even during mitosis. In the case of irreversible damage to the DNA p53 induces the activation of apoptosis (31). Mutational events form that inactivate p53 is specific for each individual tumor. It has been noted that the loss of p53 function, in patients with SCC, is most frequently associated with mutation of cytosine and thymidine dimer (so called “tandem mutation”) (32, 34).

The absence of p53 expression in tumor islands with pronounced keratinization, in well-differentiated SCC, and high index of p53 expression in poorly differentiated SCC, suggests that the presence of p53 is indicator of the immaturity of the tumor cells and the proliferative capacity of tumors. In accordance with this interpretation is the observation that high-grade tumors have a higher level of expression of p53 (35).

In this study, there were no significant differences in the prevalence of high index of expression of p53 between the SCC and the AK, and at the same time, a positive correlation between the expression of p53 in these tumors and localization in photoexposed region was shown. It was also observed that between SCC and AK there is no significant difference in the incidence of the photoexposed region. These results were supported by the concept that the exposure to UV light is the main cause of p53 mutations. This is supported with the report of Batinac et al, in which the expression of the p53 was detected in 39. 0% of cases in the normal epidermis exposed to UV radiation (35). There are suggestions in the literature that the expression of p53 is correlated with poor prognosis and / or a lack of response on therapy (25, 36).

Most keratinocyte tumors with high proliferation index were in the group with SCC and KA, which is to be expected since these two histologically similar tumors have a pattern of growth, including cytologic atypia and infiltration (37). Despite the fact that between the two types of tumor there were no differences in the prevalence of high proliferation index, however, the correlation with high proliferation index is observed only in photoexposed region in SCC. High proliferative index was significantly lower in AK than in SCC and KA, but in a high percentage (83%) of AK patients there is strong correlation between a moderate proliferation index and tumor localization in photoexposed region. UV radiation, not only damages the DNA of keratinocytes, but also reduces the immune response of the skin and reduces the body’s ability to carry out repair adverse change (37, 38).

P53 mutants indicates that the damage is caused by UV light (24, 39), and the cells which are succumbed to DNA damaging continue to grow and to divide. Typically, the exhibit keratinization disorder is clinically manifested as an area of rough skin. While the changes are localized above the basal membrane, lesions represent a squamous

cell carcinoma "in situ". At some point, malignant cells can penetrate into the dermis, to give the invasive squamous cell carcinoma. Period of evolution is about 10-20 years and probably about 8-20% of actinic keratoses develop into squamous cell carcinoma. It is believed that patients with actinic keratoses are about 200 times more likely to develop squamous cell carcinoma (1). Changes ascending and infiltration, multiple ulcerations and inflammation of the surrounding are clinical signs suggestive of malignancy (40).

Sporadically there are suggestions that solar keratosis should be considered as squamous cell carcinoma "de novo", and not as a pre-cancerous lesion that evolves into squamous cell carcinoma. (41, 8), yet in the literature there is consistent view that the disease is classified into the precursor of squamous cell carcinoma (1, 2, 37). Numerous intraepidermal proliferative disorders can be precursors of squamous cell carcinoma but actinic keratoses are characterized by a dysplastic epithelium usually on body parts exposed to the sun (19, 20).

High proliferative index in KA, observed in our study, which is no different from the index of proliferation in the SCC, was also reported by other authors, but unlike many other authors (1, 35, 37, 39) we haven't observed association of KA with exposure to ultraviolet radiation. In addition, in this study the expression of p53 in KA did not positively correlated with tumor localization on photoexposed region, such as in the case with the SCC and AK. This points to a complex phenotype and heterogeneous etiology of KA. Speaking in favor of that, also goes a recent findings in the statement that KA is a separate entity caused by alteration of the TGFβ signaling pathway (42).

In line with other reports (22, 32) are our findings that there is significant association between the age of patients and the type of tumor keratinocyte, respectively our patients with SCC are significantly older than patients AK and KA. We also observed significant correlation between age of patients and p53 expression. Specifically in subjects younger than 60 years of age, significantly more common was high index of p53 expression in SCC then in other keratinocyte tumors, but with age, expression of p53 increases in the AK which could explain the prolonged exposure to UV radiation. Similar observations have been published by Turkish authors who have furthermore pointed out a strong correlation with the p53 expression to the tumor size in AK (22). Our correlation analysis identified a significant association of the proliferation index and the expression of p53 only in the SCC, which has also been observed by other authors (43, 44). In all age groups, in this study, a high proliferative index was significantly more frequent in SCC and KA than in AK.

Between the type of tumor and gender we did not find a significant association but we have noted that the SCC is slightly more frequent in male patients, and accordingly, there are reports according to which SCC occurs in males 2: 1 to 3. 3: 1 ratio in relation to females (45, 46)

CONCLUSION

Proliferation index and the index of the expression of p53 are useful for the differentiation of precursor lesions from keratinocyte skin carcinoma. Overexpression of p53 is associated with the process of aging and significantly correlated with exposure to UV radiation, especially in the SCC and AK.

Finally, high expression of Ki67 is a good indicator of proliferative activity, and high expression of p53 in AK and SCC supports additional pathogenetic significance of this oncoprotein in carcinogenesis of the skin.

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DISCLOSURE

The authors declare they have no competing interests or other interests that might be perceived to influence the results and discussion reported in this paper.

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DECLINE IN FEMALE FERTILITY AFTER 40 YEARS

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OPADANJE FERTILNOSTI KOD ŽENA STARIJIH OD 40 GODINA

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ABSTRACT

Important factor related to the conception possibility is women age. The decline in fertility with aging is proven and evident in literature. Infertility is increasing and many couples seek help in advanced techniques such as IVF (in vitro fertilization) in order to overcome the problem caused by aging, but the quality of the oocytes is a significant limiting factor. With the aging the quantity and quality of oocytes decreases, such as the quality of the embryo after fertilization. The accelerated rhythm of life, liberty and women inclusion in all kinds of professions brought many benefits to women, but also increasingly postponing births. Each person is unique individual, and can be more or less fertile compared to the average at same age. Unfortunately, some women has a rapid decline in fertility - accelerate aging, very early, already in the early twenties and when testing them with different methods and exams, the result is very low number of oocytes, low value of anti-Müllerian hormone and also very poor quality of these oocytes, or low ovarian reserve. The problem is that when you have accelerate aging, even IVF techniques can not be of great help in achieving pregnancy. The pregnancy rate (17,65%) and the childbirth rate (5,88%) with the patients older than 40 is very low, although comparable to the data from the scientific literature and speaks in favour of the fact that the success of assisted reproductive techniques is very modest with women older than 44.

Keywords: *Infertility, anti-Müllerian hormone; antral follicle count; ovarian reserve*

SAŽETAK

Starost žene je važan faktor kada je u pitanju mogućnost začeća. Opadanje fertilnosti sa starenjem je dokazano i očigledno u našem društvu. Mnogi parovi traže pomoć u naprednim tehnikama kao što su IVF (in vitro fertilizacija), da bi prevazišli problem nastao zbog starenja. Ipak kvalitet jajne ćelije je značajan limitirajući faktor.

Pravilnije je reći da sa starenjem žene opada kvantitet i kvalitet jajnih ćelija, što dalje vodi do lošeg kvaliteta embriona nakon fertilizacije. Ubrzani tempo života, sloboda žene i uključivanje u sve vrste profesija doneli su mnoge prednosti ženama, ali i sve češće odlaganje rađanja. Svaka individua je jedinstvena, i može biti manje ili više fertilna u poređenju sa prosekom i njenim godinama starosti. Mada je retko, moguće je da neka osoba ima brzo opadanje kvaliteta jajnih ćelija (ubrzano starenje) veoma rano, već u ranim dvadesetim godinama života i prilikom izvođenja različitih testova i pregleda možemo utvrditi veoma mali broj oocita u jajnicima, nisku vrednost anti-Müllerian hormona a takođe i loš kvalitet tih jajnih ćelija tj, lošu ovarijalnu rezervu. Naime, kada postoji ubrzano reproduktivno starenje, čak ni primena IVF tehnika ne može biti od velike pomoći u postizanju trudnoće. Stopa trudnoća i stopa porođaja kod pacijentkinja starijih od 40 godina je veoma niska (stopa trudnoća 17,65%, stopa porođaja 5,88%), mada komparabilna sa podacima iz literature i govori u prilog tome da je uspeh asistiranih reproduktivnih tehnika veoma skroman kod žena starijih od 44 godine.

Ključne reči: *Infertilitet, anti-Müllerian hormon; broj antralnih folikula; ovarijalna rezerva*

ABBREVIATIONS

FSH- follicle stimulating hormone,
E2-estradiol,
AMH- anti-Müllerian hormone,
AFC-antral follicles count,
IVF- in vitro fertilization,
ART-assisted reproductive technology.

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INTRODUCTION

During the intrauterine development of the female fetus with the help of three simultaneous processes: mitosis, meiosis and oogonial atresia number of germ cells in the gonads in the 20th week of gestation reaches 6-7 million, out of which about two thirds are intrameiotic primary oocytes. From that moment and on, processes of irreversible atresia are performing that reduce the number of germ cells in the gonads predominantly through follicular (oogonial) atresia, which occurs around the 6th lunar month, and continues during the reproductive age (1, 2). In human fetuses atresia generally occurs via apoptosis in oocytes until in adult age mainly through apoptosis of granulosa cells. It takes place during each stage of folliculogenesis, but it is more common in follicles that have reached a size that could be selected for ovulation (3). Thus, some of 300000 to 400000 of follicles are in the rest period at menarche, only about 500 follicles will ovulate, and the rest are subjected to atresion. Promptness of ovarian atresia is not constant and can be said to have a biexponential shape, with the changes that primarily determine the number of remaining oocytes in the ovaries, and less – women`s age. It is normal to register a significant decline in the number of ovarian follicles after 37-38 years, when the total number of remaining follicles is approximately 25 000 (4).

Based on numerous studies conducted by reproductive biologists, optimal fertility period is between 18 and 31 years (5). Several theories was formulated to explain the decline in the oocyte quality with aging. Hypothesis "production line" indicates that the quality of oocytes is established during fetal life and oocytes that are less susceptible to errors, for example - non-disjunction during meiotic division, or, as we can say, better quality oocytes ovulate first, leaving those of lower quality to ovulate later in life (6). For this reason, frequent "reproductive errors" might be more likely consequence of the reduced number of remaining ovarian follicles than women`s age.

With ageing the risk of non-separation during metaphase II of the second meiotic division increases, and this phenomenon can be explained by the accumulation of errors in the genome as a result of compromised function of granulosa cells: defective microcirculation around the biggest follicle which leads to the reduction of oxygen levels in follicular fluid, or a gradual increase in intracellular oxidative stress (7).

In older women meiotic spindles during metaphase II (the second meiotic division) are diffuse, show a lack of bipolarity and the chromosomes are not clearly arranged along the equator of the spindle but are loosely holding for the spindle and are in different locations along the spindle (8). This less regulated localization of chromosomes in oocytes of older women can be a cause of problems encountered during the separation and segregation of chromosomes. So we get embryos with a high percentage of chromosomal abnormalities that contain all possible combinations of monosomies or trisomies (9). Monosomies in conceptus are present in the same percentage as the trisomies, but these embryos are lost in a much earlier stage of pregnancy and almost never come to terms. Thus, the incidence of genetic abnormalities is much greater than we can imagine, and certainly grows with the age of a woman as much as a number of miscarriages in advanced age (10).

Women who are approaching 40 years of age, may be diagnosed in most cases an increase in basal serum FSH (FSH measured by the 2nd or 3rd day of the menstrual cycle) and this value directly indicate a decrease in the number of antral follicles capable of ovulating (11). This increase FSH levels usually starts about a decade or more before the onset of menopause. Jump FSH values occurs as a result of the negative feedback FSH-modulating ovarian proteins, mainly inhibin A and inhibin B. Since the Inhibin B (dimeric polypeptide secreted by ovarian granulosa cells) is predominantly secreted in the early antral follicles, decrease in the value of inhibin B in serum directly indicates a decrease in antral follicles "pull" in the ovaries (12).

The increase in serum FSH may be also independent of the age (13). In patients with this problem it can certainly be established poorer ovarian response after controlled ovarian hyperstimulation with fewer obtained oocytes similarly as in older women. Also a large number of studies and expert opinions are still divided as to whether ovaries will respond better to stimulation in younger women with elevated FSH and low values of inhibin B or ovaries of older women with normal FSH and inhibin B. However, in most cases, we can conclude that the ovarian reserve is a better predictor of oocyte production capacity or the number of oocytes obtained after ovarian hyperstimulation, and patient's age is a better predictor of the quality of obtained oocytes (14, 15). We should not forget that there are patients with mutated FSH receptor in which the amino acid asparagine in the receptors protein is replaced with serine at position 680, and this change leads to a conformational change in FSH receptor which is now much less active and require much higher levels of FSH hormone for normal functioning. In these cases, serum FSH levels certainly are not a reflection of accelerated reproductive aging (16, 17, 18).

Implantation of the embryo-blastocyst is a complex process with a lot of interaction between the blastocyst and endometrium, or the embryo and the endometrium, but to be successful requires two conditions: a vital embryo and receptive endometrium(window of implantation) (19, 20, 21). Another important factor affecting fertility in the advanced age is abnormal endometrial receptivity, and it is predominantly a result of the reduced number of progesterone receptors, as a result of reduced levels of estrogen receptors(22, 23).

With aging the possibility of the occurrence of many pathological changes in the endometrium that affect its receptivity increases and these changes include endometrial polyps, fibroids, adhesions, endometriosis and hydrosalpinges (24, 25, 26, 27, 28).

Expression of estrogen receptors (ER) at the implantation time has important role in endometrial receptivity and

in healthy women estrogen receptors are down regulated at that moment, but in women with endometriosis, there is up regulation of ER, which leads to low pregnancy rate (29, 30). Endometrial thickness measured by ultrasound, more than 9-10mm, is associated with significantly higher pregnancy rates relative to thickness below 6mm, usually seen in older patients with specific hormonal status (31).

Treatment of impaired endometrial receptivity is still in focus of many studies and some of them propose endometrial gene therapy, some endometrial stimulation using local injury, or endometrium priming with instillation of granulocyte colony-stimulating factor, piroxicam and human chorionic gonadotropin (hCG) (32, 33).

Quantity of remaining oocytes in the ovaries or ovarian reserve testing, probably has not great influence on natural fertility (if a woman trying to get pregnant the natural way). However, if a woman undergoes in vitro fertilization techniques, this parameter has a lot of influence in terms of ovarian response to drugs used to stimulate ovulation (34).

The more the number of remaining oocytes in the ovaries, the more oocytes may be obtained from the stimulated cycle, and it gives a greater chance for the realization of successful pregnancy.

Thus the reproductive age is best defined by ovarian reserve, the functional ovary potential at one moment, or the current number of follicles in the ovary that are able to ovulate. Before starting ART procedures, ovarian reserve of each patient can be determined:

1. ultrasonography-measuring the number of antral follicles (AFC-antral follicle count), ovarian volume and blood flow through the ovaries,
2. determining basal hormone levels 2nd or 3rd day of the menstrual cycle: FSH, LH, estradiol-E2, AMH (antiMullerian hormone), inhibin B, inhibin A, P-progesterone, FSH: LH ratio, P: E2 ratio, testosteon -T, VEGF (vascular endothelial growth factor), IGF 1 (insulin-like growth factor), IGF-BP I (insulin-like growth factor-binding protein) (35)
3. by implementation of the ovarian stimulatory tests, such as: CCCT (clomiphene citrate challenge test), EFORT-test (exogenous FSH ovarian reserve test) and GAST (gonadotropin agonist stimulation test) (35, 36).

The aim of these tests is to precisely predict the potential success for the patient, but to warn patients with poor ovarian reserve parameters on the likely poor outcome and all of that before starting IVF treatment (37, 38).

AMH is a dimeric glycoprotein hormone which belongs to the TGF family (transforming growth factor) - and is produced by the granulosa cells of ovarian follicles. In the literature it is also called MIS or Mullerian inhibiting substance and it was first investigated in sexual differentiation of male during embryogenesis (39).

It is first produced in the primary follicles which are formed from primordial follicles. At this stage, the follicles are microscopic and can not be visualized by ultrasound. The maximum production of AMH is in preantral and small antral follicles (smaller than 4 mm in diameter). AMH production decreases and then stops as the follicle grows, so that there is almost no AMH production in follicle diameter greater than 8mm (40). Thus, AMH levels are constant, can be determined any day of the menstrual cycle and are used to estimate the pull of growing follicles in women. Size of the pull, or the quantity of growing follicles is closely dependent on the number of remaining primordial follicles, and thus the value of AMH reflects the amount of remaining oocytes, or the size of the ovarian reserve (41).

According to many researchers, AMH can be considered to be a marker for the ovarian ageing process (42). AMH values are a good predictor of the IVF outcome, so the higher concentrations of AMH are associated with a greater number of mature oocytes obtained after stimulation, consequently, a large number of embryos, and finally a higher percentage of clinical pregnancies (43, 44, 45, 46).

Interesting is fact that AMH levels are reduced in smokers or women who had consumed long term hormonal contraception (almost 30% lower values than in controls) (47, 48, 49).

Measuring the ovary volume and antral follicle count (AFC) with ultrasound is currently one of the best methods for assessing ovarian reserve. Antral follicles are small follicles with a diameter of 2-8mm, which can be seen, measured and counted by ultrasound. Using a vaginal probe is certainly the method of choice, and measurement of the antral follicle count give us indirect information of the relative number of microscopic primordial follicles remaining in the ovary, which can potentially develop in the future. Number of antral follicles is a good predictor of the number of mature oocytes that can be obtained in stimulated cycle and the number of oocytes obtained directly correlates with the success of IVF (50). By measuring the number of antral follicles at the beginning of the stimulated cycle we can predict the response after controlled ovarian stimulation.

In patient selection prior to IVF it is necessary to determine both complementary factors: AMH provides information about number of very small, non-atretic follicles, and AFC provide information about follicle sizes and discrepancies in follicle size (51).

Measuring the volume of the ovary by ultrasound is also a reliable indicator of ovarian reserve. It is already well known fact that decreased volume of the ovaries is associated with advanced age. For women with ovarian volume less than 3 ml, we can say that there is a high risk of poor response to controlled ovarian stimulation during IVF, and high rate cancellation of cycle (52, 53).

Many studies suggest that about 13 years before menopause, ovary begin to show signs of accelerated aging, even if there are regular menstrual cycles (54, 49).

Therefore, it is useful to determine whether this physiological signals has started. But there is no gold standard for this testing, so assessment of ovarian reserve is possible using many laboratory hormone levels measurements and

stimulatory tests (55), by measuring mean LH, amplitude of LH and LH response to GnRH (56, 57).

Clomiphene test (or Clomiphene challenge test) is a dynamic type of testing that can detect some cases of decreased ovarian reserve, which still show normal levels of FSH day 3 of the menstrual cycle.

EFORT-test (exogenous FSH ovarian reserve test) and GAST test (Gonadotrophin analogue stimulating test- or ovarian response to the use of GnRH agonists) is type of testing usually used for prediction of patients response during ovarian stimulation prior to IVF. It is effective and simple method for determing good and poor responders in IVF (58, 59, 60, 61).

The quality and quantity of oocytes decreases rapidly from 38 years of age.

From 44 years onwards, with the help of IVF techniques and using the woman own oocytes, the chance for pregnancy almost does not exist. In fact, if using oocytes of women older than 44 years, the chances of success are below 2% per attempt. Oocyte quality is passed on to the quality of the embryo, which is by far the most important factor that determines the IVF procedures success (62).

THE AIM OF WORK

The aim of this work is to determine the different methods of ART in the treatment of infertility with women at the age from 40 to 45 and the contribution to the development of the strategy for the successful outcome of IVF with the patients of this age.

PATIENTS AND METHODS

This study is conducted on KGA KCS, and the data are collected during the traetment of infertile female patients from the department for *In vitro fertiliztion* in the period from 2008 to 2011.Patients were included in the study according to certain criteria: the age of the patient, BMI, the cause of fertility, if there were previous ART procedures and which ones, the lenght of stimulation, the type of stimulating process, the number of aspirated follicle, the number of obtained oocytes, the number of embryos as well as the procedure of formation of embryos (ICSI or IVF), embryotransfer and the chracteristics of the returned embryos. The obtained data were statistically processesed by using the SPSS program package and represented both graphically and tabularly

a) The patients were divided into six groups according to their age (40, 41, 42, 43, 44, i 45). Apart from the basic hormonal status, the level of FSH hormone on the 2nd or 3rd day of the menstrual period was tested seperately.
b) Based on the used protocol of the controlled stimulation of the patient's ovary (agonists or anti-agonists GnRH and gonadotropins by short or long protocol) the results after the stimulation were followed and compared in terms of determining: the number of obtained oocytes and their quality (M II, M I, germinative vesicles -GV or the degenerated oocyte), the rate of fertilization, the success of embryotransfers and the number of returned embryos (with the review of the quality of embryos), the rate of pregnancies and the rate of childbirths.

Table 1: The characteristics of the cycle with embryotransfer with women older than 40.

parameter	value
Age	42,04±0,1
How many ampoules of gonadotropine were used	38,61±1
The number of obtained oocytes	4,87±0,1
% MII oocytes	83,5
% 2PN	56,22
The number of embryos in ET totally	118
% returned embryos	2,23

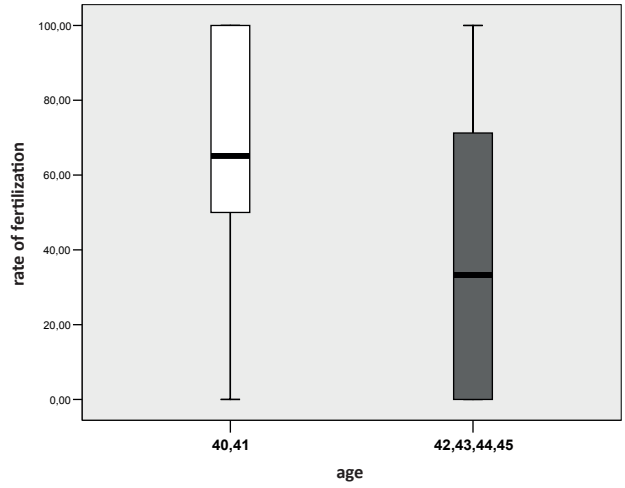
THE RESULTS

The outcome of ART technique with 68 patients of the age from 40 to 45 who were treated on KGA KCS during the period from 2008 to 2011 was analysed. Out of 68 analysed cycles of stimulation, the embryotransfer was done with 51 patients ie. in 75% of cases, and the cycle was cancelled – terminated with 17 patients (25%) for the following reasons: in 8 cycles there were no grown oocytes, in 6 there was no fertilization, in 2 there was a bad growth of embryos after the fertilization and one was cancelled due to extremely difficult embryotransfer so the embryos were frozen. (**Table 1.)**

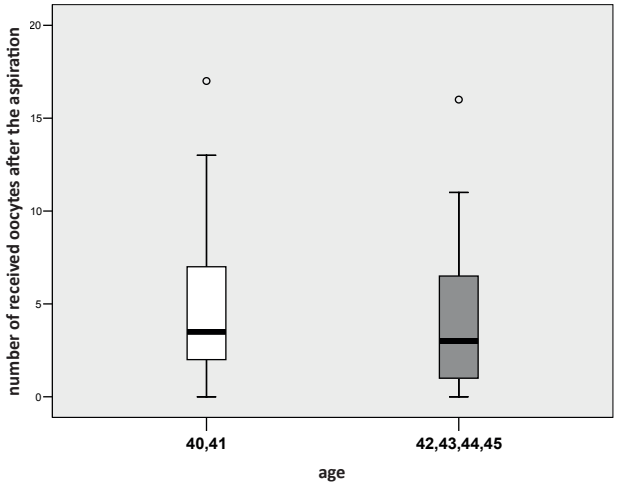
Out of 68 patients included in the research, for only 5 the clinical pregnancy was determined thereof one had ectopic pregnancy, with one patient the prenatal diagnostics that is the biopsy of chorionic cups (CVS) showed SY Down so the pregnancy was terminated and with one patient the pregnancy was cancelled before the term due to premature delivery in 8th lunar month. 51 embryotransfers were done, 5 embryos were returned with 2 patients, 4 embryos were returned with 7 patients, and 1 to 3 embryos were returned with all the others. Based on these data we have the rate of pregnancies per transfer in this research to be 17,65%, and the rate of childbirths per transfer 5,88%.

Considering the fact that the number of patients who belong to a certain age group (eg. 40, 41, 42 ...) is different, and in order to get a better interpretation of the statistical values, the patients were divided in two age groups: 1st group 40 and 41years old and 2nd group 42 and more. If we now consider the rate of fertilization of the obtained egg cells with the patients belonging to groups 1 and 2, we get a statistically significant difference. So there is a higher rate of fertilization with women aged 40 and 41 (median 65,16%) than with women older than 42 (median 33,33%) (Mann-Whitney U test (Z= -2,82, p=0,005<0,05) (**graph 1)**.

Graph 1



Graph 2



The statistical tests were done due to determining the normal distribution, and after that the non-parameter analises were done (Shapiro-Wilk).

Statistically there is a significant difference in the rate of fertilization with women aged 40 and 41 (median 65,16%) and with women aged 42-45 (median 33,33%).

If we analyse the success of the process of stimulating the ovulation ie analyse the number of received oocytes after the aspiration with the patients of these two age groups we come to the result that there is no significant difference: the group 40-41 (median 3,5) and the group 42-45 (median is 3). The analyses were done by Mann-Whitney U test (Z= -0,98, p=0,328>0,05) which showed that there is no statistically significant difference in the number of aspirated oocytes (**graph 2**)

The average values of the number of aspirated oocytes after the stimulation with the patients aged 40-41 (median is 3,5) and patients aged 42-45 (median is 3) are very similar.

If we want to prove more precisely the success of the applied ART techniques with the patients divided according to their age, it is necessary to analyse the percent of the obtained mature oocytes (MII) after the stimulation ie.one of the parameters that shows the quality of the egg cell.

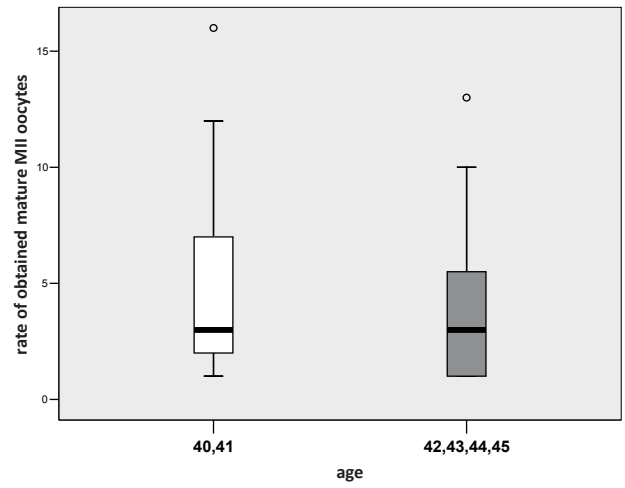
Mann-Whitney U test (Z= -0,776, p=0,438>0,05) was implemented which showed that there is no significan difference in the number of obtained mature MII oocytes with the patients aged 40-41 (median is 3) and patients aged 42-45 (median is 3) **(graph 3)**.

The implemented Mann-Whitney U test (Z= -0,776, p=0,438>0,05) did not show the significant differences in the number of obtained MII oocytes with the patients aged 40-41 and patients aged 42-45. Medians are the same and are 3.

By analysing the very procedure of stimulation, that is how many days of stimulation by gonadotropines are necessary to have a satisfyng result as well as the number of used ampoules of gonadotropinesthat were necessary for a successful stimulation , we get the results that show that there is no significant difference between these two age groups.

T test (t= -0,864, d=64,61, p=0,391>0,05) was implemented which showed that there is no statistically significant difference in the number of days of stimulation with the patients aged 40-41 (10,25±1,74) and patients aged 42-45 (10,61±1,70) **(Table 2), (Graph 4)**.

Graph 3



Graph 4

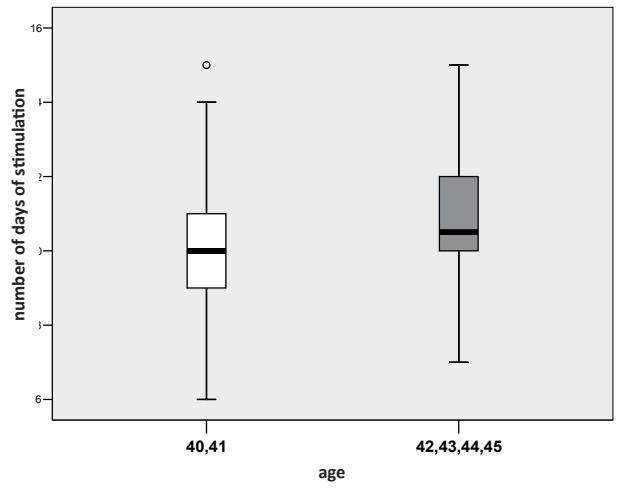


Table 2: The results of the t test which analysed the number of days of stimulation by gonadotropines related to the age of the patients in research – patients divided in two aged groups: 40-41 and 42-45.

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
number of days of stimulation	Equal variances assumed	,096	,757	-,866	66	,390	-,361	,417	-1,194	,472
	Equal variances not assumed			-,864	64,613	,391	-,361	,418	-1,196	,473

The results of the t test which analysed the number of days of stimulation by gonadotropines related to the age of the patients in research – patients divided in two aged groups: 40-41 and 42-45.

T test (t= -0,864, d=64,61, p=0,391>0,05) was implemented which showed that there is no statistically significant difference in the number of days of stimulation with patients aged 40-41 (10,25±1,74) and patients aged 42-45 (10,61±1,70).

By analysing the data obtained after the stimulation of ovulation with the mentioned age groups of the patients, we get the results which show that there is no statistically significant difference in the number of used ampoules of gonadotropines for stimulation (p>0,05). The results were tested by Mann-Whitney U test (Z= -0,025, p=0,980>0,05) **(Table 3)** which showed that there is no significant difference in the number of used ampoules of gonadotropines with patients aged 40-41 (median is 36) and patients aged 42-45 (median is 35,5) **(Graph 5)**.

There is no statistically significant difference in the number of used ampoules of gonadotropines with patients aged 40-41 (median is 36) and patients aged 42-45 (median is 35.5)

By analysing the number of obtained embrios per each cycle with the patients older than 40 (divided into the same categories ie 40-41 and 42-45), we get the statistically significant difference, ie there is a better result of IVF tehniques with the younger patients than with the older ones, which corresponds to almost all literary data.

The statistical significance was determined by applying the Mann-Whitney U test. The test results are (Z= -2,140, p=0,032<0,05) **(Table 4)** and they point out the significant differences in the number of obtained embrios with the pa-

Test Statistics[a]

	number of ampoules
Mann-Whitney U	574,000
Wilcoxon W	1102,000
Z	-,025
Asymp. Sig. (2-tailed)	,980

Grouping Variable: age22

Table 3: The results of the statistical analyses of Mann-Whitney U test (p=0,980>0,05)

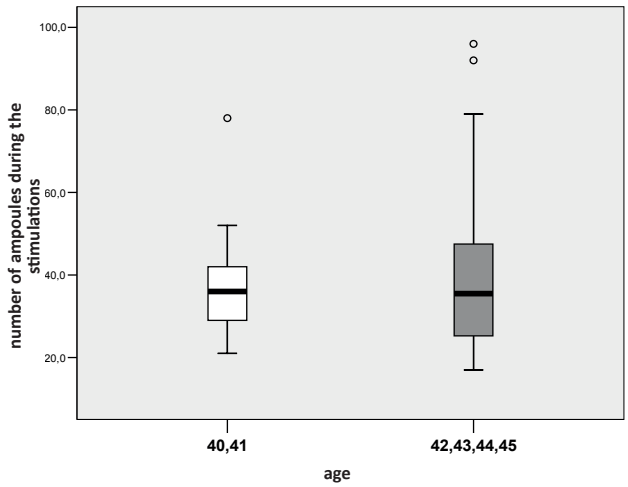
Test Statistics[a]

	number of embryos
Mann-Whitney U	320,000
Wilcoxon W	816,000
Z	-2,140
Asymp. Sig. (2-tailed)	,032

Grouping Variable: age22

Table 4: The statistical significance p=0,032<0,05 in the number of obtained embrios with patients of two age groups.

Graph 5



Graph 6

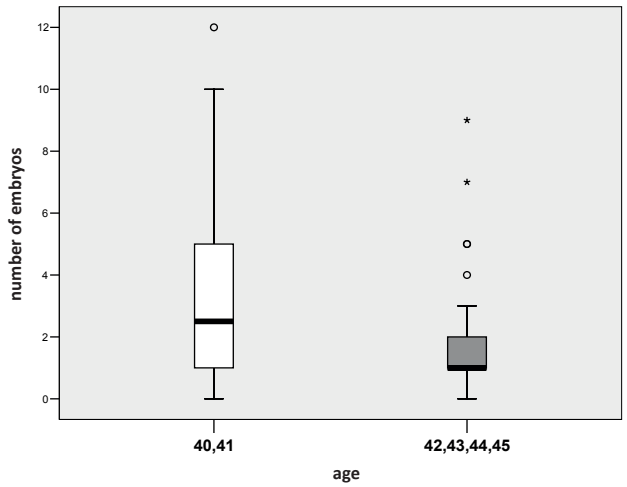


Table 5: The corellation tests which show the statistically significant negative corellation between the patients'age and the number of embryos.

Correlations

			starost	stopa_ fertilizacije	broj_ embriona
Spearman's rho	age	Correlation Coefficient	1,000	-,232	-,258*
		Sig. (2-tailed)	.	,057	,045
		N	68	68	61
	rate of fertilization	Correlation Coefficient	-,232	1,000	,467**
		Sig. (2-tailed)	,057	.	,000
		N	68	68	61
	number of embryos	Correlation Coefficient	-,258*	,467**	1,000
		Sig. (2-tailed)	,045	,000	.
		N	61	61	61

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

tients aged 40-41 (median is 2,5) and patients aged 42-45 (median is 1) **(Graph 6)**.

The median of the number of obtained embrios with patients aged 40-41 is 2.5, and with the patients aged 42-45 the median is 1 – statistically significant difference.

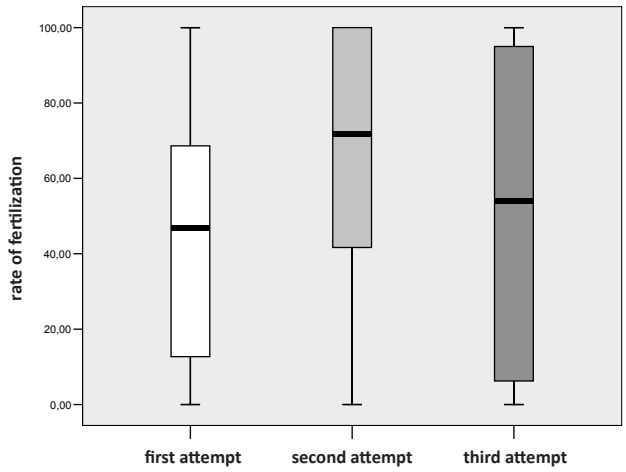
If we examine the obtained result by corellation tests, we also get the statistically significant negative corellation between the number of obtained embrios after the applied ART techniques and the age of the patients, ie the older group of patients the less number of embrios (Spearmans'rho = -0,258, p=0,045<0,05).

If we apply the corellation tests on the fertilization rate and the patients'age, we get the values which are near the significance (Spearmans'rho = -0,232, p=0,057>0,05). The results of the tests are shown in **Table 5.**

If we use the statistical analyses to test the fertilization rate related to the fact that this is the first attempt of in vitro fertilization or second, third or more, we obtain the result which points out that there is no significant difference (χ^2=2,505, d=2, p=0,286>0,05). **(Graph 7).**

Kruskal-Wallis test was implemented (χ^2=2,505, d=2, p=0,286>0,05) which showed that there is no significant difference in the fertilization rate with the patients with the first attempt (median is 46.88%) , second attempt (median is 71.82%) or more than two fertilization attempts (median is 53.98%).

Graph 7



DISCUSSION

The results of this clinical study point out that the characteristics and outcomes with the women aged 42 and older are different and mostly much worse comparing to the women aged 40 and 41 and they mostly match the majority of data available in the literature. The thing that makes the interpretation of the obtained results difficult is the relatively small number of patients involved in the research comparing to the literary data and uneven number of patients in each age group (40, 41, 42, 43, 44 and 45) so in order to obtain the decisive results during the statistical testing we divided the patients into two age groups 40 and 41 years old and 42-45 years old.

The rate of pregnancies per transfer in this study is 17.65%, as is shown in majority of studies which examined the success of ART techniques with women older than 40 where results vary from 11.6 % to 18.2 % (63, 64, 65). The rate of childbirths per transfer in this study is 5.88% whereas in the scientific literature it is 7-8.5% (66, 67). There were no multiple pregnancies and clinical pregnacies with the patients older than 43. The data from this study confirm the so far conclusions from the literature that the fertilization rate with the patients of younger age is significantly higher comparing to the older patients (in this case the statistically significant difference in the fertilization rate between age groups of 40-41 and 42-45).

However if we analyse the very process of stimulation of ovulation ie whether the short or long protocole is used, the number of days of stimulation as well as the number of ampoules of gonadotropines used for adequate stimulation of ovulation with these patients, there is a difference, but not statistically significant difference. We also do not find the significance by analysing the aspirated oocytes in these two age categories or

the number of mature MII oocytes in the total number of aspirated oocytes related to the age. All these data speak in favour taht it is possible to have a satisfying result with older patients after the stimulation in terms of good reaction of ovary on the applied stimulation protocole but the further outcome of the procedure, ie whether the clinical pregnancy and the childbirth will happen or not depend much more from the quality of the egg cell which is weakened in the older age, and which is confirmed by this study.

The statistically significan difference is also confirmed in the number of obtained embrios with women aged 40-41 and 42-45which is also proved in majority of cases in similar studies that involved much bigger number of examined patients (63).

When we analyse the success of ART techniques with women of older age, in many countries the number of embryos taht are returned by transfer is not limited (68). In this study, only one patient had 5 embryos returned, and 7 patients had 4 embryos each, and the rest 42 patients 1-3 embryos. There is no significance in the pregnancy rate or childbirths per transfer related to the number of returned embryos. Similar results were obtained in the majoruty of other recent studies (63, 64, 68, 69).

CONCLUSION

With the patients aged 40-41 we can expect relatively good response of the ovary after the stimulation by gonadotropine in terms of the number of obtained oocytes and the number of mature MII oocytes depending on the level of FSH and AMH hormones. However the successful outcome of ART techniques mainly depends on not only the number but the quality of obtained egg cells which definitely recedes with ageing.

The pregnancy rate and the childbirth rate with the patients older than 40 is very low, although comparable to the data from the scientific literature and speaks in favour of the fact that the success of ART techniques is very modest with women older than 44.

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SERBIAN TRANSLATION AND CROSS-CULTURAL VALIDATION OF THE QUESTIONNAIRE FOR ASSESSING PATIENT SATISFACTION WITH ENDOSCOPIC EXAMINATION OF THE DIGESTIVE TRACT

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SRPSKI PREVOD I MEĐUKULTURALNA VALIDACIJA UPITNIKA ZA PROCENU ZADOVOLJSTVA KOD PACIJENATA KOJI SU PODVRGNUTI GASTROINTESTINALNOJ ENDOSKOPIJI

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ABSTRACT

Patient satisfaction is a key indicator to assess the quality of gastrointestinal endoscopy. The aim of this study was to examine the Serbian translation and cross-cultural validation of the questionnaire for the assessment of satisfaction in patients who underwent gastrointestinal endoscopy.

After obtaining the consent of the author of the original questionnaire, translation and cross-cultural validation of the GESQ (Gastrointestinal Endoscopy Satisfaction Questionnaire) were carried out in accordance with the conductors of the International Society for Pharmacoeconomics and Outcomes Research (ISPOR). The study was conducted in the Center for Gastroenterohepatology (GEH) of the Kragujevac Clinical Center and included 165 patients. The reliability of the Serbian translation of the GESQ was estimated by calculating Cronbach's alpha for the whole questionnaire in order to implement the structural validation. The overall score of the questionnaire was compared and correlated with the total scores on the Short Subjective Well-being scale (KSB) and visual analogue scale (VAS), which were administered to the same patients.

The Serbian translation of the GESQ showed high reliability with a Cronbach's alpha coefficient of 0.763, good structure and homogeneity by randomly sharing the questionnaire into two parts. Exploratory factor analysis indicated the existence of four factors that explain 57.200% of the variability.

he Serbian version of the GESQ showed similar psychometric characteristics to the original English questionnaire, with a similar factor structure, and represented a valid, reliable and acceptable tool for the assessment of patient satisfaction with the endoscopic examination of the digestive tract.

Keywords: *gastrointestinal endoscopy, questionnaire, patient satisfaction, translation, cross-cultural validation.*

SAŽETAK

Zadovoljstvo pacijenata je ključan indikator za procenu kvaliteta gastrointestinalne endoskopije. Cilj ove studije je bio prevod i međukulturalna validacija upitnika za procenu zadovoljstva kod pacijenata koji su podvrgnuti gastrointestinalnoj endoskopiji.

Nakon dobijanja saglasnosti autora originalnog upitnika, prevod i međukulturalna validacija GESQ upitnika izvršena je u skladu sa vodičima Međunarodnog društva za farmakoekonomiju i ishode istraživanja (ISPOR). Studija je sprovedena u Centru za Gastroenterohepatologiju (GEH) Kliničkog centra Kragujevac i obuhvatila je 165 pacijenata. Pouzdanost srpskog prevoda GESQ upitnika je procenjena izračunavanjem parametra Cronbach's alpha za upitnik u celini u cilju sprovođenja konstruktivne validacije skale.

Njen ukupni skor je upoređen i koreliran sa ukupnim skorom Kratke skale subketivnog blagostanja (KSB) i vizuelno analognom skalom (VAS) koje su sprovedene na istim pacijentima.

Srpski prevod GESQ upitnika pokazao je visoku pouzdanost sa vrednošću Cronbach's alpha koeficijenta od 0,763, dobru konstrukciju i homogenost pitanja prilikom nasumičnog deljenja upitnika na dva dela. Eksplorativna faktorska analiza je ukazala na postojanje četiri faktora koja objašnjavaju 57,200 % varijabilnosti.

Srpska verzija GESQ upitnika, pokazuje slične psihometrijske karakteristike kao i originalni upitnik na engleskom jeziku sa sličnom faktorskom strukturom i predstalja validno, pouzdano i prihvatljivo sredstvo za merenje zadovoljstva pacijenata sa endoskopskim pregledima digestivnog trakta.

Ključne reči: *gastrointestinalna endoskopija, upitnik, zadovoljstvo pacijenata, prevod, međukulturalna validacija*

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INTRODUCTION

Endoscopic examinations of the digestive tract are commonly used safe and secure methods for the diagnosis and treatment of the gastrointestinal tract diseases (1,2). Procedures include diagnostic or therapeutic upper and lower endoscopy (3). As invasive procedures, these examinations are not without risks and risk of complications (1,4,5). Examinations can be performed with or without anaesthesia and can often be very unpleasant and painful for patients (2,6).

In today's health care system, a lot of attention has turned to patients' satisfaction with different diagnostic and therapeutic procedures. Patient satisfaction has become a key indicator of quality measures in gastrointestinal endoscopy. The European and American Society for Gastrointestinal Endoscopy gave the recommendation for the routine collection of quality indicators which include the satisfaction of patients (7-9).

Previous studies showed that patients who experienced complications during or after endoscopy were less satisfied with these procedures that and that they rarely decided to repeat this type of examination or give approval for their implementation (3). Fear, anxiety and feelings of shame may affect the submission of the examination and complicate the communication between the patient and endoscopic team. These problems will increase the dissatisfaction of the patient and may lead to possible injury and inability to complete the examination (1,7,10,11). Previous studies have indicated that approximately 5% of patients refused a proposed endoscopic examination, and an additional 10% required persuasion to undergo the examination. While some studies indicate that nearly 40% of these patients have difficulty tolerating examinations, approximately 10% of patients experience severe discomfort during the examination (5,12,13). The most common reasons for refusal of these procedures are the fear of pain and a feeling of shame. The pain intensity is associated with a variety of factors and can cause immediate and long-term adverse effects. Effects of acute pain are comprised of a variety of emotional, physical and psychological events. Fear is a normal emotional response to a real threat and is recognized as truth by individuals, while anxiety represents a state of mind that is non-rational and characterized by a sense of insecurity and the presence of various neurovegetative symptoms (14). Patients have expressed fear of the examination and possible complications more often than fear related with a possible diagnosis (15). Previous studies indicated that pain that occurred during and after completion of the examination had a lot of influence on the tolerance of examination, and was the most important factor related with patients' decision to repeat this type of examinations (16-18).

In previous studies, instruments which were used to measure the satisfaction of patients failed to show satisfactory reliability and validity (7). The modified GHAA-9 questionnaire was recommended by the American Society for Gastrointestinal Endoscopy for the assessment of satisfaction and did not include all the necessary factors for evaluation of patient satisfaction (3,7,19). Some other instruments are mainly used to examine factors affecting the toleration of the examination (2-4,12,14,16,17,20). In the Serbian language, there is no validated questionnaire for the assessment of patient satisfaction with the endoscopic examination of the digestive tract.

The aim of this study was to examine the Serbian translation and cross-cultural validation of the questionnaire for assessing patient satisfaction with the endoscopic examination of the digestive tract. To achieve better cooperation of patients with endoscopic teams, preventative measures were be applied, which increased patient satisfaction and significantly improve the diagnostic and therapeutic procedures.

MATERIALS AND METHODS

Serbian Translation and cross-cultural validation of the questionnaire

Serbian translation and cross-cultural validation of the GESQ (Gastrointestinal Endoscopy Satisfaction Questionnaire) was carried out in accordance with the recommendations of the International Society for Pharmacoeconomics and Outcomes Research (ISPOR) (20). Permission for translation and cross-cultural validation of the GESQ from English to Serbian has been obtained by the author of the original questionnaire: Professor Hayley Hutchings from College of Medicine, Swansea University, United Kingdom. The original GESQ (version 2) was developed and validated in the UK (7). This questionnaire was first translated to Serbian by two independent translators who were not members of the research team. One of the translators was Ana Braković, a lecturer of English at the Medical School in Kragujevac, and the other was Biljana Jelić, a lecturer of English at the Polytechnic School in Kragujevac. These lecturers translated the questionnaire independently of each other, and then the translations were combined to create one version in Serbian. The combined Serbian version was then translated back to English by Dr. Marko Babić, a general practitioner, who is a native English speaker and a citizen of United States of America. When translating back to English, Dr. Babić was not aware of the original English version of the questionnaire. The back-translation to English was then compared with the original English version of the questionnaire. Additionally, the translation was sent for review to the author of the original questionnaire, Professor Hayley Hutchings. Comparisons of all versions were performed, and the necessary corrections were introduced, and linguistic errors were checked. The authors' precision and clarity of the questions was considered, including whether the questions referred to the wrong answer and whether it is necessary that respondents have clinical knowledge in order to provide the answers to the

GESQ

This survey was made for the prupose of assesing YOUR personal views after having an endoscopic procedure. There are no correct or wrong answers to the following questions: just put a cross in the box that best describes how you think. Your answers will be confidential, and won't influence the way you will be treated in any way. The information will be used to determinate how many people were satisifeid with their endoscopy, and to improve the endoscopy service.

1.How easy was it for you to understand the information that was sent to you before your endoscopy?

Very easy ☐ **Easy** ☐ **Fair** ☐ **Difficult** ☐ **Very difficult** ☐

2.Was the information given to you before your endoscopy appointment useful in answering any of your questions?

Very useful ☐ **Useful** ☐ **Fair** ☐ **Not very useful** ☐ **Not at all useful** ☐

3.Before preforming your endoscopy, how much opportunity did you have to ask questions about the endoscopy procedure?

Much ☐ **A little** ☐ **Not at all** ☐

4.How easy was for you to understand the explanation of given to you before your endoscopy?

Very easy ☐ **Easy** ☐ **Fair** ☐ **Difficult** ☐ **Very difficult** ☐

5.Was the explanation you received before your endoscopy helpful in answering your questions?

Very useful ☐ **Useful** ☐ **Fair** ☐ **Not very useful** ☐ **Not at all useful** ☐

6.How would you grade the communication skills (e.g. courtesy, respect, sensitivity, friendliness) of the person who performed your endoscopy?

Very poor ☐ **Poor** ☐ **Fair** ☐ **Good** ☐ **Very good** ☐

7.How would you grade the technical skills (eg. thoroughness, carefulness, competence) of the person who performed your endoscopy?

Very poor ☐ **Poor** ☐ **Fair** ☐ **Good** ☐ **Very good** ☐

8.How would you grade the communication skills (eg. courtesy, respect, sensitivity, friendliness) of the other staff in the endoscopy unit?

Very poor ☐ **Poor** ☐ **Fair** ☐ **Good** ☐ **Very good** ☐

9.How much discomfort did you feel during your endoscopy?

Very much ☐ **Much** ☐ **Fair** ☐ **Little** ☐ **None** ☐

10.How much pain did you experience during your endoscopy?

Very much ☐ **Much** ☐ **Fair** ☐ **Little** ☐ **None** ☐

11.How much discomfort did you experience after your endoscopy?

Very much ☐ **Much** ☐ **Fair** ☐ **Little** ☐ **None** ☐

12.How much pain did you experience after your endoscopy?

Very much ☐ **Much** ☐ **Fair** ☐ **Little** ☐ **None** ☐

13.After you had your endoscopy, how much opportunity did you have to ask questions about the findings?

Very much ☐ **A little** ☐ **None** ☐

14.After you had your endoscopy, how much explanation of the findings did you receive?

Too much ☐ **About right** ☐ **Not enough** ☐

If you did not receive an explanation, then please go directly to question 21.

15.How easy was it for you to understand the explanation given to you after your endoscopy?

Very easy ☐ **Easy** ☐ **Fair** ☐ **Difficult** ☐ **Very difficult** ☐

16.Was the explanation given to you after your endoscopy useful in answering your questions?

Very useful ☐ **Useful** ☐ **Fair** ☐ **Not very useful** ☐ **Not at all useful** ☐

17.Overall, satisfied were you with your endoscopy?

Very satisfied ☐ **Satisfied** ☐ **Neither satisfied nor dissatisfied** ☐ **Dissatisfied** ☐ **Very dissatisfied** ☐

18.If, in the future, you have another endoscopy, how satisfied would you be, if it was performend by the same person?

Very satisfied ☐ **Satisfied** ☐ **Neither satisfied nor dissatisfied** ☐ **Dissatisfied** ☐ **Very dissatisfied** ☐

19.How would you grade the overall reputation of the hospital?

Very poor ☐ **Poor** ☐ **Fair** ☐ **Good** ☐ **Very good** ☐

questions. Compliance with these principles was intended to achieve similar meaning of questions between the original and translated versions of the questionnaire.

The final version of the Serbian translation of the GESQ was then tested in a pilot study on 20 respondents in order to determine the existence of any ambiguity in the questions and to identify additional questions that patients think are relevant to the assessment of their satisfaction with endoscopy. We examined whether all the questions were sufficiently clear, precise and comprehensible, and whether a correction of some questions in the questionnaire was needed. After the pilot study implementation, the necessary changes were made, and then the final version of the scale in Serbian was multiplied and prepared for testing reliability. Data obtained from the pilot study were not taken into account in the statistical analysis.

Population and sample

The final version of the Serbian GESQ was tested on patients who underwent gastrointestinal endoscopy at the Center for GEH at the Clinical Center Kragujevac. The study involved 165 patients and lasted two months, from 02.10.2016. to 10.04.2016. This study was conducted with the approval of the Ethics Committee of the Clinical Center Kragujevac. The questionnaire was offered to all patients who were referred for endoscopic examination in the Centre for GEH of Clinical Center Kragujevac to complete. After endoscopic examination the patients who agreed to participate in the study were given a questionnaire for assessing patient satisfaction with endoscopic examinations of the digestive tract. Before completing the questionnaire, patients got the informer and gave informed consent. After the completion of examinations, study participants were interviewed by the principal investigator, who then filled out the questionnaires. Question number 18 (How would you rate the comfort in the recovery area in the endoscopy suite?) was omitted from the questionnaire, since the study involved patients who had an examination done without anaesthesia, so there was no need to lie down in the recovery room where patients usually lie while recovering from endoscopy with anaesthesia. Patients who are hospitalized in the Center for GEH also did not lie in this room, but rather in their room at the hospital at the centre for GEH after the examination. Question number 15 (Did the person who performed your endoscopy give you the explanation?) was also omitted out from the questionnaire because patients who responded to this question with a NO did not complete the questionnaire and were excluded from the study. The study included all patients who were referred for endoscopic examination in the Centre for GEH, of Clinical Center Kragujevac, 18 years or older, who agreed to participate in the study and had the ability to understand and complete the questionnaire. In the study, respondents who did not answer all the questions in the questionnaire, those who refused to participate in the study, and patients with diagnosed psychiatric illness, dementia, neoplastic or other concomitant serious illness were excluded. Assessment of patient satisfaction with the specified questionnaire was conducted only among those patients in whom the examination was carried out without anaesthesia. Those patients who had examination done with anaesthesia were excluded from the study, because anaesthesia might influence their satisfaction assessment.

The original GESQ (version 2) consists of 21 questions. The questionnaire contains questions that were formulated in the form of a five-point and three-point Likert scale. The original questionnaire also contains dichotomous questions with the options YES and NO (7). Two questions are different than all others (I'm always willing to admit it when I make a mistake? I have always had trust in my doctors?) and were selected as socially desirable when offered questions on a five-point Likert scale. Socio-demographic questions offered relating to sex, place of residence, education, marital status, habits of respondents are an integral part of the questionnaire. When filling out the questionnaire respondents enter their first and last name, age and date of completing the questionnaire. This questionnaire contains information about the type of examination that was carried out, their urgency, the symptoms for which patients are referred for examination, etc. As an addition to this questionnaire, the VAS was used. On a 10-cm-long line, respondents mark the level of satisfaction with the endoscopic examination of the digestive tract. By marking the cross on the line, the respondents expressed their satisfaction. Satisfaction was then evaluated by measuring the distance from the left end of the scale, which was equal to 0, to the crosses. Satisfaction was quantified by values from 0 to 10, where 0 indicated dissatisfaction with examinations, and a maximum of 10 indicated maximum satisfaction with preformed examinations. With the first version of the questionnaire, patients responded to three open questions about whether they understood all the questions, what issues are less understood, whether there was a question which they did not want to answer, and why.

Reliability tests

Reliability of the Serbian translation of the GESQ was tested using two methods. Internal consistency was assessed by calculating the Cronbach's alpha parameter for the whole questionnaire. By the second split-half method the questionnaire was split into two equal parts with the same number of questions. Cronbach's alpha was calculated for each of the two equal parts. Using the parameters for both parts, the number of questions in both parts and the average correlation between the questions in both parts of the original questionnaire, the Spearman-Brown coefficient for the whole questionnaire was calculated through the Spearman-Brown's predictive formula (22). The collected data were statistically analysed by SPSS 18.00 for Windows (23).

Factor analysis

Eligibility of the questionnaire and sample factor analysis were tested by the Kaiser-Meyer-Olkin method that measures the adequacy of sampling and by Bartlett's sphericity test. Then, the factors were initially extracted without rotation, provided that their Eigenvalues were greater than 1, using a Scree plot (extracting factors which were above the fracture on the chart). Referential orthogonal rotation axis was performed by the Varimax method, and factors' extraction were performed by the same criteria as for unrotated solutions.

Validity

Content validation of the Serbian translation of the GESQ was tested by a three-member committee of the Center for GEH of Clinical Center Kragujevac. To implement constructive validation of the Serbian translation of the GESQ, its overall score was compared and correlated with the total score on the KSB scale, which contained 8 questions and offered answers on the Likert scale of 1 to 5 (which are categorized from 1- completely disagree to 5-strongly agree), and the VAS scale, which was implemented on the same subjects (3,7,24). Before using the scale on the same subjects, permission for use was granted by the original author - Assistant Professor Veljko Jovanović from University of Novi Sad, Faculty of Philosophy, Department of Psychology. This scale was previously validated in a Serbian population.

RESULTS

Characteristics of the sample

In a study conducted in the city of Kragujevac 165 respondents participated. Of the total respondents, 13 respondents did not provide answers to all questions in the questionnaire, and they were excluded from the study. Statistical analysis included 152 respondents. Percentages of respondents by sex, age groups and mean value of VAS are shown in Table 1. The urgency, type examination carried out, symptoms for which the respondents were send to examination, and information on whether the examination was carried out for the first time or not are shown in Table 2.

Reliability analysis

The correlation matrix was built to indicate the correlation of mutual questions, and the correlation of each question with the remaining questions from the questionnaire. Questions 6, 7 and 20 were shown to have negative correlation. By inversion of the scores of questions 6, 7, 8 and 20, the correlation of these questions becomes positive.

Table 1: Percentage of respondents by sex, age groups, mean VAS.

	Respondents	
Sex	Male	52.6 %
	Female	47.4 %
Age group	18-29	15.1 %
	30-49	19.7 %
	50-65	45.4 %
	Over 65	19.7 %
Age	Age range	19-84
	Average	50.75 +/-15.206
VAS scale-mean	7.035 +/-1.2744.	

Table 2. Urgency of examination, type examination, symptoms, patients who were first sent for examination

Urgency examination	Urgent	131	86.2 %
	Rutine	21	13,8 %
Type examination	Gastroscopy	75	49.3 %
	Colonoscopy	71	46.7 %
	FRSS, Rectosygmoidoscopy	6	3.9 %
The symptoms for which the patient is sent for examination Gastroscopy	Dyspeptic symptomos	61	81.3%
	Weht loss, anemia, anorexia	14	18.7%
The symptoms for which the patient is sent for examination Colonscopy	Bleding per rectam	32	41.6 %
	Change in bowel habit	29	37.7 %
	Stomach pain, anemia	16	20.8 %
The first examination	Yes	65	42.8 %
	No	87	57.2 %

Descriptive statistics

The mean values of most questions were in the range of 2 to 4, except in the case of questions 3 and 13, which were 1.60 and 1.65. These questions were separated by their low variance. The value of Cronbach's coefficient for the entire questionnaire was 0.612. Then, the change of its value was analysed by the elimination of some questions in the questionnaire. By eliminating the remaining questions (questions number 15 and 18) Cronbach's coefficient has a final value of 0.763. The obtained definitive version of the questionnaire contains 19 questions, which were carried out for factor analysis. The questionnaire was then divided into two parts by the split-half method, and Cronbach's coefficient was determined for each part individually. Cronbach's coefficient values were 0.649 and 0.614. The correlation between these two parts was 0.570. After the distribution of the questionnaire into two parts, the Spearman-Brown coefficient for the whole questionnaire was calculated through the Spearman-Brown's predictive formula. The Spearman-Brown coefficient had value of 0.726. Factor analysis was conducted by the principal component analysis (PCA) method with the remaining 19 questions in the questionnaire. Prior to implementation of the PCA, the adequacy of data for the factor analysis was assessed. The value of the KMO test,

as an indicator of the adequacy of the sample was 0.788, which exceeded the recommended value of 0.6. Bartlett's test of sphericity reached statistical significance (p = 0.000), indicating that the factor analysis could be carried out. Principal components analysis revealed the presence of four factors which had an eigenvalue greater than 1. Extracting these four factors explained 28.573%, 12.605%, 9.102% and 6.921% of the variance. By examining the scree plot, the existence of a clear point of fracture after the fourth factor was confirmed, as shown in Figure 1. To make it easier to interpret these four factors, the Varimax orthogonal rotation method was conducted. These four factors explain 57.200% of the variance. Share of variance, the cumulative percentage of variance and eigenvalues of these four factors after the rotation are shown in Table 3. Table 4 shows the weight factor matrix after performing the rotation. The rotated solution revealed the existence of different structures, and that all four components have different factor weights.

The values of the factor weights in Table 4 suggest that the first factor includes five questions (questions 1, 2, 3, 4, 5), the second factor includes six questions (questions 13, 14, 16, 17, 18, 20), the third factor includes four questions (questions 6, 7, 8, 19) and the fourth factor includes four questions (questions 9, 10, 11, 12). Among the questions that belong to each factor, there is a connection. These questions explain the same phenomenon, and it is reasonable to assign the structure of the questionnaire in this way. Names of factors, questions that belong to them, Cronbach's alpha coefficient and mean value of the score of each factor with the total score of the questionnaire are presented in Table 5.

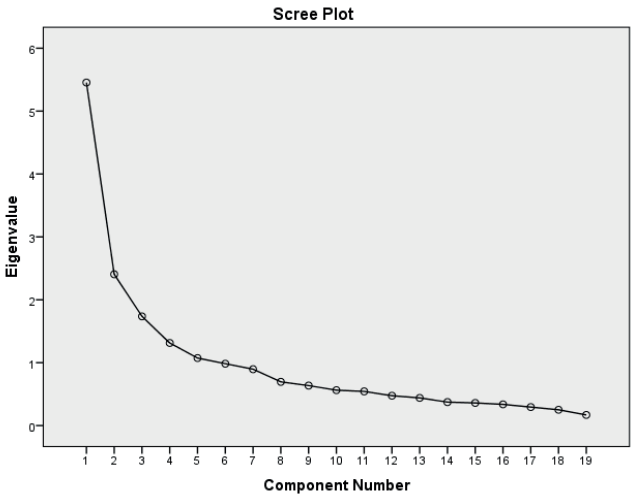

Figure 1. Scree plot

Table 3. Percentage of variance, the cumulative percentage of variance and value "eigenvalue" four factors after the rotation

Factors	„Eigenvalue"	Percentage of explained variance	The cumulative percentage of explained variance
1	3.273	17.224 %	17.224 %
2	2,932	15.434 %	32.557 %
3	2.446	12.873 %	45.530 %
4	2.217	11.670 %	57.200 %

Temporal stability

In this study, time stability of the translation was not tested (test-re-test method), because patients were supposed to undergo the same endoscopic examination twice at two different time points carried out by the same person and under the same conditions (same type, time of examination, and endoscopist). It was not feasible to contact people who live outside the city where the studies were conducted.

Table 4. Matrix factors weight after rotation

	Component			
	1	2	3	4
GESQ1	.576	.198	.362	.160
GESQ2	.803	.158	.088	-.049
GESQ3	.609	.253	-.080	-.219
GESQ4	.861	.167	.044	.101
GESQ5	.861	.160	.040	.000
GESQ6	.227	.218	.712	.023
GESQ7	.074	.145	.736	-.155
GESQ8	-.120	.107	.749	-.037
GESQ9	.043	.126	-.413	.595
GESQ10	-.031	-.068	.042	.767
GESQ11	.015	-.117	-.210	.660
GESQ12	-.021	-.067	.048	.742
GESQ13	.203	.692	.131	-.028
GESQ14	.038	.791	.086	.010
GESQ16	.181	.662	.322	.009
GESQ17	.238	.750	.196	.006
GESQ18	.362	.485	.237	-.249
GESQ19	.276	.347	.495	-.248
GESQ20	.169	.413	-.040	-.230

Divergent validity

As we did not have a questionnaire that would measure the related phenomena in this study, divergent validity was verified by respondents completing the questionnaire and scale to measure entirely different phenomena related to satisfaction with the endoscopic examination, such as the Short scale of subjective well-being (KSB) and the visual analogue scale (VAS). The correlation between the total scores of the questionnaire and these two scales was then examined. Divergent validity was tested using the non-

Table 5. Names of factors, questions pertaining to them, Cronbah's coefficient values and the mean value of each factor

	Questions	Cronbah coefficient	The mean valule of the score
Factor 1 Information before endoscopy	1. How easy to understand was the information that was sent to you before your endoscopy ? 2. Was the information sent to you before your endoscopy appointment useful in answering your questions? 3. Before you had your endoscopy, how much opportunity did you have to ask questions about the endoscopy procedure 4. How easy to understand was the explanation given to you before your endoscopy? 5. Was the explanation given to you before your endoscopy useful in answering your questions?	0,840	11,184
Factor 2 Information after endoscopy	13. After you had your endoscopy, how much opportunity did you have to ask questions about the findings? 14. After you had your endoscopy, how much explanation of the findings did you receive? 16. How easy to understand was the explanation given to you after your endoscopy? 17. Was the explanation given to you after your endoscopy useful in answering your questions? 18. Overall, how satisfied are you with your endoscopy? 20. How would you rate the overall reputation of the hospital?	0,767	13,348
Factor 3 Communicative and technical skills of the staff	6. How would you rate the communication skills (eg. courtesy, respect, sensitivity, friendliness) of the person who performed your endoscopy? 7. How would you rate the technical skills (eg. thoroughness, carefulness, competence) of the person who performed your endoscopy? 8. How would you rate the communication skills (eg. courtesy, respect, sensitivity, friendliness) of the other staff in the endoscopy unit? 19. If, in the future, you have another endoscopy, how satisfied would you be to have it done by the same person?	0,728	8,263
Factor 4 Pain or discomfort during or after endocopy	9. How much discomfort did you experience during your endoscopy? 10. How much pain did you experience during your endoscopy? 11. How much discomfort did you experience after your endoscopy? 12. How much pain did you experience after your endoscopy?	0,687	14,164

parametric correlation (Spearman correlation coefficient). Nonparametric correlation was chosen because, some of the scores did not follow a normal distribution.

Based on the correlation coefficient between the questionnaire and these two scales (r = -0.246, p = 0.02; r = -0,374, p = 0:00;), it can be concluded that there is not a high correlation between the questionnaire and these two scales, thereby supporting the divergent validity. The Multitrait-Multimethod correlation matrix is shown in Figure 2.

DISCUSSION

The final version of the GESQ for the assessment of patient satisfaction with endoscopic examinations of the digestive tract, for use in Serbian populations, contains 19 questions and shows high reliability, with a Cronbach's coefficient value of 0.763, and good structure and homogeneity of random questions while sharing the questionnaire into two parts. Exploratory factor analysis indicated

	GESQ questionare	KSB scale	VAS scale
GESQ questionare	1	-.246	-.374
KSB scale	-.246	1	.235
VAS scale	-.374	.235	1

Figure 2. Multi-trait, multi-method correlation matrix (nonparametric Spearman correlation)

the existence of four factors that explain 57.200% of the variability. The method of principal component analysis discovered four factors that are clinically relevant to the assessment of patient satisfaction with gastrointestinal endoscopy. The identified factors are as follows: the information that the participants get before endoscopy; the information that the participants get after endoscopy; communicative and technical skills of the staff; and pain and discomfort during and after endoscopy. These are the same factors that are defined by the author of the original questionnaire. Additionally, these subscales displayed similar alpha values as the subscales in the original questionnaire.

Receiving adequate information before endoscopic procedures can reduce the anxiety and fear in patients that are normally present prior to the implementation of these procedures. The study conducted by S. Pehlivan at al. showed that providing verbal or written information to patients before performing endoscopic examination significantly affects their submission to the examination, reduces anxiety and increases patient satisfaction with this kind of examination (1). Similar results were found in a study by M. Qureshi at al. (3). In our centre, before performing invasive endoscopic procedures, patients receive written information about the proposed procedure, and then patients give their written consent to undergo the procedure. Providing adequate information to patients is intended to reduce the level of anxiety, to relax patients and to answer their questions, doubts and fears (17).

A study conducted in England examined the experiences of patients endoscopic examinations and determined that respondents react differently upon completion of the examination and disclosure of the results. While patients low in pre-examination anxiety indicated satisfaction with the results of the examination and believed in the results, another group of patients, whichwho reported showed frustration and increased nervousness, did not believe in the results of the examinations (13).

A study by Sánchez del Río A et al. used a questionnaire that was approved by the American Society of Gastrointestinal Endoscopy (GHAA-9) and showed that patients who experienced these examinations for the first time could hardly estimate the technical skills of the staff, a possible cause of a stated lack of experience with patients undergoing endoscopic examinations. This study of a large number of participants was the first to encounter this type of examination (9). A study that used the same questionnaire and was carried out in Canada investigated the factors influencing patient satisfaction with endoscopic examinations and the factors that influenced patients' willingness to repeat the endoscopic examination. The study found that patients who positively evaluated the technical skills of the staff and who experienced less pain during the procedure, who were more willing to repeat endoscopic examination. Additionally, patients who had a positive assessment of the communicative skills of the staff and received quality information before and after the completion of the examination were more satisfied (19).

A study by Ussu VM et al. showed that the presence of pain during and after the completion of endoscopy had a substantial influence on the toleration of the examination and on patient satisfaction (4). A study by Campo R et al. indicated that having prior endoscopy can significantly affect the tolerance of examinations (5). Two studies conducted in Taiwan, which included respondents who had an upper endoscopy without sedation, found associations between negative experience with prior endoscopies and increased anxiety before the endoscopic examination as well as patient satisfaction (12,16). Similar results were obtained from a study conducted in Germany on patients who were included in the screening programme for colorectal cancer, where researchers tried to establish a link between the avoidance of examinations and previous bad experiences (18). A study conducted in Iran came to the opposite conclusion, that prior experience of patients with endoscopic examinations is not a reliable parameter for assessing the tolerance of the forthcoming examinations (20).

Certainly, the use of conscious sedation may increase patient satisfaction with gastrointestinal endoscopy (18). While conscious sedation is routinely used during lower endoscopies, the role of conscious sedation during an upper endoscopy is still insufficiently defined (10). The use of conscious sedation varies from country to country, continent to continent, and even in different endoscopy centres within the same country. The use of conscious sedation is associated with higher costs, an extension of the duration of the procedure, the need for the monitoring of vital functions and an increasing incidence of complications (10,12,14,16,18).

In a study conducted in the UK, constructive validity of the questionnaire with other questionnaires and scales was not checked (7). This study tested the divergent validity of the questionnaire with the Short Subjective Well-being scale (KSB) and the VAS scale.

The main limitation of this study is that it was not possible to perform retesting of patients 15-30 days after completion of the endoscopic examination to determine the temporal stability of the questionnaire. This study included only patients who underwent examinations without anaesthesia, unlike other studies which included patients who underwent examinations without anaesthesia and patients who had an examination performed in analogosedation (4). This study was conducted in a single endoscopic centre on a smaller number of specific subjects. The limitation of this study is the absence of a second questionnaire that

measured the same phenomenon. Additionally, convergent validity of the questionnaire has not been checked; however, divergent validity of the structure was checked. When interpreting the results, it should be taken into account that the answers about patient satisfaction may have been influenced by other factors, such as information derived from relatives and friends as well as attitudes about health and medical information (9).

CONCLUSION

The Serbian translation of the GESQ shows similar psychometric characteristics to the original version of the questionnaire in English, with a similar factor structure. We believe that this questionnaire is a reliable tool for assessing patient satisfaction with endoscopic examinations of the digestive tract, and can be used as an indicator of quality for this purpose.

In future work, the questionnaire would be applied to endoscopist training to determine whether the advancement of endoscopic beginners affects the satisfaction of patients and whether there is a positive correlation with increased experience of endoscopist. The authors suggest that in the future, this questionnaire should be tested in different groups of patients who underwent other endoscopic procedures, such as endoscopic ultrasound (EUS) and endoscopic retrograde cholangiopancreatography (ERCP).

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INFLUENCE OF THE LOCAL AND SYSTEMIC OXIDATIVE STRESS ON PERIODONTITIS: ROLE OF ANTIOXIDANT THERAPY

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UTICAJ LOKALNOG I SISTEMSKOG OKSIDATIVNOG STRESA NA PERIODONTITIS: ULOGA ANTIOKSIDANTNE TERAPIJE

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ABSTRACT

This study was divided into experimental part of study which was conducted on 75 Wistar rats with the modeled periodontitis and and clinical part of research which included 106 patients with the chronic generalized periodontitis (CGP). The study established an importance of the oxidative stress (both local and systemic) in development and progress of the disease. It is found out that the saliva of rats with the modeled periodontitis there is the reliable increase in the content of total protein, alkaline phosphatase (ALP) and malonic dialdehyde (MDA) in 1.2, 2.6 and 2.8 times respectively, with the reduced activity of catalase in 2.5 times (all p<0.05). It is determined that the gingiva tissue of rats with the modeled periodontitis has the reduced contents of total protein, collagen, elastin and sulfated glycosaminoglycans in 2.8, 1.5, 1.6 and 1.3 times respectively (all p<0.05). It is proved that the antioxidant (AO) therapy normalizes in the rat saliva the content of protein and MDA (decrease in 1,2 and 1,8 times accordingly, p<0.05) and increases the activity of catalase (in 2.5 times, p<0,05). Calcium D_3 normalizes the protein content and activity of ALP (decrease in 1.2 and 1.5 times, respectively, p<0.05).

It is found out that the saliva of patients with CGP in the acute phase the content of protein, ALP and MDA increases in 1.9, 2.2 and 1.5 times accordingly (p<0,05) with the reduced catalase activity in 1,1 times (p<0,05). It is revealed that the inclusion of CGP patents in AO complex therapy results jointly with the best clinical effect in the more expressed reduction in generation of reactive oxygen species and lipid peroxidation and also the increased plasma APA.

Keywords: *periodontitis, chronic generalized periodontitis, oxidative stress, free radicals*

SAŽETAK

Ova studija podeljena je na eksperimentalni deo studije koji je sproveden na 75 Wistar pacova sa modelom periodontitisa i klinički deo istraživanja, koji uključuje 106 pacijenata sa hroničnim generalizovanim periodontitisom (CGP). Studija je utvrdila značaj oksidativnog stresa (kako lokalnog tako i sistemskog) u razvoju i napretku bolesti. Utvrđeno je da je pljuvačka pacova sa periodontitisom povećanog sadržaja ukupnih proteina, alkalne fosfataze (ALP) i malonil-dialdehida (MDA) i to 1,2, 2,6 i 2,8 puta postepeno, sa smanjenom aktivnošću katalaze 2,5 puta (p<0,05). Utvrđeno je da tkivo gingiva pacova sa periodontitisom ima smanjeni sadržaj ukupnih proteina, kolagena, elastina i sulfatnih glikozaminoglikana 2,8, 1,5, 1,6 i 1,3 puta (p<0,05).

Dokazano je da se antioksidativna terapija (AO) normalizuje u pljuvački sadržaj proteina i MDA (smanjuje se 1,2 i 1,8 puta, p <0,05) i povećava aktivnost katalaze (2,5 puta, p <0,05). Kalcijum D3 normalizuje sadržaj proteina i aktivnost ALP-a (smanjenje 1,2 i 1,5 puta, respektivno, p <0,05). Utvrđeno je da pluća pacijenata sa CGP u akutnoj fazi sadrže 1,9, 2,2 i 1,5 puta veci sadrzaj proteina, ALP i MDA (p <0,05) uz smanjenu aktivnost katalaze 1,1 puta (p<0,05). Dokazano je da uključivanje antioksidativne terapije pacijentima sa CGP rezultira dobrim kliničkim efektom sto se ogleda u redukciji reaktivnih kiseonicnih vrsta i peroksidacije lipida, kao i povećanog APA u plazmi.

Ključne reči: *periodontitis, hronični generalizovani periodontitis, oksidativni stres, slobodni radikali*

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INTRODUCTION

To date, one of the most pressing problems of dentistry is inflammatory periodontal diseases (1, 2). According to the WHO, these diseases are very aggressive and almost not treatable (3). Their incidence between the ages of 35 to 44 ranges from 55% to 95%, while for people over 45 years it makes from 95% to 99% (4, 5). Currently, there is large bank of accumulated extensive studies on periodontitis. However, despite the improvement of surgical, therapeutic and orthopedic techniques, periodontal diseases are steadily progressing (6, 7). In addition, it is still a controversial issue on the priority of etiology and pathogenesis. The researchers pay more and more attention to studying endogenous causes of periodontitis, as one of the most common forms of periodontal inflammatory diseases (8, 9). However, the impact of exogenous factors on the development of such diseases, in our view, tis insufficiently viewed in the literature.

The research relevance is determined by such factors such as the growth of morbidity (10), difficulty of early diagnostics, difficulty in reaching the stable remission, relationship of the general body condition and the condition of periodontal tissues (11).

One of the first hypotheses for the development of periodontitis was the hypothesis of nonspecific infection bloom (1960-1973) which implied that inflammatory periodontal diseases developed due to nonspecific infection of dental plaques microorganisms. It was assumed that periodontitis develops due to the increased number of dental plaque bacteria. However, experimentally it was discovered that not all experimental dogs evolved periodontitis, while there was the increasing dental plaques biomass (3-5).

In 1975 leadership the leadership was captured by the plaque specific hypothesis. It was discovered that on the tooth plaque there is the specific micro flora, the so-called periodontal pathogenic bacteria (2, 6). Thus, in 1985 year a new theory of periodontitis development was introduced, the theory of opportunistic infections.

To date, the generally accepted view is that under the influence of exogenous or endogenous factors there is an activation of microorganisms in the dental plaque replacing other bacteria (12, 13). Thus the changed body defenses, local changes in the acid-base equilibrium, hypoxia, anaerobic niche etc. form the environment which is convenient for reproduction of pathogenic bacteria causing the opportunistic infection activity and development of inflammatory periodontal diseases. The pathogenesis of periodontitis, as an inflammatory periodontal disease involves free-radical processes (FRP), oxygen (generation of active oxygen forms by leukocytes (GAROFL) and lipid (peroxidation of lipids) (POL) (7). However, the number of researches devoted to FRP study for the patients with periodontal diseases is few and conflicting (14-16).

Due to that it is reasonable and actual to have a complex study of GAROFL, the content of malonic dialdehyde (MDA) in the saliva, the gum tissues and blood plasma and also the anti-peroxide activity (APA) in the rat plasma with the modeled periodontitis and the patients with the chronic generalized periodontitis (CGP) and to correct their disturbances by the antioxidant (AO) therapy.

MATERIALS AND METHODS

Experimental part of study

The experiments were carried out on 75 Wistar male rats with the weight of 180-200 g at the age of 16-18 weeks. The animals were divided into five groups: 1. Control (n=15); 2. Comparison group (n=15); 3. Group of animals treated with AO therapy (citoflavin dose of 130,0 mg/kg/day, mexidol dose of 100 mg/kg/day and the ascorbic acid dose of 4 mg/kg/day (n=15); 4. Group of animals treated with calcium D_3 dose of 130,0 mg/kg/day by calcium, 51,4 ME/kg/day by vitamin D_3 (n=15); 5. Group of animals treated with AO therapy and calcium D_3 (n=15).

For animals of 2-5 groups the periodontitis was modeled by adding to the fodder mix of experimental animals the oxidized sunflower (10 % of the ration weight, approximately 2 ml/day per rat) (5). It is proved that this aterogenous diet causes periodontal changes, which according to the morphological manifestations correspond to periodontitis of the human. The sunflower oxidation was made by its heating within 40 minutes at the temperature of 130–150°C with the air blowing under the presenting catalyst - oxidizer (0,1% $CuSO_4$).

The animals were observed for 45 days, after which 60 rats receiving the oxidized diet were divided into 2, 3, 4 and 5 groups and marked. 1st group of rats (n=15) received the common sunflower and was a group of the negative control. Further on animals from 3-5 groups were treated according to their marking by the intra-ventral introduction of researched preparations each day for 14 days.

To evaluate the therapy we assessed the local (gum biochemistry) and system action of the drugs (saliva biochemistry).

At the end of the research protocol the animals were subjected to euthanasia under the ethereal anesthesia by the instant decapitation. 24 hours before that the rat saliva stimulated by pilocarpin was sampled (0,1% pilocarpin hydrochloride subbualno). The collected samples were centrifuged at 6000 rpm for 20 minutes to obtain supernatants, with the determined content of protein (by biuret method), alkaline phosphatase (ALF, by Bessei-Lowry-Brock method), MDA (reaction with 2-thiobarbituric acid) and catalase (by reaction with the molybdenum reagent) (8,9,10,15). After euthanasia, the animal tissues were sampled for the biochemical analysis. In the gum tissue the protein content was evaluated by the biuret method and also components of the connective tissue matrix: collagen, elastin and sulfated glycosaminoglycans spectrophotometrically with using the readymade sets of "BIOCOLOR" firm (UK).

Clinical part of study

The clinical research included 106 patients (51 men (48,1%) and 55 women (51,9%) with CGP at the age from 17 to 77 years (the average age made 49,81±9,04).

When included in the study the patients were randomized by the random sampling method into three groups: 1 comparison group was 32 (30,2%) patients at the age from 30 to 73 (the average age made 45,76±9,13), receiving the traditional therapy including the standard complex of surgical, therapeutic and orthopedic measures. 2 main group included 39 (36,8%) patients at the age from 28 to 76 (the average age made 50,33±10,62). All patients in this group in the traditional therapy structure received additionally AO therapy (cytoflavin of 1—2 pills 2 times per day for 25 days, mexidol of 1 pill (125 mg) 3 times per day and ascorbic acid of 1 bean (100 mg) 3 times per day). 18 (16,7%) patients received additionally calcium (calcium D_3) of 1 chewing pill 2 times per day for 30 days. 3rd group included 35 (33%) patients at the age from 28 to 76 (the average age made 49,76±9,20), receiving the traditional therapy (10 persons; comparison group) and additionally the treatment - prophylactic paste (LPP) based on medicinal herbs (15 persons). They cleaned their teeth with it at home in the usual way 2-4 times per day for 2-3 weeks. The patient groups were homogeneous and statistically not different from each other.

The patient treatment tactics corresponded to the standards and included a complex of surgical, therapeutic and orthopedic measures. Dental deposits of all patients were removed, with smoothing and polishing the root naked part and immobilization of movable teeth by splinting. As the local therapy they used antiseptic rinsing, applications on the gingival edge mucous membrane and introduction of anti-inflammatory agents in the periodontal pocket.

The patient condition was made in dynamics (at admission, on the 4th and 30th day of the therapy) based on the complex of clinical, laboratory, instrumental research methods: studying the anamnesis and complaints, examination of the dental edge on the mucous membrane with evaluating the recession degree of the dental edge and teeth mobility, researching the content of total protein, ALT, MDA and catalas in the saliva, examinating FRP in the blood plasma by GAROFL indices - basal rate of the chemiluminescence intensity (PICLb) and the chemiluminescence intensity rate stimulated by zymosan (PICLs), by indicators of APA plasma, MDA, the roentgen imaging based on the orthopantomogram with analysis of the reduced intra-alveolar septum height and the level of teeth root exposure, measuring the depth of periodontal pockets by the periodontal probe, periodontal index (PI), Federov-Volodkin index of hygiene (IH) and expression index of the inflammatory phenomena, gingivitis, papillary-marginal-alveolar index (PMA).

Chemiluminescence (CL) indexes of GAROFL were researched on the chemiluminescencemeter LCB "Wallac" (Sweden) adapted for the chemiluminescencemetry at the standard temperature of 36,9°C. The detected parameters were the level of basal (spontaneous) CL (PICLb) in the standard mist volume of leucocytes with their standard concentration (2500 in 1 μl). After adding a non-specific activator (0,1 ml of 1% zymosan solution) PICLs were detected. MDA - is the secondary product of POL, determined with using the methods described by Douest J.C. (1983). The research method of plasma APA is based on measuring and comparing indices of the CL plasma induced by the hydrogen peroxide and its spontaneous CL (Ind/Sp CL). The calculated ratio is the value which is inversely proportionally to plasma APA. The less is this ratio, the more is APA and vice versa.

STATISTICAL ANALYSES

The statistical processing of the results was made by using the programs of Microsoft Excel and the data statistic analysis package of Statistica 8.0 for Windows (StatSoft Inc., USA) and SPSS 15.0. The differences were considered statistically important at the error level of $p<0,05$. For the quantitative variables the test was made on the normality of distribution by means of Shapiro- Wilk criterion. To assess the factual results the statistic analysis methods were used: c^2- Pearson criterion (analysis of mating tables), t-criterion of Student, Newman - Keils criterion for multiple comparisons. For independent non-parametric samples we used Mann-Whitney criterion, for the multiple comparison - Kraskel - Wallis criterion. For the dependent non-parametric samples we used the Wilcoson criterion, for the multiple comparisons - Friedman.

RESULTS

We have detected that in the experiment the rat saliva with the modeled periodontitis is known to have the increased total protein for 1,2 times ($p<0,05$) due to inflammation and ALP in 2,6 times ($p<0,05$) due to cytolysis with the simultaneous increased content of MDA in 2,8 times ($p<0,05$) and less catalase activity in 2,5 times ($p<0,05$), which proves activation of FRP and less AO protection (Table 1).

In the gum tissues of rates with the modeled periodontitis there is the less content of total protein, collagen, elastin and sulfated glycosaminoglycans in 2,8, 1,5, 1,6 and 1,3 times accordingly (all $p<0,05$), which proves the predominance of catabolic processes in development of periodontal disease (Table 2).

The similar data were obtained for the patients with the CGP. In the saliva there was the protein increased in 2,2 times ($p<0,05$), ALP in 3,3 times ($p<0,05$) and MDA content in 1,7 times ($p<0,05$) at the simultaneously reduced catalase in 3,1 times ($p<0,05$ times; Table 3).

For the same patients in the blood there was the increased PICLb in 1,6 times ($p<0,05$) and PICLs in 3,9 times

Table 1. The content of protein, MDA and ALP activity and rat saliva catalase in the control, comparison group and after treatment of AO therapy, calcium D_3 and combination of AO therapy with calcium D_3.

Researched groups	Protein (g/l)	ALP (ME/l)	MDA (µ/l)	Catalase (µcat/l)
Control (n=15)	6,28±0,29	522,60±4,26	1,21±0,14	192,7±8,1
Comparison (n=15)	7,79±0,34*	1308,6±125,0*	3,27±0,22*	72,8±6,2*
AO (n=15)	6,42±0,38**	1199,8±176,5* ##	1,72±0,36**	179,3±12,8** #
Calcium D_3 (n=15)	6,47±0,53**	872,4±44,5* **^	2,32±0,19* **^	114,3±11,0* **#^
AO+ Calcium D_3 (n=15)	6,35±0,38**	558,3±81,3**##^	1,36±0,38**^	191,0±9,3**^

*Note: *- p≤0,05 as compared with the Control group; ** - p≤0,05 as compared with the comparison group; # - p≤0,05 the difference of rat groups receiving AO therapy and Calcium -D_3; ## - p≤0,05 the difference of rat groups receiving AO therapy and AO therapy + Calcium -D_3; ^ - p≤0,05 the difference of rat groups receiving Calcium -D_3 and AO therapy + Calcium -D_3*

Table 2. The content of protein, collagen, elastin and sulfated glycosaminoglycans (GAG) in the gum tissues of rats in the control, comparison group and after treatment of AO therapy, calcium D_3 and in combination of AO therapy with calcium D_3.

Researched groups	Protein (g/l)	Collagen (mcg/g)	Elastin (mcg/g)	Sulfatated GAG (mcg/g)
Control (n=15)	201,0±4=5,1	65,7±5,1	33,8±2,6	107,0±7,9
Comparison (n=15)	71,8±2,9*	42,7±1,9*	23,1±2,3*	81,1,8±1,9*
AO (n=15)	161,2±8,8* ** ##	56,7±2,6** ##	31,3±2,1** #	89,1±2,9* ** ##
Calcium D_3 (n=15)	148,4±6,8* **^	61,4±2,0**^	24,2±1,6* #^	82,4±3,8*^
AO+Calcium D_3 (n=15)	215,7±10,4** #^	71,5±1,4** #^	36,1±2,5** ^	108,9±3,9** #^

*Note: *- p≤0,05 as compared with the Control group; ** - p≤0,05 as compared with the comparison group; # - p≤0,05 the difference of rat groups receiving AO therapy and Calcium -D_3; ## - p≤0,05 the difference of rat groups receiving AO therapy and AO therapy + Calcium -D_3; ^ - p≤0,05 the difference of rat groups receiving Calcium -D_3 and AO therapy + Calcium -D_3*

Table 3. The content of protein, MDA and ALP activity and saliva catalase for healthy donors and patients with CGP before treatment and on the 14th and 30th day after treatment

Researched indicator	1 day	14 day	30 day
Protein (N: 2,00±0,2 g/l)			
CGP comparison group (n=10)	4,38±0,56*	3,41±0,33*	2,30±0,21**
CGP of LPP group (n=15)	3,78±0,20*	2,70±0,27**	2,10±0,22** #
ALP (N: 1,0±0,1ME/L)			
CGP comparison group (n=10)	3,27±0,3*1	2,91±0,29*	2,31±0,21* **
CGP of LPP group (n=15)	2,21±0,38*	1,30±0,14**	1,10±0,12**
MDA(N: 7,0±0,7 mmol/l)			
CGP comparison group (n=10)	11,73±0,67*	10,87±0,62*	9,39±0,56* **
CGP of LPP group (n=15)	10,21±0,48*	8,80±0,90*	7,70±0,80**
Catalase (N: 250,0±20,0ME/l)			
CGP comparison group (n=10)	80,24±5,41*	89,05±5,52*	91,86±5,15*
CGP of LPP group (n=15)	219,98±9,30*	231,00±11,37*	245,00±12,55**

*Note: *- p≤0,05 –as compared with the norm; **- p≤0,05 – as compared with 1st day; # p≤0,05 – as compared with 14 day;*

(p<0,05), and also MDA content in the plasma in 1,4 times (p<0,05) and the reduced plasma APA in 2,1 times (p<0,05).

The revealed changes of FRP objectify the relevance of prescribing antioxidant, energy-correcting drugs to the patients suffering periodontitis which is mostly efficient in the early stages of the disease. The additional prescription of pathophysiological treatment will prevent FRP transfer from the deficient stage of O_2 and energy into the stage of necrosis. To restore the cellular energy deficit the amber acid was used, stimulating the Krebs cycle and production of ATP additional amount, citoflavin in combination with mexidol and ascorbic acid. To improve the results treatment was supplemented with calcium and vitamin D_3 (calcium D_3).

According to data obtained when researching the rat saliva it is clear (Table 1) that AO therapy normalizes the

content of protein and MDA (it is reduced in 1,2 and 1,8 times accordingly; p<0,05 and p<0,05 accordingly) and increases the catalase activity (in 2,5 times; p<0,05). Calcium D_3 normalizes the protein content and ALP activity (they are decreased in 1,2 and 1,5 times accordingly; p<0,05 and p<0,05 accordingly). The combined treatment of AO therapy and calcium D_3 completely normalized all researched parameters of the rat saliva. The research of the rat gum tissue demonstrated (Table 2), that AO therapy increases the content of protein, collagen and elastin (in 2,3, 1,2 and 1.4 times; all p<0,05) but they remained significantly lower than the norm. Calcium D_3 caused the further increase in the content of protein and collagen (in 2,1 and 1,4 times accordingly; p<0,05 *и* p<0,05 accordingly). The combined introduction of AO therapy and calcium D_3 completely normalized all researched parameters of the rat gum tissue. Thus the antioxidant therapy resulted in the regressed inflammation and reduced FRP with the simultaneous improvement of AO protection in the saliva and rat gum tissues with the modeled periodontitis.

Based on the saliva indicators of CGP patients (Table 3) LPP resulted in less content of pro-inflammatory substances in the saliva (protein, MDA and ALP in 1,8, 1,3 and 2,0 times accordingly; all p<0,05) and increased the anti-inflammatory agents (catalase in 1,1 times; p<0,05), which was more expressed than in the comparison group. LPP caused both improved biochemical parameters and better clinical picture of the disease (index of RMA, PI and IG reduced in 3,4, 1,6 and 1,5 times accordingly; all p<0,05).

The impact results of the traditional therapy, AO therapy and AO therapy in combination with calcium D_3 on PICLb are given on Fig.1.

For CGP patients receiving the traditional therapy (n=32) PICLb reduced in 1,2 times (p>0,05), and for CGP patients, receiving AO therapy (n=39), and AO therapy in combination with calcium D3 (n=18) in 1,4 and 1,6 times accordingly (p<0,05 and p<0,05 accordingly). Thus, PICLb reduced reliably only under the impact of AO and AO therapy in combination with calcium D_3.

The impact results of the traditional therapy, AO therapy and AO therapy in combination with calcium D_3 on PICLs are given on Fig.2.

For CGP patients receiving the traditional therapy (n=32) PICLs reduced in 2,8 times (p<0,05), and for CGP patients, receiving AO therapy (n=39) and AO therapy in combination with calcium D_3 (n=18) in 3,1 and 3,7 times accordingly (p<0,001 and p<0,001 accordingly). Thus, PICLs was reliably reduced for CGP patients, receiving AO therapy and AO therapy in combination with calcium D_3 as compared with CGP patients receiving only the traditional therapy.

The impact results of the traditional therapy, AO and AO therapy in combination with calcium D_3 on MDA content in the plasma are given on Fig.3.

For CGP patients receiving the traditional therapy (n=32) MDA content in the blood plasma was reduced in 1,2 times (p>0,05), and CGP patients receiving AO therapy (n=39) and AO therapy in combination with calcium D3 (n=18) in 1,3 and 1,3 times раза accordingly (p<0,05 and p<0,05 accordingly). Thus, MDA content in the blood plasma was reliably reduced only under the influence of AO therapy and AO therapy in combination with calcium D_3.

The impact results of the traditional therapy, AO therapy and AO therapy in combination with calcium D_3 on the plasma APA are given on Fig.4.

For CGP patients receiving the traditional therapy (n=32) the ratio of the induced to spontaneous plasma CL reduced in 2,0 times (p<0,05). Similar results were obtained for CGP patients receiving AO therapy (n=39) and AO therapy in combination with calcium D_3 (n=18; in 2,0 and 2,0 times accordingly; p<0,05 and p<0,05 accordingly). Thus, the plasma APA was increased for all patients regardless the method of treatment.

The positive dynamics of FRP parameters was accompanied with the between clinical picture of the disease and treatment results. The patients receiving AO therapy to a greater degree demonstrated the reduced featured of inflammation, the mucous became pink, with its more density. Already in 14 days of the therapy the periodontal pocket depth decreased significantly - for 0,8—2,3 mm, on average for 24%. The patients treated with the traditional therapy had the average reduced depth of the periodontal pocket of 16% (for 0,2—1,3 mm). Differences between the groups are reliable (p<0,05). But for that the positive dynamics at the additionally received AO therapy was more expressed with the better image of orthopantogram. These positive changes were accompanied by the dental scale dynamics of the group which is more relevant and surpassing the comparison group (index of RMA, PI and IG reduced in 4,9, 1,5 and 1,5 times; all p<0,05).

DISSCUSION

Periodontitis is an inflammation of periodontal tissues characterized by the progressive destruction of periodontal ligament and bone. Etiology and pathogenesis of inflammatory periodontal diseases has not been established. It is proved that periodontitis can't be without the plaque. It is now believed that the primary etiological factor in the development of periodontal diseases is bacteria and their toxins. The main pathogenetic factor - is the inflammation that occurs in response to the invading periodontal pathogenic micro flora in the periodontal tissue, with the expression depending on a number of systemic and local factors (17).

Today it is believed that under the certain forms of periodontitis the specificity of bacteria is stimulated by the fact that dental plaque microorganisms evolve

under the exogenous or endogenous influence and crowd out other bacteria. Therefore the inflammatory periodontal diseases are now viewed as an opportunistic infection, depending on both available pathogenic bacteria and the environment promoting their reproduction ($_{p}$H local changes, anaerobic niche, resistance changes, etc.) (18).

Causes of periodontitis can be the following: malocclusion, violated teeth shapes, poor oral hygiene, violated diet (lack of protein, vitamins). In the development of periodontitis the structure of food is also important - too soft food, not promoting the teeth cleaning and normal load during the chewing which can also be a cause of periodontitis. Bad habits such as chewing on one side of the jaws, i.e. the functional overload of some jaw areas, can also contribute to the development of periodontitis. It develops often after the gum inflammation - gingivitis or periodontal disease. The special role is played by chronic illnesses, poor ecology, occupational hazards and metabolic disorders (19).

An important etiological factor in the development of periodontal disease is periodontal pathogenic bacteria. According to WHO recommendations their representatives are such species which along with the predominantly anaerobic respiration differ by high adhesives, invasive and toxic properties in relation to periodontal tissues (20).

The dental plaque, as one of the main instruments for microorganisms to affect the paradontium accumulates in the margin areas and inter-dental spaces.

When there is inflammation in periodontal tissues due to the increased permeability of blood vessels, it is to be the increased flow of the dental liquid, with the increased migration of polymorphic nuclear leucocytes, which are an essential element of nonspecific blood protective system. Periodontitis results in hyper activation of leucocytes, macrophages, and platelets. Process of accumulating hyper activated leucocytes and platelets in areas of inflammation is the base to develop the tissue destruction (21).

Leucocytes phagocytize bacteria, decomposition products of tissues and destroy them by their lysosome enzymes (such as peptidase, proteases, oxidases, deoxyribonuclease and lipase). The activated cellular membranes of polymorphic nuclear leucocytes excrete the arachidonic acid - an unsaturated fatty acid, which serves as a precursor of leucotrienes, thromboxanes and prostaglandins. This group of substances plays an important role in the launch of inflammation, regulation of the lumen and permeability of blood vessels (20, 21).

Degraded trophy of periodontal tissues leads to changes in the energy process that ensures the viability of cells. It activates primitive ways to generate energy using peroxide and free radical oxidation with formation of large amounts of highly toxic products: reactive oxygen species, MDA, etc. The change environmental acidity disrupts maturation of osteoblasts with the active formation of osteoclasts (19, 20).

We have found experimentally that in the saliva of rats with modeled periodontitis there is an increase in total protein due to inflammation and ALP due to cytolysis with simultaneous increase of MDA content and lower catalase activity which proves FRP activation and less AO protection. Along with that in the saliva of rats with modeled periodontitis there is the decreased content of protein, collagen, elastin and sulfated glycosaminoglycans demonstrating the predominance of catabolism in inflammation.

The similar results were obtained for patients with CGP. In the saliva there is the increased protein, ALP and MDA content in the plasma with the simultaneous reduction of catalase. In the blood of the same patients there are increased indicators of PICLb and PICLs, and also the reduced plasma MDA content and less plasma APA.

Thus, CGP pathogenesis is a very complex process. With the low level of oral hygiene, insufficient teeth self-cleaning, changes in the qualitative and quantitative composition and increased pathogenic micro flora there is the stronger pathogenic potential of the “dental” plaque. Microorganisms can affect the paradontium through extraction of toxins: exotoxins, endotoxins, metabolites and enzymes. The released enzymes can provide a lytic effect on the connective tissue fiber carcass of the paradontium (collagenase, protease), epithelial structures (cearatase), and surface structure of cells (neuraminidase). This especially occurs when common protective factors are poor (atherosclerotic damage of vessels, disturbed neurohumoral regulation, changed immunological activity, diseases of internal organs, chronic psycho – emotional stress, intoxications, hypo – and vitamin deficiency, genetic predisposition) and when local protective paradontium factors are weaken (local traumatic factors, functional overload or insufficiency of periodontal tissues, development abnormalities of teeth-maxilla system, qualitative and quantitative changes in saliva and oral fluid).

These substances activate leucocytes that begin to generate intensively reactive oxygen species. They themselves cause damage and inflammation of paradontium, as well as through POL initiation. These processes are taking place under the declining AO protection.

The revealed changes in FRP objectify the relevance of prescribing AO, energy correction drugs for the patients with periodontitis that is most effective in the early stages of the disease.

The use of AO therapy contributed to reversing the oxidative stress and activating the catalase of saliva, as well as restoration of the protein level. Preparation of calcium and vitamin D_3 increasingly reduced ALP activity enhanced in pathology, but poorly compensated the system disorders of antioxidants — pro-oxidants. The combined introduction of mixed AO and calcium D_3 fully normalized biochemical parameters of the rat saliva with periodontitis: protein, ALP activity, catalase and MDA levels. AO therapy provid-

ed the increased levels of total protein, collagen and elastin. For rats with periodontitis the introduction of calcium D3 also contributed to the increase in the level of protein and collagen in tissues, however, revealed positive changes were significantly higher than such only for animals from the comparison group, but differed from those in the control group of rats. Calcium D_3 did not influence the level of elastin and sulfated glycosaminoglycans. The combined introduction of calcium D_3 and AO therapy fully normalized the revealed pathological changes in gingival tissues of rats with periodontitis.

CONCLUSION

When evaluating the influence of AO therapy in patients with CGP the results were obtained positively evaluating the dynamics of both oxygen and lipid spectrum of FRP. It is important to note that the existing imbalance of FRP oxygen cascade is tracked up to 30 days of observation for the patients with CGP, which proves the long-term persistence of oxidative stress ongoing processes in these patients.

This positive dynamics in the group of AO therapy is combined with a more positive indicator of plasma APA than in the comparison group by the 30 day of treatment. Positive dynamics of oxidative stress parameters correlated with an improvement of the clinical picture and treatment results.

Thus, the identified violations of the local and systemic oxidant stress in periodontitis required the immediate correction of AO preparations.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

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BIOLOGICAL MECHANISMS OF CHRONIC WOUND AND DIABETIC FOOT HEALING: THE ROLE OF COLLAGEN

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BIOLOŠKI MEHANIZMI ZARASTANJA HRONIČNIH RANA I OZDRAVLJENJA DIABETIČNOG STOPALA: ULOGA KOLAGENA

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ABSTRACT

The treatment of chronic wounds is a continuously developing research focus. The problems of excessive mechanical forces, infection, inflammation, reduced production of growth factors, and lack of collagen will affect the results of treatment. The purpose of this study was to analysse the elements that lead to long-term non-healing of chronic wounds and trophic ulcers, including diabetic foot syndrome, by determining the optimal treatment algorithm. The paper presents an analysis of the world literature on the etiopathogenesis and principles of chronic wound treatment in diabetic foot syndrome. The epidemiology of chronic wounds of different genesis is presented. The issues of physiological and metabolic disorders in chronic ulcers affecting the process of wound healing are discussed. Particular attention is paid to collagen, which is a protein that forms the basis of connective tissue; collagen ensures the strength and elasticity of the skin, which confirms the importance of its role not only in aesthetics but also in the process of wound healing. Different types of collagen and their roles in the mechanisms of chronic wound healing in diabetic foot syndrome are described. The results of clinical studies evaluating the effectiveness of medical products and preparations, consisting of collagen with preserved (native collagen) and fractionated structures, in treating chronic wounds of diabetic foot syndrome are analysed. It has been shown that the use of native collagen preparations is a promising treatment for chronic ulcers and wounds, including diabetic foot syndrome, which makes it possible to increase the effectiveness of treatment and reduce the economic costs of managing these patients.

Keywords: *chronic wounds, diabetic foot syndrome, collagen.*

SAŽETAK

Protokoli lečenja hroničnih rana su u stalnom razvoju. Problemi prekomernih mehaničkih sila, infekcije, upale, smanjenja proizvodnje faktora rasta, nedostatka kolagena će uticati na rezultate tretmana. Svrha ovog rada je da analizira razloge koji su doveli do dugoročnog neizlecenja hroničnih rana, trofičkih ulkusa, uključujući sindrom dijabetičkog stopala, određivanjem optimalnog protokola tretmana. U radu je prikazana analiza dostupne literature o etiopatogenezi i principima lečenja hroničnih rana kod sindroma dijabetičkog stopala. Prikazana je epidemiologija hroničnih rana različite geneze. Razmatrana su pitanja fizioloških i metaboličkih poremećaja u hroničnim ulkusima koji utiču na proces zarastanja rana. Posebna pažnja se posvećuje kolagenu - proteinu koji čini osnovu vezivnog tkiva, osiguravajući snagu i elastičnost kože, što potvrđuje važnost njegove uloge ne samo u kozmetologiji, već posebno u procesu zarastanja rana. Opisani su različiti tipovi kolagena i njegova uloga u mehanizmima zarastanja hroničnih rana kod sindroma dijabetičkog stopala. Analizirani su rezultati kliničkih studija koje ocenjuju efikasnost medicinskih proizvoda i preparata na bazi kolagena sa očuvanom strukturom (nativni kolagen) i frakcionisanog kolagena u pogledu perspektive hroničnih rana kod dijabetičke terapije stopala. Pokazalo se da je upotreba nativnog kolagenskog preparata obećavajući pravac u lečenju hroničnih ulkusa i rana, uključujući i sindrom dijabetičkog stopala, što omogućava povećanje efikasnosti lečenja i smanjenje ekonomskih troškova tretmana ovim pacijentima.

Ključne reči: *hronične rane, sindrom dijabetičkog stopala, kolagen.*

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INTRODUCTION

The significant increase in the number of patients with chronic wounds, especially those with complications of type 2 diabetes mellitus and poor effectiveness of combination therapy, has created a challenge for practising surgeons, endocrinologists and other specialists to search for superior and more effective treatments for such patients. Long-term and continuous therapy often ends results in amputations, which produces a plethora of disabled individuals who are excluded from a productive social life; this outcome imposes an extra financial burden at all levels.

The pathophysiology of diabetic foot syndrome (DFS) includes many mutually potentiating components, such as neuropathies, vascular disorders, impaired immunity, and infections, creating a vicious cycle (1-3). Numerous studies have been dedicated to the treatment of patients with chronic wounds, which has helped to work out recommendations for evidence-based approaches that are now recognized by the world community (Table 1).

Unfortunately, even when a whole set of recommended treatments accompanied by continuous glucose monitoring and pharmacological support of adequate tissue perfusion is used, wound healing occurs within 12 weeks in only 24-50% of patients (4-7). The absence of an adequate understanding of the physiology of wound healing hampers the development of new wound care products for chronic wound treatment and, until now, did not allow for the development of evidence-based evaluation of the comparative effectiveness of different types of wound healing devices in patients with protracted, non-healing chronic ulcers.

Therefore, despite all the achievements in the health care system, chronic ulcers, especially in patients with diabetes mellitus, still remain the most topical medico-social issue, and the solution requires an in-depth investigation of common factors involved in the activation of physiological defence mechanisms and objectivization of the most promising methods of treatment.

EPIDEMIOLOGY OF CHRONIC WOUNDS

A wound is considered to be chronic (also known as protracted, non-healing wound or trophic) if it does not heal within 6 weeks of its existence or show any signs of healing despite appropriate standard wound care. The most common causes of chronic wounds include chronic lower extremity venous diseases (venous trophic ulcer), chronic arterial insufficiency (obliterating atherosclerosis) and diabetes mellitus (diabetic foot syndrome), as well as pressure ulcers (decubitus trophic ulcers), which occur as a result of pressure on weight-bearing areas for too long and/or disturbed innervation due to disorders of the nervous system. All these pathological conditions represent a very complex and most topical medico-social problem.

In this light, 1-2% of the population is suffering from chronic venous trophic ulcers of the lower extremities, which are the cause of 50-70% of all leg chronic wounds (8-9). Only half of venous trophic ulcers heal within 4 months, while 20-25% of those fail to undergo epithelialization even within 2 years (10-11). A quarter of leg ulcer cases are caused by atherosclerotic lesions of the lower limb arteries with haemodynamically significant reduction of tissue perfusion and disturbed oxygenation of the skin and soft tissues of the leg.

However, the two abovementioned conditions can be approached by fairly effective, primarily surgical treatments. The narrowing of the magistral blood vessels is eliminated using modern intravascular technologies. Unfortunately, diabetic foot syndrome (DFS) manifesting in the appearance of chronic ulcers on the weight-bearing areas of diabetic patients poses a far more serious problem with no effective solution to date. The occurrence of DFS is manifested clinically due to neural influences on trophic changes, and lesions in the microvascular bed occur due to glycation of the vessel wall proteins.

The global prevalence of diabetic foot syndrome is 6% (12). Every fourth patient with diabetes mellitus bears a heavy burden of DFS (13). That said, every year, 2-10% of diabetic patients develop DFS, and approximately 10% of affected lower limbs are amputated due to the occurrence of suppurative and gangrenous changes (14-16), making DFS a highly debilitating illness leading to global economic losses. According to the European Study Group on Diabetes and the Lower Extremity (Eurodiale), a high-level amputation surgery is performed in 5% of patients with DFS each year (17, 18). That said, a 5-year survival rate following amputation ranges between 30% and 70% (19). In patients with DFS, the arteries of the microcirculatory bed are affected in 50-80% of cases, and the severity of this injury correlates with the healing potential and determines the prognosis (20, 21), which translates into a colossal economic impact. The Eurodiale study has shown that the average cost of treatment of uninfected ulcers is 10 000

Table 1. Recommended approaches for the treatment of patients with diabetic foot syndrome (ranked by the level of evidence)

Treatment	Diabetic foot ulcer (primary)	Chronic diabetic foot ulcer
Plantar pressure redistribution	Recommended (1A)	Recommended (1A)
Surgical treatment of the wound	Recommended (2A)	Recommended (2A)
Antibiotics	Treatment of infection caused by aerobic Gram-positive bacteria (2A)	Broad-spectrum systemic antibiotics (2A)
Wound dressings using wound-healing agents	Recommended (2A)	No sufficient evidence available

euros versus 17 000 euros for infected ulcers with concurrent peripheral artery disease (18). In the USA,, the total treatment cost for protracted, non-healing diabetic ulcers with severe infection (complex surgical and conservative treatment and, due to its failure, below knee amputation) the is $190 000, according to a 2012 estimate (22).

PHYSIOLOGICAL AND METABOLIC DISTURBANCES IN PATIENTS WITH CHRONIC WOUNDS

The course of wound healing includes the following phases: inflammation (exudation), proliferation/regeneration (granulation tissue formation) and scar regeneration (epithelialization). Macrophages play a key role in wound healing. They phagocytize pathogenic organisms and tissue degradation products and stimulate formation of granulation tissue. Fibroblasts are required during the proliferation phase, as they produce the important structural elements including collagen, elastin and extracellular matrix proteins.

Wound healing depends on the particulars of the wound (aetiology, depth, and size) and the patient's general condition. In normal circumstances, the healing wound has a low level of bacterial contamination, inflammatory cytokines, proteases and active oxygen forms, and it also has a functionally active extracellular matrix and high mitotic activity of epithelial cells (23). However, to ensure the synthesis of granulation tissue, collagen and the ground substance of the extracellular matrix, energy is required in the form of adenosine triphosphate (ATP) molecules, which areis mainly produced in the mitochondria, provided a sufficient amount of oxygen is available. For that reason, any disturbance of the oxygen supply to cells leads to a several-fold reduction of ATP synthesis and a corresponding decrease of collagen synthesis. This is the main pathophysiological mechanism leading to the disturbance of normal tissue regeneration and formation of chronic wounds / trophic ulcers (1, 2).

Studies conducted in different countries demonstrated glycosylation of collagen in diabetes mellitus; therefore, despite the upregulation of type I collagen gene expression, there is an impaired synthesis of fully functional collagen molecules in the wound. Degraded tissue components and superactive matrix metalloproteinases (MMP) present in chronic non-healing wounds promote a protracted inflammatory response (24). Fibroblast physiology is changing as well (25). As a response to ischaemia/hypoxia fibroblast migration and proliferation decreasing, it reduced collagen production occurs (26, 27).

For this reason, along with basic methods of therapy, the administration of collagen is considered an important adjunct therapy in patients with various chronic wounds, protracted non-healing trophic ulcers and bed sores.

COLLAGEN-TYPES AND ROLES

Collagen (kolla - glue, genes - producing) is the most common mammalian protein representing 25-35% of all proteins in the body; i.e., approximately 6% of body weight (28, 29). Collagen forms the mechanical backbone of the connective tissue and ensures durability and resilience of the bones, ligaments, skin, vessels and other tissues. As with any other protein, even more so the ones with natural load-bearing function, collagen is constantly synthesized and catabolized. Allegedly, in young subjects, the amount of newly synthesized collagen is equal to 6 kg per year. In the second half of life, the rate of all synthesis reactions decreases, and the amount of newly synthesized collagen is reduced 2-fold. It is important to note that approximately 40% of the total collagen amount is present in the skin, which confirms its role in skin care and especially in the process of wound healing.

To date, scientists identified more than 40 genes that collectively encode 28 different types of collagen designated with roman numerals from I to XXVIII (28, 30). Such variability of collagen types is required to ensure different physiological functions in different tissues and organs. Characteristics of the most common types of collagen I – IV are presented in Table 2.

Table 2. The most common types of collagen I – IV.

Type	Genes	Tissues and organs	Composition	Associated pathology
I	COL1A1, COL1A2	Ubiquitously, especially in skin (dermis), ligaments, bones, fascias, dentin, cornea	1% - hydroxylysine, 33% - glycine, 13% - proline; low glycosylated, large-diameter fibrils	Rheumatism, dysplasias, Marfan syndrome, Ehlers-Danlos syndrome
II	COL2A1	Cartilages, vitreous body, cornea	>1% hydroxylysine, highly glycosylated fibrils thinner than in type I collagen	Collagenopathy, achondrogenesis, Stickler syndrome
III	COL3A1	Skin (mostly foetal skin), uterus, blood vessels, reticular fibres of haematopoietic organs	Excess of hydroxyproline, hydroxylysine deficiency, low-glycosylated	Aortic aneurism, dysplasia, Ehlers-Danlos syndrome
IV	COL4A1, COL4A2, COL4A3, COL4A4, COL4A5, COL4A6	Basal membranes	Excess of hydroxylysine and hydroxyproline, alanine deficiency, nearly completely glycosylated	Goodpasture syndrome

Based on its supramolecular organization, collagen can be divided into two categories; i.e., fibrillar and non-fibrillar collagen (29-32). The main fibrillar types of collagen are types I, II and III. The most common in adults is type I collagen, which possesses the greatest tensile mechanical strength due, among other things, to fibrils of the largest diameter. The non-fibrillar collagens may form networks with various topological properties (e.g., type IV collagens (basal membranes), type VIII collagens (cornea, blood vessels), type VI collagens (beads-on-string-type structures; cartilage, blood vessels, skin, uterus, lungs, kidneys); types XXVI and XXVIII (many tissues and organs), and type VII collagens (anchor filaments; skin, oaoesophagus, cornea, chorion). Many non-fibrillar collagens are bound to the surface of collagen fibrils (types IX, XII, XIV, XIX-XXII), and some of these are transmembrane proteins (types XIII, XVII, XXIII, XXV).

Therefore, collagen fibrils are the macromolecular compositions containing several types of collagen and their bound proteins (33). The composition of different fibrils depends on their stage of development and type of tissue, which is why the definition of specific structures, such as "type I collagen fibrils" or "type II collagen fibrils", is an oversimplification. In this regard, adult skin mostly contains type I collagen (80-85%). That said, up to 20% of all collagens belong to type III collagen (which dominates during embryogenesis but in adulthood makes up 5-10%), including types V, VI, VII, XII, XIV and other collagen types. Collagen fibrils in ligaments mostly comprise types I and III collagen; cartilage comprises types II and XI and the cornea comprises types I and V collagen (29, 32).

The direction of collagen fibrils is important for providing tensile strength and resilience to tissues and organs. Considering the direction of collagen, in the central part of human skeleton bones (pipe bones and flat bones), collagen fibrils appear to run parallel in the longitudinal direction, whereas in the peripheral part they run in a transverse direction. The parallel fibrils in the ligaments secure resistance to mechanical loading, whereas the collagen fibril network does so in the cartilage. In the dermis, collagen fibrils form the network and the level of development is proportional to the applied load or pressure; hence, the most developed network is present in the skin of the heel. In the skin of the healing wound, the collagen fibril network shows a peculiar kind of randomness.

Collagen is synthesized and secreted mostly by fibroblasts inat different degrees of maturity and by cells producing the intercellular substances that enhance wound healing. Fibrillar collagens are primarily synthesized in the form of a soluble precursor, procollagen (25, 34). Procollagen molecules are converted to collagen by proteinases during or after their secretion (35). Mature collagen molecules pack together to form fibrils, and the process is regulated by superficial and extracellular proteins such as other collagens, integrins, and fibronectin (33, 36-38).

It is important to emphasize that collagen is not merely the passive structural component of the molecular backbone. The biological function of collagen is to mediate interaction with different cell surface receptors and other extracellular matrix proteins. Interaction of collagen with specific cell receptors triggers signal events that regulate cell migration, adhesion or proliferations. There are three main families of collagen receptors: collagen-binding integrins, collagen-binding immune receptors and discoidin domain receptors. The latter are kinase receptors that bind the main fibrillar collagens of types I-III and regulate cell proliferation, differentiation and matrix modulation (39-41), thereby ensuring the wound/ulcer healing process.

COLLAGEN-BASED MEDICAL DEVICES AND PRODUCTS

The above-mentioned studies convincingly demonstrate the enormous roles of collagen, collagen-based products and medical devices in the stimulation of the regenerative and proliferative phase of the wound healing process. Collagen-based products mainly differ from each other by the degree of purity and cross-linking, relative percentage of different collagen types, source and form of collagen presentation. Sources of collagen may include skin, intestine and other organs. After collection of source material, the manufacturers prepare each product according to specific individual processes by removing cell components and retaining the natural matrix. The degree and methods of purification of collagen products are different, which directly affects the preservation of the collagen structure. In some cases, the collagen structure is preserved (native collagen), and in other cases it is not (fractionated collagen). It is supposed that cleaved collagen with a degraded collagen matrix still maintains properties inherent to a three-dimensional spiral molecule (42). To increase the tensile strength of fibrillar protein, some companies use collagen cross-linking or multiple lamination methods. Each approach has its own potential advantages and drawbacks.

Manufacturing of biomaterials based on ***native collagen*** allows complete preservation of its natural structure and removal of cell elements carrying specific cellular markers (melanocytes, macrophages, and lymphocytes) as well as portions of blood vessels and hair follicles that ensure low antigenicity of collagen devices (43).

When introduced into the wound, native collagen plays the role of an exogenous matrix, which stimulates fibroblast/macrophage chemotaxis, providing the basis for directed migration of cell components of the wound bed. As a result, the collagen implant activates fibroblast proliferation and secretion, which secures angiogenesis and stimulates activity of immune cells (lymphocytes and macrophages) that take part in the regulation of regeneration. Finally, the native exogenous collagen gradually resorbs and is substituted by the recipient's own connective tissue. Therefore, native collagen is a kind of "stencil" for the formation of the recipient's own tissue, which makes it far more superior compared to products containing cleaved collagen (29, 43, 44).

Examples of products containing native collagen include the following: (1) Integra (Integra Life Sciences Corp, Plainsboro, New Jersey, USA) – pigskin collagen matrix; (2) Collost biomaterial (BioPHARMAHOLDING LLC) – cattle dermis collagen containing mostly type I collagen; and (3) Primatrix (TEI Biosciences Inc, Boston, Massachusetts, USA) – acellular collagen derived from bovine foetal dermis containing mostly type III collagen.

Type I collagen is the most studied species. In the *in vitro* experiments, it accelerated formation of the intercellular matrix by dermal fibroblasts (45). The native type I collagen binds a number of proteases and inflammatory cytokines (including neutrophil elastase, MMP-2, interleukins (IL) IL-6, IL-8 and IL-1, superoxide-anion, and peroxynitrate), which are abundant in the wound exudate from a chronic wound/ulcer (46, 47). In an *in vivo* study on epithelial cells derived from dermal microvessels, type I collagen was shown to activate angiogenesis (48). These studies demonstrated that topical application of type I collagen modulates the milieu of the chronic wound showing the effect as soon as 2 weeks after collagen application (47).

Type III collagen is a homotrimeric fibrillar collagen that dominates at the first step of wound healing, ensuring the initial cell migration and differentiation. Studies with a COL3-negative mouse model have shown that type III collagen modulates scar formation through the effect on migration and differentiation of myofibroblasts. The absence of type III collagen resulted in disruption of structure, and its relative deficit led to an increased scar size and faster wound closure compared to normal COL3-positive mice (49). The results of these studies call into question the higher effectiveness of type III collagen in the healing of chronic wounds. However, there are data attesting to the possible transformation of type III collagen to type I collagen (50). In addition, the experimental study using the pig model and recombinant type III collagen gel demonstrated its wound healing effectiveness (51). However, the gel was used as a delivery agent for cultured autologous skin cells (keratinocytes and fibroblasts), which promoted the formation of wound granulation tissue.

Fractionated collagen acts as a bioactivator that employs collagen molecule fragments to recruit macrophages and fibroblasts for wound healing. Fractionated collagen functions as a pseudosubstrate for MMPs; hence, fractionated collagen binds and inactivates MMPs, thereby suppressing proteolysis of the intercellular matrix proteins.

An example of fractionated collagen is Cellerate Rx (Wound Care Innovations, Ft. Lauderdale, FL, USA), within which collagen fibrils are cleaved to approximately 1/100 the length of the original fibrils, which results in disruption of virtually all internal bonding and an increased rate of collagen integration. However, the structural advantages of collagen are lost. Fractionated collagen is supposed to act as a bioactivator that employs collagen fragments to recruit macrophages and fibroblasts. The other examples are the so called "foamed" collagens, which manufacturers claim have a production method that is based on collagen degradation with subsequent blending of collagen with cellulose. This enables preservation of a classical triple-helical collagen structure that promotes binding of elastase, activating the MMP action (52, 53). Products of this kind include Prisma and Promogran (Systagenix, North Yorkshire, United Kingdom), which contain approximately 55% collagen and 44% cellulose and, in the case of Prisma, an additional 1% of silver ions bound to the cellulose matrix. Biostep and Biostep Ag (Smith & Nephew, Largo, FL, USA) are also products of this kind. They contain collagen, carboxymethyl cellulose and sodium alginate, which facilitate cell migration and tissue regeneration. Biostep additionally contains ethylenediaminetetraacetic acid, which inhibits proteolytic enzymes in chronic wounds thereby improving wound healing. Puracol Plus and Puracol Plus AG (Medline Industries, Mundeline, IL, USA) contain pure collagen without additives.

Tarlton J.F. et al. in 2013 showed that fractionated collagen Promogran can decrease the MMP activity in all types of chronic wounds. However, the effectiveness of this compound depends on the acidity of the milieu as any modulation of proteolytic activity is lost at a neutral pH (54).

It was suggested that cleavage of collagen increases the rate of its integration. However, during collagen fractionation, the structural advantages of the collagen matrix are lost. In addition, the benefits of an increased rate of collagen integration are arguable as it entails the necessity of frequent repeated use of the compound. In contrast, the intact collagen is resistant to lymphocyte action and is retained longer in the wound bed acting as an exogenous matrix (55). Another shortcoming of fractionated and subsequently cross-linked collagen is its densely packed structure that prevents penetration of fibroblasts and their interaction with the collagen matrix (56).

In 2016, Wiegand C. et al. conducted a study to evaluate the physiological effect of the native and fractionated collagen on a chronic wound healing process (57). It was shown that fibroblasts seeded onto the native collagen matrix demonstrated exponential growth, whereas the proliferation rate of fibroblasts on the fractionated collagen matrix was very low. The use of native collagen is accompanied by a more effective and significant MMP sequestration. In addition, native collagen causes a marked *in vitro* stabilization of the platelet-derived growth factor BB (PDGF-BB).

Considering the results of the above-mentioned studies, one can surmise that native but not fractionated collagen, mostly of type I, is preferable for the treatment of trophic ulcers and protracted non-healing wounds.

USE OF NATIVE COLLAGEN IN THE TREATMENT OF DIABETIC FOOT SYNDROME

It was shown that the use of native collagen products promotes chronic wound area reduction (58). Native collagen products (Collost) promote faster wound pro-

cess transition to an active regeneration stage and lower wound contamination by microorganisms (59, 60). In a study by Ivanus S.Ya., by day 12 of treatment with Collost, the wound's level of contamination did not exceed 10^5 CFU/g (61). Use of Collost led to a 1.8-fold reduction in time of wound preparation for autodermoplastic closure (32.0±4.6 days versus 56.8±8.7 days in the comparator group) (62). Preliminary results of a multicenter, randomized prospective clinical trial encompassing 71 patients with diabetic foot syndrome Wagner II – III showed that native collagen (Collost) effectively decreased the length, width, area and volume of diabetic ulcers so that epithelialization could occur faster (63, 64). After 4 weeks of treatment, complete epithelialization was observed in 22.2% of patients from the main group (Collost) and in 8.6% of patients from the comparator group (standard therapy). In the main group, they managed to achieve reduction of the wound area by 67% versus 39% in the comparator group, compared to corresponding values obtained on day 1 (65).

The other multicenter, randomized, controlled clinical trial that enrolled 307 patients also demonstrated a shorter time to complete diabetic ulcer closure in patients receiving native collagen product (Integra) (66). Use of native collagen reduces the pain syndrome in patients with chronic wounds including those with DFS (58, 59, 67).

It is important to emphasize that the use of native collagen products does not produce side effects (58, 59, 63, 64, 68), which attests to high safety of this treatment.

Inclusion of native collagen-based biomaterials in the treatment regimen for DFS also demonstrated its cost-effectiveness. For instance, it was shown that Collost brings down the resulting necessary cost to the treatment effectiveness ratio (69).

Comparative evaluation of native collagen products versus other up-to-date medical products used for topical treatment of DFS demonstrated high relative effectiveness and safety of the former. In a comparative clinical study, the use of Collost membrane and epidermal growth factor-based formulation Heberprot-P in patients with DFS showed the superiority of the collagen-based biomaterial (70). There was a higher rate of positive dynamics in the course of the wound process, a higher incidence of complete epithelialization (complete healing or positive dynamics in 89% of patients in the Collost group and 19% in the comparator group), fewer amputations (0% and 32%, respectively) and fewer cases of individual intolerance to native collagen products (Collost biomaterial) (70).

A comparative study of Apligraf living cell therapy and PriMatrix native collagen in the treatment of diabetic (n=40) and venous (n=28) foot ulcers revealed high effectiveness of both methods. It was found that ulcers in patients receiving PriMatrix healed faster than in patients treated with Apligraf despite a larger wound area in the former group (56).

Russian scientists have developed a patented method for DFS therapy that comprises the sequential use of conservative therapy, which includes surgery combined with an ultrasound hydrosurgery procedure and application of a native collagen-based product; this method allowed the authors to reduce the incidence of organ removal surgery and complications by 15-33%, shorten duration of the inpatient stay by 20% and decrease the number of return visits by 13.4-18.5%, which has been demonstrated in a large study in 1195 patients (60, 71, 72).

USE OF FRACTIONATED COLLAGEN IN PATIENTS WITH DIABETIC FOOT SYNDROME

Lobmann R. et al. (2006) assessed the effects of Promogran in 33 patients with DFS and found no statistically significant differences in the levels of MMP mRNA, IL-1β and TNF-α compared to those in the control group. In addition, the MMP levels in the wound measured by an ELISA also did not differ significantly between the groups. However, the IL-1β level was elevated on day 8 only in the Promogran group (p=0.01), and a significant reduction in the MMP-9/TIMP-2 ratio was found in the collagen treatment group. Nonetheless, the latter group demonstrated a higher rate of wound healing (73).

Similar results were obtained by Motzkau M. et al. (2011) in a randomized study in 19 patients with DFS of whom 13 patients received fractionated collagen (74). A 26-day observation did not reveal any differences in the expression of MMP mRNA, TNF-alpha and other MMPs in the wound tissue. At the same time, the authors' statements regarding statistically significant reduction of the wound area in the group receiving collagen (p = 0.003) werewas more than doubtful considering the small sample size.

The effectiveness of fractionated collagen products can be improved. In this regard, Kakagia D.D. et al. showed that the efficacy of Promogran alone is lower than that of Promogran combined with growth factors (75).

The largest trial on the effectiveness of fractionated collagen in patients with DFS is the study by Veves A. et al. (76), which was carried out in 276 patients aged between 23 and 85 years who had been randomly assigned to the Promogran group (n=138) and the mock-treated group (wet dressing, n=138). After 12 weeks of treatment, 37% of patients from the Promogran group demonstrated complete wound closure versus 28% in the mock-treated group (p=0.12). More pronounced differences were found for patients with ulcers of < 6 months duration (45% and 33%, respectively, p=0.056); however, the difference did not reach statistical significance. Therefore, this large multicenter study failed to demonstrate the significance of effectiveness of fractionated collagen in the treatment of patients with DFS.

Therefore, the analysis of results obtained with products of fractionated collagen did not reveal conclusive benefits of its use in patients with DFS compared to studies employing native collagen products.

CONCLUSION

Treatment of chronic wounds is in a continuously advancing direction. The issues of excessive mechanical forces, wound infection and inflammation, decreased production of growth factors and, of course, collagen deficits, will have an impact on treatment outcomes. Numerous studies have shown that collagen-based products are bioactivators and promote regeneration of the recipient's own tissues through integration with the surrounding tissues. Their major benefits are the regulation of the biochemical milieu of the wound and the stimulation of chemotaxis and angiogenesis. These products resemble a thin layer of the natural skin but are free from the shortcomings inherent in foreign cell elements associated with skin graft rejection.

However, not all collagen-based products and devices are the same. They differ in composition and degree of preservation of the natural collagen matrix. Native (noncleaved) collagen serves as a matrix for directed migration of macrophages and fibroblasts into the wound bed, and it activates chemotaxis, proliferation and secretory activity of cell elements. During the course of wound healing, the collagen is dissolved and replaced by the recipient's own connective tissue. The native collagen-based products modulate protease activity and stimulates production of the recipient's own collagen.

Unlike native collagen, fractionated (cleaved) collagen interacts less actively with fibroblasts because of its densely packed components; accordingly, it has a less pronounced influence on the rate of cell migration and proliferation. However, the effectiveness of collagen products depends on the acidity of the milieu and it goes down at a neutral pH.

The advantages of physiological action of native collagen-based products are confirmed by their clinical effectiveness. Native collagen-based products promote accelerated transition of the wound process to the regeneration stage, shorten the chronic wound healing time, help to decrease the chronic wound area, reduce the extent of bacterial contamination, alleviate the pain syndrome and reduce the recurrence rate. The administration of native collagen-based products enables ingthe reduction of the resulting necessary cost tothe treatment effectiveness ratio.

Therefore, administration of native collagen-based products is a fairly promising direction in the treatment of chronic ulcers and wounds, including those in patients with diabetic foot syndrome. This enables improvement ofin the effectiveness of treatment and reduces the cost of providing medical care for such patients.

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UPDATE OF ANTIPLATELET THERAPY IN PATIENTS WITHOUT KNOWN CARDIOVASCULAR DISEASE

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ANTITROMBOCITNA TERAPIJA KOD BOLESNIKA KOJI NEMAJU POZNATU KARDIOVASKULARNU BOLEST

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ABSTRACT

Platelet activation and aggregation play a critical role in thrombosis, a fundamental pathophysiologic event responsible for the acute clinical manifestations of atherothrombotic events such as acute coronary syndrome, myocardial infarction, ischemic stroke/transient ischemic attack and peripheral artery disease. Dual antiplatelet therapy (low-dose aspirin plus ADP-P2Y12 receptor blockers) has become the cornerstone of therapy for the management of acute and chronic coronary artery disease and the prevention of ischemic complications associated with percutaneous coronary intervention. However, dual antiplatelet therapy in primary prevention of cardiovascular disease in patients without known cardiovascular disease did not significantly reduce the risk of cardiovascular events, such as myocardial infarction, stroke or death, but significantly increased the rate of bleeding. Furthermore, despite multiple randomized controlled trials evaluating the efficacy and safety of aspirin use in patients without known cardiovascular disease, its role in primary prevention is still unclear, especially in patients with a higher risk of cardiovascular disease (non-diabetic individuals with ≥2 risk factors for coronary artery disease, elderly ≥60 years with additional risk factors, and patients with diabetes). Currently, there are four ongoing randomized controlled trials aiming to fill the missing gap in the efficacy and safety of aspirin therapy for primary prevention in these patients. The current European and United States Guidelines agree that primary prevention of cardiovascular disease is essential, but there are some substantial differences in risk estimation and treatment strategies among patients without known cardiovascular disease. This short review is focused on these differences and practical treatment approach to these patients based on present European and United States recommendations.

Keywords: *Antiplatelet, aspirin, primary prevention, cardiovascular disease, coronary artery disease.*

SAŽETAK

Aktivacija i adhezija trombocita imaju glavnu ulogu u procesu tromboze koja predstavlja osnovni patofiziološki događaj odgovoran za razvoj akutnih kliničkih manifestacija aterosklerotske bolesti arterija, kao što su akutni koronarni sindrom, infarkt miokarda, ishemijski moždani udar/tranzitorni ishemijski atak ili periferna arterijska bolest. Dvojna antitrombocitna terapija (aspirin plus blokatori trombocitnih ADP-P2Y12 receptora) predstavlja kamen temeljac u lečenju bolesnika sa akutnim i hroničnim oblicima ishemijske bolesti srca, kao i u prevenciji neželjenih ishemijskih kardiovaskularnih događaja, naročito nakon elektivne ili primarne perkutane koronarne intervencije. Ipak, primena dvojne antitrombocitne terapije u primarnoj prevenciji kod bolesnika koji nemaju kardiovaskularnu bolest nije značajno smanjila rizik od neželjenih kardiovaskularnih događaja, kao što su infarkt miokarda, šlog ili smrt, ali je značajno povećala rizik od krvarenja. Takođe, uprkos brojnim randomizovanim studijama koje su analizirale efikasnost i bezbednost primene aspirina kod bolesnika koji nemaju kardiovaskularnu bolest, njegova primena u primarnoj prevenciji još uvek nije dovoljno jasna, naročito kod bolesnika koji imaju visok rizik za pojavu neželjenih kardiovaskularnih događaja (nedijabetičari sa >2 faktora rizika za koronarnu bolest, starije osobe >60 godina života sa pridruženim faktorima rizika za koronarnu bolest, i pacijenti sa dijabetesom). Trenutno su u toku četiri randomizovane studije čiji je primarni cilj analiza efikasnosti i bezbednosti primene aspirina u primarnoj prevenciji kod visoko rizičnih bolesnika. Važeće evropske i američke preporuke slažu se da je kod svih individua neophodna primarna prevencija od pojave kardiovaskularnih bolesti, ali postoje određene razlike između ovih preporuka koje se odnose na procenu kardiovaskularnog rizika i terapijskog pristupa kod visoko rizičnih bolesnika koji nemaju poznatu kardiovaskularnu bolest. Ovaj pregledni članak fokusiran je na ove razlike, kao i na terapijsku stategiju u primarnoj prevenciji kod ovih bolesnika, u skladu sa važećim evropskim i američkim preporukama.

Keywords: *Antitrombocitna terapija, aspirin, primarna prevencija, kardiovaskularna bolest, koronarna bolest.*

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ABBREVIATIONS

ADP - adenosine-diphosphat;
ACS - acute coronary syndrome;
CAD - coronary artery disease;
CHD - coronary heart disease;
CV - cardiovascular;
CVD - cardiovascular disease;
DAPT - dual antiplatelet therapy;
GI - gastrointestinal bleeding;
MI - myocardial infarction;
NSAIDs - non-steroid anti-inflammatory drugs;
PAD - peripheral artery disease;
PCI - percutaneous coronary intervention;
RCT - randomized controlled trial;

INTRODUCTION

Platelet activation and aggregation play a critical role in thrombosis, a fundamental pathophysiologic event responsible for the acute clinical manifestations of atherothrombotic events such as acute coronary syndrome (ACS), myocardial infarction (MI), ischemic stroke/transient ischemic attack and peripheral artery disease (PAD)(1). Inhibition of platelet -function by combined use of aspirin (acetylsalicylic acid, ASA) and adenosine-diphosphat (ADP)-P2Y12 receptor blockers is an important strategy for preventing ischemic cardiovascular (CV) events in patients with acute and chronic coronary artery disease (CAD), including those undergoing percutaneous coronary intervention (PCI)[1]. Therefore, dual antiplatelet therapy (DAPT) has become the cornerstone of therapy for the management of acute and chronic CAD and the prevention of ischemic complications associated with PCI (1-2). However, DAPT for primary prevention of cardiovascular disease (CVD) in individuals with multiple risk factors for CAD did not significantly reduce the risk of CV events, such as MI, stroke, or CV death[3]. Only one study has evaluated the efficacy and safety of DAPT for primary CVD prevention in individuals with multiple risk factors for CAD or in patients with documented CAD, PAD or cerebrovascular disease (3). The CHARISMA trial showed that DAPT (clopidogrel 75 mg/d in combination with low-dose aspirin75-100 mg/d) was not significantly more effective than aspirin alone (75-100 mg/d) in reducing the rate of total CV events (MI, stroke, or CV death) among these patients (OR, 1.20 [95% CI, 0.91-1.59] for individuals with multiple risk factors for CAD; OR, 0.88 [95% CI, 0.77-0.998] for patients with documented CAD, PAD or cerebrovascular disease)[3]. Contrary, this study suggested that DAPT was associated with a significant increase in the rate of GUSTO-defined moderate bleeding (OR, 1.62 [1.27–2.08]), especially in patients with documented CAD, PAD or cerebrovascular disease (3).

The role of aspirin in the treatment of acute CV events as well as for secondary prevention of future CV events has been well established (4-9). Despite multiple randomized controlled trials (RCTs) evaluating the efficacy and safety of aspirin use in patients without known CVD, its role in primary prevention is still unclear (10). The minority of RCTs evaluating the use of aspirin in primary CVD prevention included patients with a higher-risk for CVD, and failed to show a significant benefit of aspirin therapy in primary prevention (10). Currently, there are four ongoing RCTs aiming to fill the missing gap in the efficacy and safety of aspirin therapy for primary prevention in these patients (10-14).

ANTIPLATELET THERAPY IN PRIMARY PREVENTION OF CARDIOVASCULAR DISEASE

Aspirin is the only antiplatelet drug investigated for primary prevention in cardiovascular disease (CVD) (15). Aspirin is irreversible non-selective inhibitor of cyclooxygenase enzyme (COX-1 and COX-2, respectively) that is responsible for formation of prostanoids, including prostaglandins, prostacyclins and platelet thromboxane (TxA2) (1-2). It prevents synthesis of TxA2 from arachidonic acid and therefore TxA2-induced platelet aggregation (2, 16). Although efficacy and safety of aspirin in the treatment of patients without known CVD have been studied in 10 RCTs, the role of aspirin for the primary CVD prevention is still unclear (2, 10, 15-16). Meta-analysis of six RCTs with more than 95,000 patients without known CVD showed that aspirin monotherapy is associated with 12% proportional reduction (odd ratio [OR], 0.88 [95% CI, 0.82-0.94]) in serious CV events (MI, stroke, or CV death), mainly due to a 23% proportional reduction in non-fatal MI (OR, 0.77 [95% CI, 0.67-0.89]), with no clear reduction in stroke (OR, 0.95 [95% CI, 0.85-1.06]), and CV mortality (OR, 0.95 [95% CI, 0.78-1.15]) (17). The efficacy of aspirin monotherapy in reduction of CV events, especially non-fatal MI, was even higher in patients at moderate (10-20%) and high (>20%) Framingham coronary heart disease (CHD) risk, without significant reduction in stroke and CV mortality regardless of the patient CHD risk (15, 18). In another meta-analysis of six RCTs comparing benefits and risks of aspirin treatment for primary CVD prevention by sex, aspirin was associated with a significant 14% reduction in CV events for men (OR, 0.86 [95% CI, 0.78-0.94]) and 12% reduction in CV events for women (OR, 0.88 [95% CI, 0.79-0.99]), respectively (10, 19). Men were shown to have a 32% reduction in MI (OR, 0.68 [95% CI, 0.54-0.86]), but without sig-

Table 1. 10-years risk for CAD and stroke in men and women, respectively, at which the number of CV events prevented is closely balanced to the number of serious bleeding events (Modified from Ann Intern Med 2009;150:396-404).

Men		Women	
Age, years	10-year CAD risk, %	Age, years	10-year stroke risk, %
45-59	≥4	55-59	≥3
60-69	≥9	60-69	≥8
70-79	≥12	70-79	≥11

CAD - coronary artery disease

nificant reduction in stroke and CV mortality (19). Women were shown to have a 17% reduction in stroke (OR, 0.83 [95% CI, 0.70-0.97]), especially ischemic stroke (OR, 0.76 [95% CI, 0.63-0.93]), but without significant reduction in CV mortality and MI (19). However, there was a significant increase in risk of major bleeding in both men and women ([OR, 1.72 [95% CI, 1.35-2.20] in men; OR, 1.68 [95% CI, 1.13-2.52] in women) (19).

The optimum dose of aspirin for preventing future CV events is still unclear (20). Although RCTs for primary CVD prevention have demonstrated benefits with various regimens (75 mg and 100 mg per day, and 100 and 325 mg every other day), it seems that 75-100 mg dose of aspirin per day is effective as higher dosages (10, 20).

Conversely, the risk of bleeding events, such as major gastrointestinal (GI) and extracranial bleedings, may increase with aspirin dose (20). Additionally, bleeding risk is higher in patients at moderate and high CHD risk, as the main risk factors for CAD are also risk factors for bleeding (15, 17, 20). Recently, U.S. Preventive Services Task Force (USPSTF) Systematic Review of RCTs, cohort studies and meta-analyses comparing aspirin with placebo or no treatment for primary CVD prevention was published[21]. Cardiovascular primary prevention studies of aspirin, used over 3.8 to 10.1 years, showed a 59% increased risk for major GI bleeding (OR 1.59 [95% CI, 1.32-1.91]) and 33% increased risk for hemorrhagic stroke (OR, 1.33 [95% CI, 1.03-1.71]), regardless of aspirin dose. The only study with significant increase in hemorrhagic stroke (OR, 1.84 [95% CI, 1.01-3.35]) was conducted in an older hypertensive patients (21-22). Meta-analysis of six primary prevention RCTs showed that the odds of hemorrhagic strokes were significantly increased in men (OR, 1.69 [CI, 1.04 to 2.73]), but not in women (OR, 1.07 [CI, 0.42 to 2.69]) (19). Furthermore, conventional risk factors for CAD, such as age, male sex, diabetes, smoking and high blood pressure were also identified as significant risk factors for major bleeding as well (21-22). The study by Hernandez et al. has determined that significant risk factors for GI bleeding were history of peptic ulcer disease or complications, and concomitant use of non-steroid anti-inflammatory drugs (NSAIDs), anticoagulants, or other antiplatelet drug (21-23).

According to previous statements, it is essential to estimate the individual baseline risk for CV events and the absolute risk for bleeding with aspirin use, and determining its net benefit (15, 17, 21-22). The most commonly used tools for assessing baseline risk for CV events are the Framingham CHD risk score preferred by USPSTF, American Heart Association (AHA), American College of Cardiology (ACC), and American College of Chest Physicians (ACCP), and the Systematic Coronary Risk Evaluation (SCORE) preferred by European Society of Cardiology (ESC). The Framingham CHD risk score predicts the 10-year risk of CV events (composite of MI and CV death), while the SCORE system estimates the 10-year risk of fatal CVD event (death from CAD, stroke or aneurysm of the abdominal aorta) (17-18, 24-25). It is estimated that the risk of total (fatal and nonfatal) CVD events is approximately 3-times higher than the risk of fatal CVD for men, and 4-times higher in women (26).

Recently, a simple risk prediction tool for upper GI complications has been proposed (27). This tool has several disadvantages, including the incorporation of approaches to modifying the bleeding risk that are not empirically proven in a patients without known CVD and insufficient external validation to confirm its readiness for clinical application (21, 28-29).

According to USPSTF guidelines for primary CVD prevention, the net benefit of aspirin use in men depends on the initial risk for CAD events and GI bleeding, while the net benefit in women depends on the initial risk for ischemic stroke events and GI bleeding (20). Based on this data, USPSTF declared that „aspirin use for the primary CVD prevention provides more benefits than harms in men or women whose risk for MI or ischemic stroke, respectively, is high enough to outweigh the risk for GI hemorrhage" (20). The USPSTF encourage both men (age 45 to 79 years) and women (age 55 to 79 years) to use aspirin when the potential benefit of a reduction in MI and ischemic stroke, respectively, outweighs the potential harm of an increase in GI hemorrhage (Table 1). Evidence on the benefits in men <45 years and in women <55 years is limited, and the potential benefit in this age group is probably low because the risk for MI in men as well as the risk for stroke in wom-

Table 2. Fundamental recommendations for aspirin use for the primary CVD prevention from the U.S. Preventive Services Task Force (USPSTF), the American College of Chest Physicians (ACCP), the American Heart Association (AHA), the European Society of Cardiology (ESC), and the American Diabetes Association (ADA)/AHA/American College of Cardiology (ACC) Guidelines.

<table>
<tr><th></th><th colspan="4">Recommendations for aspirin use in primary CVD prevention</th></tr>
<tr><td>2009 USPSTF Guidelines[20]</td><td>Encourage men age 45 to 79 years to use aspirin when the potential benefit of a reduction in MI outweighs the potential harm of an increase in GI hemorrhage (A recommendation)</td><td>Encourage women age 55 to 79 years to use aspirin when the potential benefit of a reduction in ischemic strokes outweighs the potential harm of an increase in GI hemorrhage (A recommendation)</td><td>Do not encourage aspirin use for MI prevention in men younger than 45 years and for stroke prevention in women younger than 55 years (D recommendation)</td><td>There are no recommendations for aspirin use in primary CVD prevention in men and women 80 years or older (I statement)</td></tr>
<tr><td>2012 ACCP Guidelines[18]</td><td colspan="4">Suggest the use of low-dose aspirin (75 to 100 mg daily) for persons aged 50 years or older. (Grade 2B)</td></tr>
<tr><td>2002 AHA Guidelines and 2011 Update[24-25]</td><td colspan="2">The use of low-dose aspirin (75-160 mg daily) is reasonable in persons with moderate (10-20%) and high (>20%) 10-year CHD risk (Class IIa; Level of Evidence B)</td><td colspan="2">Aspirin therapy can be useful in women aged 65 years or older if blood pressure is controlled and benefit for ischemic stroke and MI prevention is likely to outweigh risk of GI bleeding and hemorrhagic stroke (Class IIa; Level of Evidence B); and may be reasonable for women aged 65 years or younger for ischemic stroke prevention (Class IIb; Level of Evidence B)</td></tr>
<tr><td>2016 ESC Guidelines[26]</td><td colspan="2">Antiplatelet therapy is not recommended in individuals without CVD due to the increased risk of major bleeding (Class III; Level of Evidence B)</td><td colspan="2">Antiplatelet therapy (e.g. with aspirin) is not recommended for individuals with DM who do not have CVD (Class III; Level of Evidence A)</td></tr>
<tr><td>2010 ADA/AHA/ ACCF Guidelines for aspirin use in primary CVD prevention in people with diabetes[39]</td><td colspan="4">1. Low-dose (75–162 mg daily) aspirin use for primary CVD prevention is reasonable for diabetic patients without known CVD whose 10 year risk of CV events is >10%, and who are not at increased risk for bleeding (no history of previous GI bleeding or peptic ulcer disease, or concurrent use of other medications that increase bleeding risk, such as NSAIDS or warfarin). Those adults with diabetes at increased CVD risk include most men over age 50 years and women over age 60 years who have 1 or more of the following additional major risk factors: smoking, hypertension, dyslipidemia, family history of premature CVD, and albuminuria (ACCF/AHA Class IIa; Level of Evidence B) (ADA Class IIa; Level of Evidence C)

2. Aspirin should not be recommended in diabetes patients at low risk of CV events (men under age 50 years and women under 60 years with no major additional CVD risk factors; 10-year CVD risk under 5%) because the potential adverse effects from bleeding offset the potential benefits (ACCF/AHA Class III; Level of Evidence C) (ADA Class III; Level of Evidence C)

3. Low-dose (75–162 mg dialy) aspirin use for primary CVD prevention might be considered for diabetic patients without known CVD at intermediate CVD risk (younger patients with 1 or more risk factors, or older patients with no risk factors, or patients with 10-year CVD risk of 5–10%) (ACCF/AHA Class IIb; Level of Evidence C) (ADA Class IIb; Level of Evidence E)</td></tr>
</table>

CHD - coronary heart disease; CVD - cardiovascular disease; MI - myocardial infarction; GI - gastrointestinal bleeding.

en is very low (20). The risk of MI and stroke is high in patients older than 80 years as well as the risk of GI bleeding, and thus, the net benefit of aspirin use in octogenarians is probably much better in those without concomitant risk factors for GI bleeding (20). The ACCP guidelines for primary CVD prevention have followed similar approach to the use of aspirin (18). The 2002 AHA guidelines and 2011 update to the AHA guidelines for primary prevention of CVD and stroke recommended that both men and women with a ≥10% 10-year risk of CHD consider taking a daily low-dose aspirin (Table 1) (24-25).

Four additional RCTs that evaluated aspirin use in asymptomatic high CVD risk patients with pre-existing diabetes, PAD, or both showed that there were no differences in the reduction of total CV events (MI, stroke, or death) with and without use of low-dose aspirin (81-100 mg daily) (30-34). Four meta-analyses of the most aforementioned RCTs have been published recently and suggested superiority of aspirin for total CV events and non-fatal MI, but without significant reduction in stroke, CV mortality and all-cause mortality (35-38). However, there was no clear evidence of benefit in aspirin use for primary CVD prevention in these patients because of increased risk of major bleeding and hemorrhagic stroke (35-38). According to meta-analysis by Berger et al., for every 1,000 individuals treated with aspirin over a 5-year

period, aspirin would prevent 2.9 total CV events and cause 2.8 major bleeds (36). Other meta-analysis by De Berardis et al., evaluated the use of aspirin in individuals with diabetes and no CVD, and demonstrated no significant reduction in the risk of major CV events (OR, 0.90 [95% CI, 0.81-1.00]), CV mortality (OR, 0.94 [0.72 to 1.23]), or all-cause mortality (OR, 0.93 [0.82 to 1.05]) (38). Based on these data, the 2016 ESC guidelines on CVD prevention did not recommend the use of aspirin in high CVD risk patients regardless of the pre-existing diabetes (Table 2) (26). Conversely, the American Diabetes Association (ADA), the AHA, and the ACC recommend the use of low-dose aspirin (75-162 mg daily) for primary CVD prevention in diabetic patients whose 10-year risk of CV events is >10% (men age >50 years and women age >60 years with at least 1 additional risk factor: smoking, hypertension, dyslipidemia, family history of premature CV events, or albuminuria) and who are not at increased risk of bleeding (no history of previous GI bleeding or peptic ulcer disease, no concurrent use of other medications that increase bleeding risk, such as NSAID or warfarin) (Table 2) (39). Currently, there are four ongoing RCTs (ASCEND, ACCEPT-D, ASPREE, ARRIVE) aiming to fill the missing gap in the efficacy and safety of low-dose aspirin therapy for primary CVD prevention in high CHD risk individuals (non-diabetic individuals with ≥2 or ≥3 risk factors for CAD, elderly ≥60 years with additional risk factors, and patients with diabetes) (11-14).

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JUVENILE TYPE GRANULOSA CELL TUMOR

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JUVENILNI TIP GRANULOZA ĆELIJSKOG TUMORA

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ABSTRACT

Granulosa cell tumor is a type of neoplasm, which represents 2-5% of all ovarian cancers. About 5% of these tumors are juvenile-type and usually occur to girls before puberty and to women younger than thirty years of age. There are signs premature puberty or premature emergence of secondary sexual characteristics with irregular vaginal bleeding that occur to these kind of patients. To the rare cases, like this, the occurrence of granulosa cell tumors can cause the appearance of hyperandrogenism with high levels of plasma testosterone, leading to virilization which happened to this female patient. We will present the female patient who was 35 years old and which was originally hospitalized to the Clinic for Haematology Clinical Center Kragujevac, because of extreme fatigue accompanied by dizziness. During diagnostics the patient underwent to the complete gynecological examination. After gynecological examinations and necessary diagnostic procedures, it was decided continuing the treatment at the Clinic of Gynecology and Obstetrics Clinical Center Kragujevac, where she underwent a total abdominal hysterectomy with bilateral salpingo-oophorectomy for suspected uterine neoplasm. Histopathological analysis of the obtained material confirmed the presence of follicular cysts of both ovaries and juvenile type granulosa cell tumor on the right ovary; the uterus was enlarged with multiple fibroid tumors. Granulosa cell tumor should be suspected in the cases of girls and young females if there is present an ovarian cyst paired with signs of preterm puberty or hyperestrogenism. In this case, the presence of granulosa cell tumor was masked by signs of hyperandrogenism, which is not so typical, as well as the presence of uterine fibroids who have actually been the main cause for surgical treatment.

Keywords: *juvenile-type granulosa cell tumor, hyperandrogenism, virilization.*

SAŽETAK

Tumor granuloza ćelijskog tipa je neoplazma koja predstavlja 2-5% svih karcinoma ovarijuma.Juvenilni tip čini 5% ovih tumora i obično se javlja kod devojčica pre puberteta i kod žena mlađih od trideset godina.Kod ovakvih pacijentkinja javljaju se znaci preveremenog puberteta odnosno prevremena pojava sekundarnih polnih karakteristika uz iregularna vaginalna krvarenja.U veoma retkim slučajevima pojava granuloza ćelijskog tumora može usloviti pojavu hiperandrogenizma sa visokim vrednostima testosterona u plazmi,koji dovode do virilizacije kao kod naše pacijentkinje.Ovde je prezentovan slučaj pacijentkinje koja ima 35 godina i koja je inicijalno primljena na Kliniku za Hematologiju KC Kragujevac,zbog izrazite malaksalosti praćene vrtoglavicom. U toku dijagnostike konsultovan je ginekolog.Nakon obavljenog ginekološkog pregleda i neophodnih dijagnostičkih procedura, odlučeno je da se lečenje nastavi na Klinici za Ginekologiju i Akušerstvo KC Kragujevac,gde je urađena totalna abdominalna histerektomija sa obostranom adneksektomijom zbog sumnje na neoplazmu uterusa.Histopatološka analiza dobijenog materijala je potvrdila obostrano prisustvo mioma u uvecanoj materici,folikularnih cista jajnika kao i juvenilni tip granuloza ćelijskog tumora na desnom jajniku. Granuloza ćelijski tumor se javlja kod devojčica i mlađih adolescentkinja uglavnom sa znacima hiperestrogenizma i prevremenog puberteta. U prezentovanom slučaju prisustvo tumora je bilo zamaskirano izraženom maskulinizacijom koja nije toliko karakteristična, kao i prisustvom mioma uterusa koji su u stvari bili glavni razlog operativnog lečenja.

Ključne reči:*juvenilni tip granuloza ćelijskog tumora,hiperandrogenizam,virilizacija.*

ABBREVIATIONS

HE4- human epididymis protein 4
MRI-Magnetic Resonance Imaging
DHT- dihydrotestosterone

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INTRODUCTION

Ovarian cancers represent fourth most common malignancy in women, after breast, lung and colorectal cancer. The incidence of their appearance ranges from 2-13 per 100,000 women; it affects women of all ages, although more than 75% of affected women are over 45 years old.[1] Ovarian cancers are divided into three histological groups:

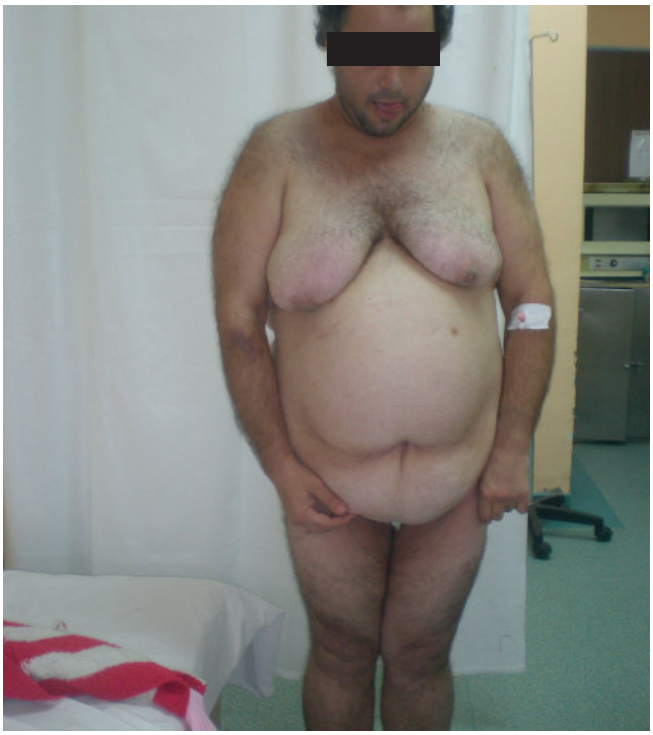
- Ovarian cancers deriving from germline cells which affects mainly young women
- Ovarian cancers of the epithelial origin typical for the elderly women
- Ovarian cancers of the stromal origin which can occur in all age groups

Granulosa cell tumor represents 2-5% of all ovarian cancers.[2,3] It originates from the granulosa cells, hormonally active ovarian stromal component, primarily responsible for the secretion of estradiol; thus, the main characteristic of these tumors is hyper production of estrogen.[4,5] Based on the histopathological and clinical characteristics granulosa cell tumor is divided into juvenile and adult type. Juvenile type of the granulosa cell tumor usually occurs to the young girls or women before thirty years of age. The presence of the juvenile form of the tumor can lead to premature puberty, or premature emergence of secondary sexual characteristics with irregular vaginal bleeding.[6] Adult form of the granulosa cell tumor can also lead to irregular vaginal bleeding but with connection to endometrial or breast carcinoma.[7] In very rare cases, granulosa cell tumor can cause hyperandrogenism, with high level of plasma testosterone which results in overt virilization of the patient.[8,9,10] We represent the case of a patient with juvenile form of granulosa cell tumor , with extreme virilization which is seen in the clinical presentation of the illness.

CASE REPORT

Thirty-five years old patient, with visible hirsutism and severe obesity, was hospitalized at the Clinic of Hematology, Clinical Center in Kragujevac, because of extreme fatigue, accompanied by vertigo and dizziness. According to the medical history, abovementioned ailments occurred a few months ago and got worse in the last couple of weeks. Furthermore, medical history showed that the patient had a menarche at the age of eighteen, and since then she had frequent, heavy and prolonged menstrual cycles. The routine blood test, taken upon submission, showed severe secondary anemia (Er 1.97, Hgb 36), while the immunochemical analysis of the concentration of the plasma sex hormones, as well as other steroids, showed only high levels of testosterone (198 ng / ml). Because of the severe hirsutism and virilization, a karyotype from the peripheral white blood cells has been done and it showed a female sex (46 XX). Afterwards, the patient underwent to the gynecological examinations, which showed hypertrophy of the clitoris with diminished vaginal opening. Considering that the patient was a virgin, gynecological exam had to be done via rectal route, and it showed uterine myomatosis with enlarged uterus, about twenty centimeters in diameter. Adnexal examination showed that both sides were physiologically available, insensitive on palpation. Following ultrasonography of the pelvis showed the enlarged uterus, 180x155x125 mm in diameter, with numerous fibroid nodes of which the largest was 6x5 cm in diameter. Dimensions of the right ovary were 45x25mm, while left was 35x20mm in diameter, with no pathological findings. Values of the tumor markers CA 125 (0-35 / ml) and HE4 (below 73 pmol/L) were within normal range. Radiography of the abdomen and thorax showed no presence of pathological changes. The MRI findings matched the ones obtained by ultrasound; it also showed enlarged uterus 185x157x125mm diameter, with numerous intramural fibroids. Both ovaries were of normal morphology, with dimensions 43x25mm for the right, and 33x19mm for the left. MRI examination also showed the absence of enlarged lymphatic nodes in the parametrical or near pelvic main blood vessels. After all examinations, a consilium's decision has been to carry on the patient treatment on Clinic of Gynecology and Obstetrics, Clinical Centre Kragujevac. Upon adequate preparation, the patient underwent operative treatment; a classic abdominal hysterectomy with bilateral salpingo-oophorectomy was done, on suspicion of uterine neoplasm. Surgery and postoperative course passed without complications, and the patient was discharged from the hospital in due course, with corresponding hormone replacement therapy. Immunochemical analysis of the concentration of the plasma sex hormones which has been done a week after surgery, were in the domain of reference values (testosterone 0.6 ng / ml).

Histopathological analysis of the obtained material was performed at the Department of Pathology of the Clinical Center in Kragujevac. Uterus weight was 1880 grams, and it diameter was 180x150x125mm, while lower part was filled with blood cloths. The tissue cross section demonstrated diffusely thickened walls composed of swirling arranged muscle cells, with proliferation of the endometrium. The right ovary was 47x34mm in diameter, while cross section showed cystic amended stroma, with cysts ranging in diameter from 4 to 8 mm, filled with clear content; and one solitary brown node 30mm in diameter. Left ovary was 48x38mm in diameter, with cross section also showing cystic changes, with cysts ranging in diameter from 3 to 9 mm, all filled with clear content. The microscopic analysis of the uterine tissue confirmed the presence of multiple fibroid tumors, while microscopically ovaries were interleaved with mutual follicular cysts, with juvenile type of granulosa cell tumor in the right ovary.



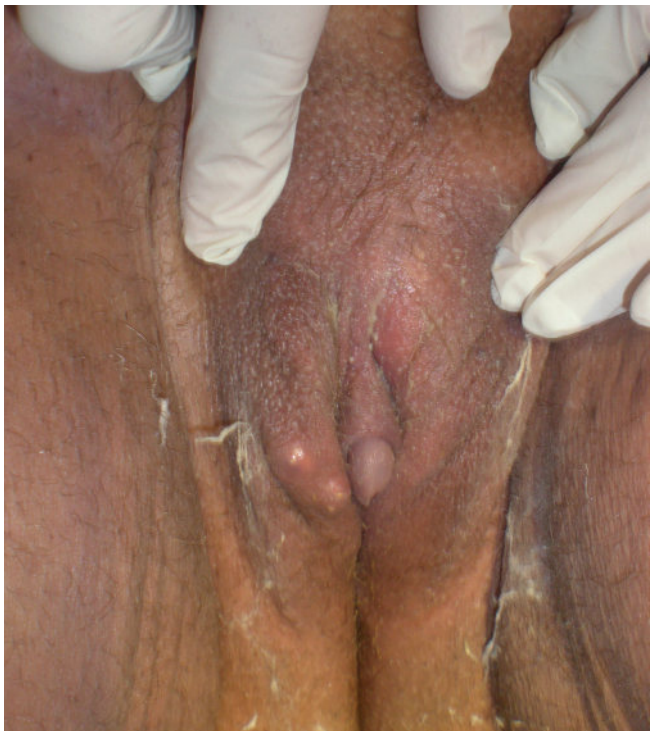
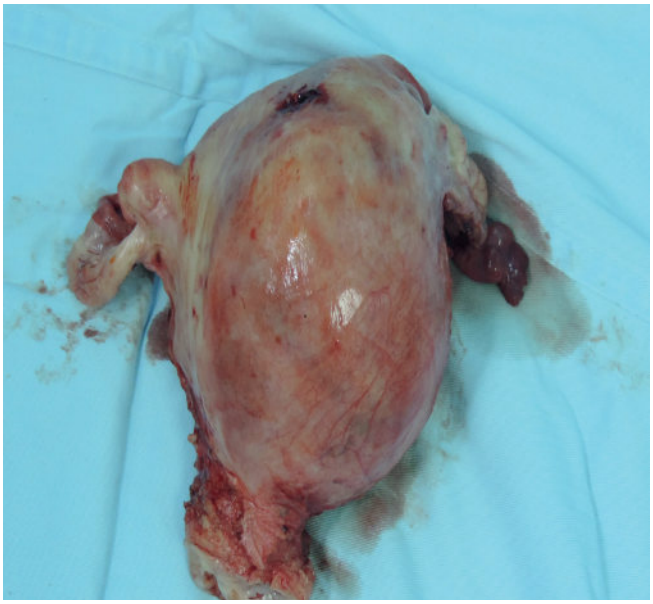


Figure 1. Severe obesity with visible hirsutism . Clitoromegaly.

DISCUSSION

Ovarian tumors are rare occurrence in girls and younger women. Granulosa cell tumor make up only 2% of all malignancies of the ovary, while juvenile type granulosa cell tumor constitutes 5% of juvenile ovarian cancers and is usually manifested in the first thirty years of life.[11,12] The granulosa cell tumors are associated with the occurrence of premature puberty, menstrual cycle disorder and in the rare cases it causes virilization. Masculinisation and virilization of the patient can be explained by the insufficiency of the enzyme aromatase in the granulosa cells of the ovarial stroma, and the overproduction of androgens instead estrogens.[8,9,10] In the rare and the most extreme cases, hyperadrogenism can lead to the appearance of hirsutism, accelerated growth and clitoromegaly, to a such degree that it raises the suspicion of genetically determined sex, as it was shown in this case report. The effect of the androgen hyper production is increased by conversion of testosterone to much more



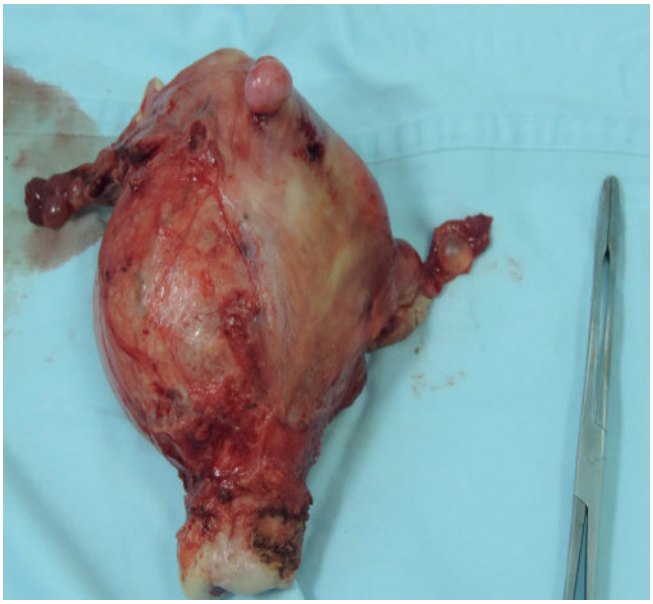


Figure 2. Multiple fibroid tumors of uterus.Mutual folicular cysts both ovary.

potent dihydrotestosterone (DHT), in the androgen-dependent tissues, by the enzyme 5-alpha-reductase.[13] Apart from converting testosterone into a more potent form, enzyme 5-alpha-reductase increases the sensitivity of cell receptors for androgens, which may explain severe masculinisation in our patient (figure 1). The granulosa cell tumors are often unilateral, solid, well-differentiated and have a small malignant potential.[14] The diagnosis of granulosa cell tumors is based on the clinical presentation of the illness, and additional diagnostic procedures such as ultrasonography, magnetic resonance imaging or computed tomography. The differential diagnosis should exclude diseases of the other parts of the endocrine system, especially the pituitary and adrenal glands.[15,16] The treatment of the juvenile type of granulosa cell tumor is exclusively surgical, by ophorectomy or adnexectomy, after which the symptoms of masculinisation can spontaneously disappear.

CONCLUSION

Granulosa cell tumor should be suspected in girls and young females cases if there is present an ovarian cyst paired with signs of preterm puberty or hyperestrogenism. In this case, the presence of granulosa cell tumor was masked by signs of hyperandrogenism which is not so typical, as well as the presence of uterine fibroids who have actually been the main cause for surgical treatment.

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EPIDURAL ANESTHESIA FOR CAESAREAN SECTION IN A PATIENT WITH RISK OF MALIGNANT HYPERTHERMIA

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EPIDURALNA ANESTEZIJA ZA CARSKI REZ KOD PACIJENTKINJE RIZIČNE NA MALIGNU HIPERTERMIJU

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ABSTRACT

Malignant hyperthermia is a hypermetabolic disorder of skeletal muscle that occurs in genetically susceptible individuals after exposure to anesthetic. Basic disorder is an increase of calcium ions inside the skeletal muscle, increasing metabolism and reducing cell energy supplies leading to development of acidosis, cell membrane destruction and cell death. Due to the increased metabolism occurs hypercarbia and strong stimulation of the sympathetic nervous system (tachycardia, hypertension, ventricular arrhythmia, tachypnea dropped for the neuromuscular blockade). Sweating, cyanosis, muscle rigidity and hyperthermia are also present.

This work presents the case of a female patient aged 32 who was heterozygous for the mutation RYR1 gene and therefore has an increased risk of malignant hyperthermia. Per anamnesis we got data that patient's brother suffers from central core disease (myopathy). Patient has no muscle disease, 41st week of pregnancy and was admitted to the hospital for childbirth. Vaginal delivery in epidural analgesia was planned. Epidural catheter is placed in the space L3 - L4, through which she received 0.25% levobupivacaine 10 ml. Due to adverse obstetric findings cesarean section underwent after two hours. Given the increased risk of malignant hyperthermia, the safest type of anesthesia for cesarean is epidural anesthesia. Over the epidural catheter has received 0.5% levobupivacaine 18 ml. Anesthesia machine was verified, hoses were replaced with new ones, CO_2 absorber system was replaced, and whole system is flushed with pure oxygen, before surgery started. During the operation the patient had stable vital parameters that are monitored. She got a male child Apgar score of 9/10 and saw her child at birth. After the operation was transferred to the intensive care unit where we monitored the vital parameters, laboratory analysis, the amount and color of urine. Since all parameters were satisfactory, following day she was transferred to the ward, and she was discharged with a child on the fourth day after the surgery.

Keywords: *Anesthesia, caesarean section, malignant hyperthermia*

SAŽETAK

Maligna hipertermija je hipermetabolički poremećaj skeletnih mišića koji nastaje u genetski osetljivih osoba, posle izlaganja anestetičkim pokretačima. Osnovni poremećaj je povećanje kalcijumovih jona unutar skeletnih mišića, povećanje metabolizma i smanjenje energetskih zaliha ćelija sa razvojem acidoze, destrukcije membrane ćelije i ćelijske smrti. Zbog povećanog metabolizma nastaje hiperkarbija i snažna stimulacija simpatičkog nervnog sistema (tahikardija, hipertenzija, ventrikularna aritmija, tahipneja izostaje kod neuromišićne blokade). Prisutno je znojenje, cijanoza, mišićni rigiditet, hipertermija.

U ovom radu prikazan je slučaj pacijentkinje stare 32 godine koja je heterozigotni nosilac mutacije RYR1 gena, te ima povišen rizik od maligne hipertermije. Rođeni brat boluje od centronuklearne miopatije. Ona nema mišićnu bolest, u 41. nedelji trudnoće je i primljena je u bolnicu radi porođaja. Planiran je vaginalni porođaj u epiduralnoj analgeziji. Plasiran je epiduralni kateter u prostoru L3 - L4, preko koga je dobila 0.25% levobupivakain 10 ml. Posle dva sata,zbog nepovoljnog akušerskog nalaza urađen je carski rez. S obzirom na povišen rizik od maligne hipertermije, najbezbednija vrsta anestezije za carski rez je epiduralna anestezija. Preko epiduralnog katetera je dobila 0.5 % levobupivakaina 18 ml. Pre operacije je proveren aparat za anesteziju. Stavljena su nova creva, zamenjen CO_2 apsorber, sistem ispran čistim kiseonikom. U toku operacije pacijentkinja je stabilnih vitalnih parametara koji se prate na monitoru. Dobila je muško dete Apgar score 9/10. Videla je svoje dete na rođenju. Nakon operacije je prevedena u jedinicu intenzivnog lečenja gde su praćeni vitalni parametri, laboratorijske analize, količina i boja urina. Kako su svi parametri bili zadovoljavajući sutradan je prebačena na odeljenje i četvrtog dana od operacije otpuštena kući sa detetom.

Ključne reči: *Anestezija, carski rez, maligna hipertermija*

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ABBREVIATIONS

MH - Malignant hyperthermia

INTRODUCTION

Malignant hyperthermia (MH) is a hypermetabolic disorder of skeletal muscle that occurs in genetically susceptible individuals after exposure to anesthetic. Basic disorder is an increase of calcium ions inside the skeletal muscle, increasing metabolism and reducing cell energy supplies leading to development of acidosis, cell membrane destruction and cell death (1). Due to the increased metabolism occurs hypercarbia and strong stimulation of the sympathetic nervous system (tachycardia, hypertension, ventricular arrhythmia, tachypnea dropped for the neuromuscular blockade). Sweating, cyanosis, muscle rigidity, hyperthermia and rhabdomyolysis are also present (2, 3, 4).

Triggers of MH in patients with a genetic predisposition are all inhalation anesthetics (excluding N_2O), and depolarizing muscle relaxant succinylcholine (1, 2).

MH crisis can occur at any time during the perioperative period.

The earliest described examples of MH are deaths of two members of the same family, mother and son (on 1915 and 1919) who received anesthetic chloroform and diethyl ether. Muscle rigidity is described during the operation (5). MH was described in more details and it was explained that it is a hereditary disease by Deuborgh 1962 (6).

The incidence of MH differs between countries from 1:10.000 – 1:250.000 anesthesia (2). Incidence in children is 1:15.000, and it is more often in men. In Denmark, the incidence of fulminant MH is 1:250.000 in general anesthesia, but it is significantly increased when combined inhalation anesthesia with succinylcholine, to 1:62.000 (7). It is estimated that in France 1:2.000- 1:3.000 people carry a mutation susceptibility to MH. The prevalence in the Japanese population can be more than 1:2.000 of people (8).

Mortality rate of MH ranges from 5-30% (1).

CASE REPORT

The patient who is 32, in 41st weeks of pregnancy was admitted to the hospital for delivery. Since a large percentage of deliveries performed in epidural analgesia in our hospital, patient was interested in such a manner childbirth. Prior to labor epidural analgesia it was required for patient to be examined and informed about epidural analgesia by the anesthesiologist. The patient had anesthetic examination at the beginning of the ninth month of pregnancy.

The personal history of the patient states that chronic diseases glomerulonephritis is present. She has regularly nephrology examinations and she has not receiving therapy. At the age of five she had an appendectomy, anesthesia was without complications. She claims that she had examination for "lesser controlling ability of the fourth and fifth fingers of her left hand", four years ago. Electromyoneurography finding was described as clear. She is allergic to penicillin.

In the family history says that her brother was suffering from central core disease (myopathy). When her brother did genetic testing, he found that he is the holder of two variants of the gene RYR1, both paternal and maternal one. The patient's mother had a caesarean section, without anesthesiology complications. At the time of examination the patient was in the process of genetic tests, expecting results.

In laboratory analyzes of blood count was in normal values, sterile urine culture, the examination of urine protein +/-, sediment Le 2-3, 3-4 shriveled Er., Proteinuria Esbach at 0,160g /24h.

Due to family history of the patient, she was asked to perform examination by a neurologist and to bring the results of genetic tests before admission to the hospital. Patient brought required reports. Genetic testing determined that patients is heterozygous for the mutation RYR1 gene, causing elevated risk of malignant hyperthermia. Neurological examination did not gave clinical signs of neurological diseases, except that hypoesthesia of fourth and fifth fingers of her left hand is still present.

After 41 weeks pregnant patient was admitted to our hospital with painful contractions. The obstetrician report states dilation of the cervix 3-4 cm., cardiotocography (CTG) was made and oxytocin stimulation was included. Given that the patient wanted to give birth in epidural analgesia, reexamine by the anesthesiologist and access to medical files were necessary. It was required for her to sign off before delivery that she is informed on epidural anaesthesia, confirming that she wants vaginal delivery in epidural analgesia and that she is familiar with the procedure and possible complications. The patient was connected to the monitor, peripheral intravenous cannula was sited, and 500ml crystalloid included. Patient's status: TA 120/80 mmHg, ECG - sinus rhythm f 82/min, $SatO_2$ 99%, afebrile, auscultatory findings of the heart and lungs normal, Visual Analogue Scale (VAS) score of 6, body weight 65kg, height 157cm, BMI 25.6, ASA II. Upon expiry 500ml crystalloid continued with crystalloid infusion and the patient was placed in a sitting position, with spinal flexion. Anestesiologist identified the space L3 - L4. In aseptic conditions, surgical cleaning and garnish of lumbar spine was performed. In the space L3 - L4 local anesthesia was given

to patient, 2 ml of 2% lidocaine, and then the epidural space was identified using loss of resistance technique. For the test we gave 3ml dose of 1% lidocaine and epidural catheter was placed. We gave 10ml 0.25% levobupivacaina on the epidural catheter after five minutes. The pain was reduced, VAS score of 1 after 15 minutes, without motor blockade, with sensory blockade Th11-Th12. Monitored vital parameters were all the time without deviations. TA about 110/65 mmHg, pulse f 75/min, CTG monitored. Obstetric examination, two hours later, established adverse obstetric finding, dilation phase stasis, and decision to perform caesarean section was made. TA 130/80 mmHg at that moment, and f around

110 probably due to patients upset. Through the epidural catheter we gave 18ml 0.5% levobupivacaine, iv ranitidine 50mg, urinary catheter was placed and patient was transfered into the operating room. In the operating room the vital parameters were monitored on a monitor, patient receives O_2, crystalloid infusion, and motor block appears. TA ranges from 100/60 mmHg to 115/70 mmhg, pulse 75-85 f /min, SatO_2 100%, urine clear. The baby was born five minutes from the start of the operation, male, Apgar score of 9/10. The mother saw her newborn baby at birth, and in the further course of the operation wanted to sleep, and received iv. 2mg of midazolam. Upon completion of the operation, which lasted 30 minutes epidural catheter was removed, the patient was feeling well and was extremely satisfied with the anesthesia. Cesarean section was performed in the afternoon, and the patient first was verticalized the next morning. Laboratory analyzes were repeated - blood count, glucose, electrolytes, urea, creatinine, and CK were at the reference values. On the fourth day after the caesarean section the patient was discharged from the hospital with her child.

DISCUSSION

MH is a hypermetabolic disorder of skeletal muscle, it includes central core disease, multi- minicore myopathy, King Denborough syndrome (9).

MH is a heterogeneous disorder where in 80% of cases, a mutation of the gene for the ryanodine receptor (*RYR1*) with the location on chromosome 19q13,1. The main problem in the skeletal muscle of an increase myoplasmic Ca2+ concentration, which is responsible for the calcium channel in terminal tanks sarcoplasmic reticulum, known as the ryanodine receptor. It is a dominant type of inheritance with variable penetrance (1).

Clinical status and its severity of the syndrom may be different. Although muscle rigidity and extreme temperature jump are most dramatic signs of the crisis, the crisis itself starts with unexplained and progressive tachycardia, followed by ventricular tachyarrhythmia. Tachycardia almost always occurs prior to raising the temperature and muscle rigidity. Cardiac arrhythmias are caused by the heart muscle rigidity, acidosis, electrolyte imbalance and increased body temperature. Blood pressure becomes unstable. In patients on spontaneous breathing occurs tachypnea. Increase in the concentration of the expiratory carbon dioxide occurs, which is the result of hypermetabolism musculature. Canister with absorbent for carbon dioxide is hot, and the blood from surgical wound becomes dark, and cyanosis is present. Muscle rigidity usually develops on the extremities, chest and masseter, but sometimes it may be absent (non-rigid form of malignant hyperthermia). One of the alternative forms MH is masseter muscle spasm that occurs soon after giving succinyl-choline in endotracheal intubation (6). Body temperature increases every five minutes to 1-2 degrees (Celsius). The most specific and the most sensitive sign is the increase in carbon dioxide at the end of expiration (ETCO2). Clinical picture is accompanied by pH value decline due to the associated metabolic and respiratory acidosis (1, 6).

Body temperature may exceed 43 degrees (Celsius), $PaCO_2$> 100mmHg, pH <7.0 (10).

Start of MH crisis was accompanied by a high increase serum calcium and potassium, and then their significant decline after 1-2 hours. Serum phosphorus, magnesium, sodium, glucose, lactate and pyruvate are growing. Large molecules leave the muscle cell few hours after the onset of the crisis: creatinine phosphokinase (CPK), lactic dehydrogenase (LDH), glutamine-oxaloacetic transaminase (SGOT), aldolase and myoglobin. Myoglobin causes vasoconstriction and acute renal insufficiency. Myoglobinuria appears. Later complications include acute renal insufficiency, disseminated intravascular coagulopathy, cerebral edema, seizures, liver insufficiency and recurrence of MH crisis with an incidence of 25% within 24-36 hours (1).

The 'gold standard' for the diagnosis of malignant hyperthermia is in vitro contracture test which is based on contracture of muscle fibers in the presence of halothane and caffeine (2).

Discovering various RYR1 caused mutation of MH, molecular genetic test are introduced to clinical practice (1).

The first and most important in the process of MH crisis is immediate interruption of all anesthetics and muscle relaxants which are known to be triggers reactions, followed by hyperventilation of lungs with pure oxygen, at a flow rate of 10 l/min. Surgery supposed to be finished as soon as possible. In addition to standard monitoring, which includes ECG, pulse oximetry, and capnography, central venous catheter and arterial line need to be placed, body temperature and diuresis to be monitored. If used inhalation anesthetic, replacement the corrugated hose, balloon, and canister absorber is required. In the treatment of MH, following guidelines, we should use dantrolene which inhibits the release of calcium from the sarcoplasm reticulum. This is a muscle relaxant, a derivate of hydantoin. Given doses are 2,5 mg/kg, to be repeated if necessary to 10mg/kg. Continuation of dantrolene therapy is 1 mg/kg every 6 hours over the

next 24-36 hours. In addition to dantrolene therapy involves rehydration, correction of metabolic and respiratory acidosis, control heart rhythm (Ca blockers should not be given because of the interaction with dantrolene), cooling, treatment of hyperkalemia, maintenance of diuresis and coagulation factors. Laboratory analyzes need to be repeated and corrected (1, 2).

It is recorded that the muscular abnormalities are present in 67% of patients with genetic defects in MH and 36% of the first line of inheritance. Kyphosis, kyphoscoliosis, lumbar lordosis, multiple hernias, ptosis and strabismus can be included. (6). Our patient did not have any of these diseases, but she cannot clench her left fist, and she has glomerulonephritis. Since her brother has central core disease, and her genetic testing determined to be heterozygous for the mutation RYR1 gene, she was treated as a patient susceptible to MH. Pregnant women who know they are at risk for MH are advised to consult the anesthesiologist before labor (11). Thus, our patient was examined by anesthesiologist at the beginning of the ninth month of pregnancy. It is recommended that an anesthetic team is notified when such a patient is admitted to the hospital, in order to prepare. With our patient, the vaginal delivery in epidural analgesia was planned. When she was admitted to the maternity ward anesthesia machine was prepared in case of emergency caesarean section, which later happened. It is recommended that the apparatus for anesthesia replace all hoses and CO_2 absorber, anesthetic machine to be flushed with high flow rate oxygen 10 l/min, duration 1 hour (1, 12). The use of the active carbon filter is recommended in apparatus preparation for anesthesia (13).

Epidural analgesia reduces stress associated with labor and reduce the demand for oxygen during childbirth (14). Therefore we placed epidural catheter and gave 0.25% levobupivacaine 10ml, leading to patient's pain relief and reducing fear of childbirth. On the monitor, we tracked blood pressure, heart rate and frequency, periodically measured body temperature. To women with MH susceptibility, it is recommended regional anesthesia for caesarean section, in cases it is not an emergency caesarean section. Local anesthetics of the amide group are considered safe. Dantrolene is not given in the prophylaxis (12). Our patient received 18ml of 0.5% levobupivacaine for cesarean section and there were no complications during and after surgery.

In medicical journals it has been discussed case of anesthesia for elective Caesarean section at 21 year old patient with central core disease. Central core disease is characterised by muscle weakness, skeletal deformities and susceptibility to malignant hyperthermia. Total intravenous anaesthesia was used because of the combination of potential malignant hyperthermia, severe kyphoscoliosis and extensive spinal scarring. Administrated anesthetic agents were propofol, remifentanil i rocuronium as muscle relaxant. Surgery went without complications. Recovery and extubation proceeded smoothly, and the patient was disharged to the ward under midwife care. The baby was initially apnoeic and required two doses naloxone at 3 and 5 min. His apgar scores were 3, 7, and 9 at 1, 5, 10 min respectively (15).

Another interesting case has been described in Japan at 26 year old patient with elective Caesarean section, with past history of fulminant malignant hyperthermia. Hyperthermia and subsequent cardiac arrest occurred during general anesthesia when she had been planned to perform hip joint arthroplasty at the age of 7. The Caesarean section was performed under spinal anesthesia with hyperbaric tetracaine. Prophylactic oral dantrolene administration started 4 days before the operation and on the day of the operation. Intravenous dantrolene was administered during the operation and the day after surgery. No clinical symptoms of MH occurred during perioperative period (16).

In the United States, the Malignant Hyperthermia Association of the United States (MHAUS) provides Newsletters, printed information, website (17) to meet the needs of the various groups interested in MH. The European MH group (18) coordinates testing procedures throughout Europe and is made up of professionals investigating MH. Patient supported MH associations exist in France, Germany, Switzerland, Japan, United Kingdom and several other countries.

These organizations have been crucial to the education of anesthesia providers in diagnosing and managing MH and helping patients better understand the disorder.

Cocerning the above our patient was informed about the risk for MH and possible complications.

CONCLUSION

MH is a serious condition with a high mortality rate. MH susceptibility patients, who need to undergo surgery should be considering regional anesthesia, as much as possible. In case that general anesthesia is necessary, safe medicines needs to be chosen, and anesthesia machine needs to be particularly prepared for such patients. Dantrolene is not given prophylactically.

Wider introduction of the practice of genetic tests reduces unexpected and unwanted events in anesthesia.

All interested and informed clinicians and families are the best patients allies against the MH complications.

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GENETIC METHODS FOR DETECTING ASTROCYTES, NEURONS AND NEUROGENESIS

Natalia Nikolaevna Shusharina, Ekaterina Vladimirovna Silina, Victor Aleksandrovich Stupin, Tatiana Georgievna Sinelnicova, Maksim Vladimirovich Patrushev

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Author(s). (Year of publication). *Title*. Information, Place of publication.

Example:

Begg, M. M. (2001). *Dairy farm women in the Waikato 1946-1996: Fifty years of social and structural change*. Unpublished doctoral dissertation, University of Waikato, Hamilton, New Zealand.

g. Report

Format:

Author(s). (Year of publication). *Title*. Place of publication: Publisher. (Report number)

Example:

Osgood, D. W., & Wilson, J. K. (1990). *Covariation of adolescent health problems*. Lincoln: University of Nebraska. (NTIS No. PB 91-154 377/AS)

h. Government publication

Format:

Institution name. (Year of publication). *Title.* Place of publication: Publisher.

Example:

Ministerial Council on Drug Strategy. (1997). *The national drug strategy: Mapping the future*. Canberra: Australian Government Publishing Service.

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Tables should be typed on separate sheets with table numbers (Arabic) and title above the table and explanatory notes, if any, below the table.

FIGURES AND FIGURE LEGENDS

All illustrations (photographs, graphs, diagrams) will be considered as figures, and numbered consecutively in Arabic numerals. The number of figures included should be the least required to convey the message of the paper, and no figure should duplicate the data presented in the tables or text. Figures should not have titles. Letters, numerals and symbols must be clear, in proportion to each other, and large enough to be readable when reduced for publication. Figures should be submitted as near to their printed size as possible. Figures are reproduced in one of the following width sizes: 8 cm, 12 cm or 17 cm, and with a maximal length of 20 cm. Legends for figures should be given on separate pages.

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For figures published previously the original source should be acknowledged, and written permission from the copyright holder to reproduce it submitted.

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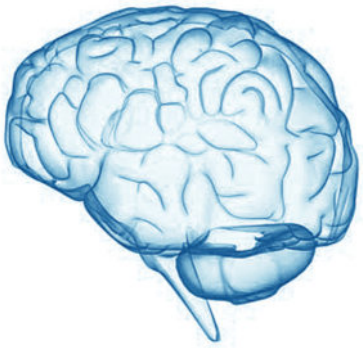
LETTERS TO THE EDITOR

Both letters concerning and those not concerning the articles that have been published in Serbian Journal of Experimental and Clinical Research will be considered for publication. They may contain one table or figure and up to five references.

PROOFS

All manuscripts will be carefully revised by the publisher desk editor. Only in case of extensive corrections will the manuscript be returned to the authors for final approval. In order to speed up publication no proof will be sent to the authors, but will be read by the editor and the desk editor.



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61

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