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THE TWO FACES OF GALECTIN-3: ROLES IN VARIOUS PATHOLOGICAL CONDITIONS

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DVA LICA GALEKTINA-3: ULOGE U RAZLIČITIM PATOLOŠKIM STANJIMA

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ABSTRACT

Galectin-3, a unique chimaera-type member of the lectin family, displays a wide range of activities. This versatile molecule is involved in fundamental biological processes, including cell proliferation, cell-cell adhesion, apoptosis and immune responses.

This review is aimed at providing a general overview of the biological actions and diverse effects of Galectin-3 in many pathological conditions, with a specific focus on autoimmunity, inflammation and tumour progression. We report herein that Galectin-3 exerts deleterious functions determined by promotion of tumour progression and liver inflammation or aggravation of T cell-mediated autoimmune diseases. On the other hand, Galectin-3 exhibits a protective role in metabolic abnormalities and primary biliary cirrhosis.

The paradoxical "yin and yang" functions of Galectin-3 depend not only on its tissue and cellular localization but also on its availability, glycosylation status and the expression level of its ligands.

Keywords: *galectin-3, tumour, inflammation, autoimmune disease*

SAŽETAK

Galektin-3 je jedinstveni himerični član familije lektina i ostvaruje širok spektar aktivnosti. Ovaj svestrani molekul je uključen u fundamentalne biološke procese kao što su ćelijska proliferacija, međućelijska adhezija, apoptoza i imunski odgovor.

Pregledni članak ima za cilj opšti pregled bioloških efekata Galektina-3, kao i njegovih različitih uticaja na mnoga patološka stanja sa specifičnim fokusom na autoimunost, inflamaciju i progresiju tumoura. U ovom radu razmatrani su štetni efekti Galektina-3 koje ostvaruje u određenim patološkim stanjima: promoviše progresiju tumoura i inflamaciju u jetri ili pogoršava neka autoimunska oboljenja izazvana T limfocitima. Suprotno, Galektin-3 igra protektivnu ulogu u patogenezi metaboličkih poremećaja i primarne bilijarne ciroze.

Paradoksne "Jin-Jang" funkcije Galektina-3 zavise od tkivne i ćelijske lokalizacije ovog molekula, a takođe i od dostupnosti, glikozilacionog statusa nivoa ekspresije njegovih liganada.

Ključne reči: *galektin-3, tumour, inflamacija, autoimunske bolesti*

INTRODUCTION

Galectin-3 is one of the best-studied galectins. Like all members of the lectin family, Galectin-3 has a high affinity for binding β-galactoside and shares a conserved carbohydrate recognition domain (CRD) (1). As a chimeric protein with unique structure, Galectin-3 contains three distinct structural regions: 1) an NH_2 terminus containing a serine phosphorylation site that is important for regulation of intracellular signalling; 2) a repetitive, proline-rich, collagen-α-like sequence cleavable by matrix metalloproteases (e.g., MMP-2 and MMP-9); and 3) a globular COOH-terminus containing a carbohydrate recognition domain and the anti-death motif NWGR. Upon binding to multivalent carbohydrates, Galectin-3 can oligomerize through its NH_2-terminus or form a pentameric lattice structure on the cell surface (Figure 1A) (1). Thus, it is involved in modulation of intracellular signalling pathways.

As a small molecular weight protein (approximately 30 kDa), Galectin-3 has multiple cellular localizations in many types of cells, in particular, epithelial and immune cells. In general, Galectin-3 serves as a regulator of fundamental biological processes, such as cell proliferation and differentiation, adhesion, migration, survival, apoptosis and immune responses (2). Intracellularly, Galectin-3 occurs mainly in the cytosol, but is also found in the nucleus

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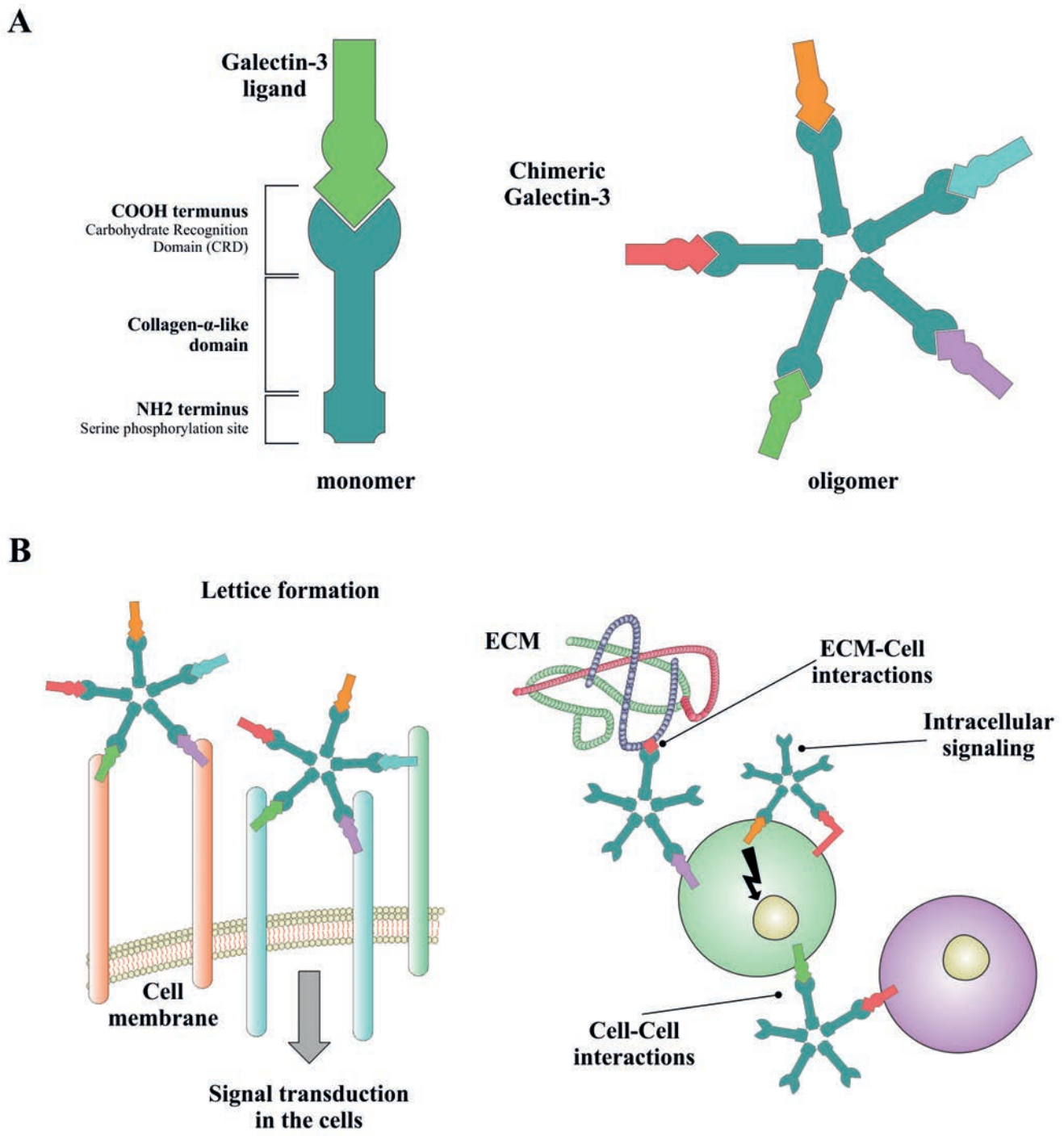

Figure 1. (A) Schematic representation of Galectin-3 monomer (left panel) and Galectin-3 oligomerization through its N-terminus in the presence of its binding ligands (right panel). (B) At the cell surface, Galectin-3 forms a pentameric lattice structure upon binding glycoproteins, thus participating in modulation of intracellular signalling pathways (left panel) as well as cell-cell and extracellular matrix (ECM)-cell interactions.

(3) and mitochondria (4, 5). Finally, it can be transported to the cell surface or secreted to the outside of cells. Galectin-3 interacts and co-operates with numerous intra- and extracellular ligands, thus participating in processes that are essential for basic cellular functions. For example, nuclear Galectin-3 acts as an mRNA splicing promoter and modulator of cell growth and proliferation (1, 6, 7). However, depending on its cellular localization, Galectin-3 may display dual effects, acting as both a negative and positive regulator of apoptosis. Cytoplasmic Galectin-3 functions as an apoptosis inhibitor by maintaining the membrane integrity of mitochondria. In contrast to cytoplasmic Galectin-3, it has been demonstrated that nuclear and extracellular Galectin-3 promote apoptosis (8-10). After bind-

ing to cell surface molecules (e.g., CD29, CD7, CD95 and the T-cell receptor), extracellular Galectin-3 may mediate apoptosis, induced by cytochrome-c release and caspase-3 activation (11-13).

Many reports suggest that extracellular Galectin-3 can act as a modulator of cellular adhesion. The multivalent characteristic of Galectin-3 enables it to act as a bridge between adjacent cells, as well as between cells and a plethora of extracellular matrix components (1, 14, 15), by simultaneously binding to carbohydrates on two adhesion ligands (Figure 1B). These adhesive interactions between cells of the same and different types that occur by binding extracellular Galectin-3 allow Galectin-3 to promote homotypic and heterotypic aggregation, in particular in metastatic processes (15-17).

Galectin-3 is expressed on numerous immune cells and possesses several immunomodulating activities, such as promoting chemotaxis (18), inducing cell-cell adhesion (for instance, dendritic cell-T cell) and cell-matrix glycoprotein adhesion (19), regulating cell proliferation and survival (8, 20), and favouring superoxide production and phagocytosis by macrophages. It has been suggested that Galectin-3 also influences the strength of antigen activation in dendritic cells (21, 22) and controls acquired immunity, including both T-helper cell 1 (Th1) (23, 24) and T-helper cell 2 (Th2) responses (21, 25) depending on the context of the host immune response. Along with its role in inflammation and immune responses in noninfectious conditions, Galectin-3 can detect certain microorganisms by binding specific carbohydrate structures of glycoproteins and glycolipids from many pathogens (26). Recently, it has been reported that Galectin-3 acts as a novel alarmin by augmenting the inflammatory response in sepsis development during bacterial pneumonia following *Francisella novicida* infection (27). The alarmin properties of Galectin-3 include stimulation of an oxidative burst in neutrophils and inflammatory cytokine production in macrophages (27).

Taken together, these studies show that Galectin-3 is a versatile molecule that binds a plethora of intra- and extracellular ligands, thus activating different physiological processes inside and outside the cells. Further, Galectin-3 exerts diverse and sometimes opposing functions under various pathological conditions, depending on the specific tissue and cellular milieu. We will discuss how this molecule contributes to the immunopathology of different diseases, with a specific focus on autoimmunity, inflammation and tumour progression.

GALECTIN-3 IN INFLAMMATION AND AUTOIMMUNITY

Inflammation is generally protective and serves to maintain tissue homeostasis and repair. However, unbalanced inflammation becomes deleterious to the host and can lead to a variety of pathological conditions. For example, metabolic inflammation, referred to as metaflammation, is a chronic inflammation generated from expanding adipose tissue and is induced by metabolic danger signals during obesity. Metaflammation precedes the development of metabolic abnormalities such as insulin resistance, type 2 diabetes and nonalcoholic fatty liver disease. Despite the fact that Galectin-3 exhibits deleterious effects under inflammatory conditions, various studies have demonstrated its protective role in the pathogenesis of obesity-induced inflammation triggered by accumulation of different metabolic stressors, such as advanced glycation end products (AGEs) (28). Namely, AGEs and the receptor for AGEs (RAGE) have been linked to enhanced apoptosis and dysfunction of pancreatic β cells and also to the pathogenesis of diabetic complications (29). Galectin-3 has been identified as an AGE receptor (30) that binds AGEs with high affinity, thus acting as a scavenger receptor for these glucose adducts that are elevated in animals on a lipid-rich diet (29). Galectin-3 protects β cells in rats from the cytotoxic effect of IL-1β (31). The expression levels of Galectin-3 in adipocytes and macrophages of adipose tissue and in serum are elevated during obesity in both humans and experimental animals (29).

To examine the role of Galectin-3 in obesity and type 2 diabetes, we used the model of high fat diet (HFD)-induced obesity in Galectin-3-deficient mice (32). We showed that lack of Galectin-3 accelerates HFD–induced obesity and type 2 diabetes by increasing visceral adiposity, hyperglycaemia and insulin resistance and by upregulation of inflammatory pathways at both local (metabolic tissue) and systemic levels. Visceral adipose tissue of obese Galectin-3-deficient mice was infiltrated with type 1 $CD3^+$ T lymphocytes, $CD3^+NK1.1^+$ NKT lymphocytes expressing IFN-γ and proinflammatory M1 macrophages, as well as $F4/80^+CD11b^+CD11c^+$ bone marrow-derived cells. In addition, regulatory T cells and, in particular, alternatively activated M2 macrophages were markedly reduced. These findings are complementary to a previous study demonstrating that Galectin-3 promotes M2-polarized macrophages (33). In addition to visceral adipose tissue, we found that a lack of Galectin-3 is also associated with inflammation in pancreatic islets, which is reflected by the presence of severe insulitis (32). This finding supports the concept that Galectin-3 has protective functions in metaflammation during obesity (29).

Principal mechanisms of obesity-induced inflammation include nuclear factor-κB (NF-κB)–dependent production of proinflammatory cytokines and increased activation of the NLRP3 (NOD-like receptor family, pyrin domain containing 3) inflammasome, leading to caspase-1-mediated cleavage and release of active proinflammatory IL-1β (34). In our study, increased expression of the NLRP3 inflammasome and IL-1β in macrophages was present in visceral adipose tissue and pancreatic islets of obese Galectin-3-deficient mice in comparison with wild type mice fed with an HFD (32). A key point of our work is that pancreatic islets of obese Galectin-3-deficient mice had increased deposition of AGE and RAGE expression,

suggesting that deletion of the Galectin-3 gene impairs their removal, leading to accelerated inflammation and subsequent damage of pancreatic β-cells. Additionally, we noticed that this increased deposition of AGE and RAGE was accompanied by higher expression levels of phosphorylated NF-κB p65 and mature caspase-1 in pancreatic tissue and visceral adipose tissue (32). It appears that the NF-κB-mediated proinflammatory pathway operates in the enhanced metaflammation observed in Galectin-3-deficient mice.

Recently, it has been suggested that type 2 diabetes is an autoinflammatory disease with a central role for NLRP3-ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) inflammasome-mediated IL-1β production (35). In this regard, we noticed a trend towards increased expression of the NLRP3 inflammasome and ASC adaptor proteins in pancreatic and visceral adipose tissue, respectively. Additionally, peritoneal macrophages from Galectin-3-deficient mice released higher amounts of IL-1β and had increased NLRP3 inflammasome expression and caspase-1 activity in response to stimulation with lipopolysaccharide and/or saturated fatty acid palmitate compared with peritoneal macrophages from wild type mice *in vitro*. We also noticed that silencing of the NLRP3 inflammasome attenuated IL-1β production by macrophages from Galectin-3-deficient mice, suggesting that the release of IL-1β was mediated by NLRP3 inflammasome activation. Finally, we demonstrated that obese Galectin-3-deficient mice had increased systemic inflammation, as shown by elevated serum levels of proinflammatory IL-6 and IL-1β, followed by significantly decreased serum levels of immunomodulatory IL-13 and IL-10. Altogether, the results obtained from our study suggest an important protective role of Galectin-3 in obesity-induced inflammation and type 2 diabetes (32).

Nonalcoholic fatty liver disease (NAFLD) is a common chronic metabolic complication of obesity and type 2 diabetes (36). Liver steatosis is a benign condition that progresses to nonalcoholic steatohepatitis (NASH), which is characterized by chronic liver inflammation and fibrosis (37). The liver is the main catabolic site for AGEs and ALEs (advanced lipoxidation end-products) (38). Recently, it has been reported that Galectin-3 is involved in the regulation of fatty acid and glucose metabolism in the liver (39). RAGE, found predominantly in hepatocytes, and Galectin-3, which is highly expressed in sinusoidal liver endothelial and Kupffer cells, are the principal scavenger receptors for AGEs/ALEs and are involved in their removal without initiation of inflammation (40). To clarify the role of Galectin-3 in the pathogenesis of obesity-related NASH, Galectin-3-deficient mice were placed on the obesogenic HFD (41). The data obtained show that Galectin-3 attenuates steatosis but promotes liver injury, inflammation and fibrosis, thus regulating disease progression in the obesogenic mouse model of NASH. In addition, we show here for the first time that the newly described profibrotic IL-33/ST2/IL-13 pathway in macrophages is Galectin-3-dependent (41).

The notion that Galectin-3 has a proinflammatory role in T-cell-mediated disease has been demonstrated in animal experimental models, such as Concanavalin A (Con A)–induced fulminant hepatitis (42). Con A is a potent, hepatotropic T cell mitogen, and it induces acute hepatitis in a model of T lymphocyte-mediated liver damage in mice (43). Our results demonstrate that ablation of Galectin-3 markedly attenuated liver injury by reducing the number of effector cells, such as T lymphocytes, natural killer (NK) and natural killer T (NKT) cells, and increasing the number of M2-polarized macrophages. Thus, our data support the assertion that Galectin-3 promotes inflammation in the liver following Con A injection. Further, apoptosis of liver-infiltrating cells contributes to the lower number of mononuclear cells in the livers of Galectin-3-deficient mice, supporting the concept that Galectin-3 has an antiapoptotic role, particularly if it is localized within cells (42). Similar to the effects of deleting the Galectin-3 gene, pretreatment of wild type mice with a selective Galectin-3 inhibitor (TD139) also significantly reduced Con A-induced liver injury by suppressing infiltration of IFN-γ-, IL-17- and IL-4-producing $CD4^+$ T lymphocytes and IFN-γ-producing $CD8^+$ T lymphocytes, and increasing the number of IL-10-producing $CD4^+$ T lymphocytes, as well as alternatively activated macrophages (42). Altogether, our findings indicated that reduced inflammation in the liver of Con A-treated Galectin-3-deficient mice and TD139-pretreated wild type mice could be the result of attenuation of macrophage and T cell activity.

Primary biliary cirrhosis (PBC) is considered a progressive autoimmune liver disease with immune-mediated destruction of intrahepatic biliary epithelial cells and frequent appearances of autoantibodies against the major mitochondrial autoantigen, PDC-E2 (pyruvate dehydrogenase complex component E2) (44, 45). It appears that biliary epithelial cells (BECs) are the major targets of injury and thus they are active participants in the initiation and perpetuation of autoimmunity in the pathogenesis of PBC. It is assumed that a unique apoptotic feature of biliary epithelial cells may contribute to epitope presentation to the immune system, causing unique tissue damage (46, 47). In this scenario, both acquired and innate immunity have been proposed as contributors in autoimmune-mediated destruction (48).

Our laboratory has decided to examine the role of Galectin-3 in PBC pathogenesis by using a murine model of autoimmune cholangitis following immunization of Galectin-3-deficient mice with 2-octynoic acid (2OA) coupled to BSA (2OA-BSA). In contrast to the results from our previous studies, which indicated that deletion of the Galectin-3 gene reduces several T cell-mediated autoimmune diseases, such as diabetes (49) and experimental autoimmune encephalomyelitis (50), we demonstrate here that another autoimmune disease, PBC, is aggravated by Galectin-3 deficiency (data have been accepted for pub-

lication). This autoimmune disease is characterized by increased periportal infiltration, bile duct damage, granulomas and fibrosis. It is well established that intracellular Galectin-3 is a critical negative regulator of apoptosis. Thus, the cause of these differences appears to be the fact that in PBC, the ablation of Galectin-3 leads to increased availability of autoantigen(s). In actuality, lack of Galectin-3 may affect mitochondrial membrane integrity and resistance to apoptosis in BECs and, consequently, cause the release of putative antigen(s) that induce a stronger activation of DCs, a higher influx of inflammatory lymphocytes, and enhanced bile duct damage and liver fibrosis. In addition, we did not detect Galectin-3 expression in BECs from healthy wild type mice. However, the expression of Galectin-3 was significantly increased in BECs from 2OA-BSA-immunized mice. Finally, we assume that increased expression of Galectin-3 in BECs in the murine model of autoimmune cholangitis is probably a compensatory mechanism that is used to protect the BECs from apoptosis induced by different stimuli.

PROTUMOURIGENIC ROLE OF GALECTIN-3

Galectin-3 expression is altered or abnormally localized in cells of various human solid tumours and blood malignancies, suggesting that this multifunctional molecule may modulate tumour progression and influence disease outcome (1, 51). Some immunohistochemistry studies have indicated that overexpression of Galectin-3 may be a prognostic factor for poor survival of patients with gastric cancer, hepatocellular carcinoma, thyroid cancer and leukaemia (52-56). It seems that the translocation of Galectin-3 from the cytoplasm to the nucleus of melanoma cells results in a more aggressive phenotype (57). Interestingly, tumour hypoxia upregulates the expression of Galectin-3 and also leads to changes in its subcellular localization (58). Under hypoxic conditions, the expression of Galectin-3 shifts from a nuclear location to cytoplasmic and membranous locations, suggesting that this shift favours resistance to apoptosis and malignancy of mammary tumour cells (58). Circulating Galectin-3 is also increased in the bloodstream of cancer patients. Thus, markedly higher serum concentrations of Galectin-3 were found among patients with many types of cancer (59-61), compared with those of healthy individuals. Additionally, patients with advanced melanoma have higher concentrations of circulating Galectin-3 than those with localized tumours. It appears that the source of increased Galectin-3 concentrations in the serum of patients with cancer may be the tumour cells, as well as the peritumoural inflammatory and stromal cells (16, 59).

Despite its involvement in physiological processes, Galectin-3 is also a key player in many steps of tumour development and metastasis. It has been demonstrated that Galectin-3 favours a broad range of cancer cell activities, such as malignant cell transformation and tumour growth (62-64), cell adhesion, migration and invasion (65-69), anoikis resistance (70), apoptosis inhibition (71), (72) and angiogenesis (73).

Galectin-3 can promote malignant transformation due to simultaneous stimulation of cell growth and prevention of apoptosis. For example, nuclear Galectin-3 can interact with β-catenin to enhance the expression of cyclin D and c-Myc (74) and thus promote cell cycle progression (14). This galectin can upregulate β-catenin expression in the nucleus of human colon cancer cells and augment Wnt/β-catenin signalling by regulating glycogen synthase kinase-3β (GSK-3β) phosphorylation and activity via the PI3K/Akt pathway (75). Additionally, cytoplasmic Galectin-3 may cause constitutive activation of Ras-dependent PI3K and Raf-1 activation by its interaction with activated K-Ras (63). Altogether, these results suggest that Galectin-3 is a pivotal player in the regulation of cancer-related gene expression and the activation of many signalling pathways. One of the main hallmarks of cancer is the evasion of apoptosis that contributes to cancer cells' survival. An additional function of Galectin-3 that is relevant to tumour progression is inhibition of apoptosis. Following apoptotic stimuli, Galectin-3 translocates from either the cytosol or the nucleus to the mitochondria (4), where it interacts with Bcl-2 and blocks the alteration of mitochondrial membrane potential and cytochrome-c release (71). It has also been reported that Galectin-3 heterodimerizes with Bax, mediated by the carbohydrate recognition domain of Galectin-3, which leads to attenuation of apoptosis in human thyroid carcinoma cells (76). Further, this galectin is an important antiapoptotic effector molecule that confers resistance to conventional cancer chemotherapy. Thus, when leukaemia cells were treated with cisplatin, Galectin-3 expression was upregulated and caused resistance to apoptosis in surviving cells (77). In contrast, silencing of Galectin-3 in gastric cancer cells augments apoptosis induction by chemotherapy by decreasing the expression of cell survival molecules (e.g., survivin and cyclin D1) (78). Additionally, the silencing of Galectin-3 with RNA interference also sensitizes multidrug-resistant cells to epirubicin by activation of the mitochondrial apoptosis pathway through modulation of the β-catenin/GSK-3β pathway in human colon cancer cells (79).

The migratory and invasive potential of tumour cells is associated with tumour progression. The invasion and metastasis processes involve changes in several proteins engaged in cell–cell and cell–matrix adhesion (2), and cell surface expression of Galectin-3 seems to contribute to these processes, thus promoting the metastatic spread of cancer cells from primary to secondary tumour sites (51, 80). Adhesive interaction with components of the extracellular matrix (ECM) is important for migration of malignant cells (81). It is well established that Galectin-3 interacts with glycoproteins of the ECM, such as fibronectin, collagen IV, elastin and laminin (19, 82). It appears that increased levels of Galectin-3 in the bloodstream of cancer patients can be critical in malignant cell metastasis

(16). One of the Galectin-3 binding partners is oncofoetal Thomsen-Friedenreich antigen, which is on the transmembrane mucin protein MUC1 expressed by many metastatic cells (83). This interaction results in increased tumour cell homotypic aggregation to form microtumour emboli that prolong metastatic cell survival in the circulation by prevention of anoikis and enhanced heterotypic adhesion of the metastatic cells to the vascular endothelium (83-85).

A critical step in successful establishment of organ-specific metastasis is the adhesion interactions of tumour cells with the host microenvironment, especially interactions with components of the ECM and vascular endothelium in target organs. It appears that the degree of organ-specific metastasis may be associated with Galectin-3 expression in the lungs. In this regard, we reported that Galectin-3 ablation in the host markedly decreases lung melanoma metastasis. In an experimental model of B16-F1 murine melanoma, we demonstrated that Galectin-3-deficient mice were more resistant to metastatic melanoma, as evidenced by markedly reduced number and size of lung metastatic colonies compared with wild type mice. In addition, by *in vitro* assay we noticed lower numbers of attached malignant cells in lung tissue sections of Galectin-3-deficient mice, suggesting that host-derived Galectin-3 plays a pivotal role in tumour cell adhesion to the metastatic target (86). Recently, it has been shown that Galectin-3, which is expressed in lungs (especially on the vascular endothelium), cooperates with poly-N-acetyl-lactosamine on N-glycans on B16-F1 murine melanoma cells, as a ligand for Galectin-3 (87). This interaction between Galectin-3 and its glycoprotein ligand not only facilitates initial adhesion of tumour cells to the vascular endothelium but also participates in subsequent metastatic processes such as extravasation, degradation of the matrix and organ colonization (88). Finally, metastatic cells continue to proliferate in the target organ. Tumour angiogenesis is involved in the metastatic cascade both at the primary site and at downstream sites of metastasis. In addition to its role in dissemination of tumour cells, angiogenesis is also required for expansion of the metastatic colony in the target tissue. It appears that Galectin-3 has proangiogenic activity via interactions with several endothelial cell surface receptors. For example, Galectin-3 interacts with αvβ3 integrin on endothelial cells and induces promotion of VEGF- and bFGF-mediated endothelial migration and, consequently, vessel branch formation (89).

Immune evasion and suppression are associated with tumour progression through inhibition of effector immune cells or via expansion of immunosuppressive cells (90, 91). Galectin-3 is an important modulator of immune responses through the regulation of homeostasis and immune cell function. For example, Galectin-3 reduces the affinity of the T-cell receptor (TCR) for major histocompatibility complex (MHC) Class I and peptide ligand by segregating the TCR from its CD8 coreceptor (92), disrupts the immunological synapse by internalizing the TCR (93), and induces apoptosis of T cells (94).

It is well established that NK cells and $CD8^+$ cytotoxic T cells are crucial players in the control of melanoma growth and metastasis. Recently, Kouo et al. (95) suggested that Galectin-3 modulates antitumour immune responses by suppressing effector $CD8^+$ T cells exclusively in the tumour microenvironment via lymphocyte activation gene 3 (LAG-3), an inhibitory receptor that is associated with regulation of terminal T-cell activation/exhaustion. Our data support a link between Galectin-3 and the cytotoxic capacity of NK cells, but not that of $CD8^+$ T cells. In fact, we demonstrated that lack of Galectin-3 is associated with enhanced tumouricidal activity of NK cells directed against B16-F1 melanoma cells (86). The antitumour capacity of NK cells is dependent on their development and maturation. We observed that Galectin-3-deficient mice constitutively have a significantly higher percentage of effective cytotoxic $CD27^{high}CD11b^{high}$ NK cells and immature $CD27^{high}CD11b^{low}$ NK cells, regardless of reduced numbers of $NK1.1^+$ cells in the spleen, compared with wild type mice. However, the percentage of less functionally exhausted $CD27^{low}CD11b^{high}$ NK cells and NK cells bearing the inhibitory KLRG1 receptor was markedly lower in Galectin-3-deficient mice (86). It is believed that Galectin-3 interferes with binding to regulatory molecules on the cancer cell that serve as ligands for receptors of NK cells (96). Additionally, the results from Wang et al. (97) suggested that expression of membrane KLRG1 receptors on NK cells impairs their activation and IFN-γ production, but increases apoptosis of these cells in chronic hepatitis C virus infection.

Some evidence suggested that Galectin-3 is associated with a decrease in regulatory T (Treg) cell frequency and thus influences the course of experimental autoimmune encephalomyelitis and *Leishmania major* infection (98, 99). These results raise the question of whether Galectin-3 deficiency also impaired the number of Treg cells in an experimental model of B16-F1 murine melanoma. We noticed that injection of melanoma cells resulted in marked increases in the percentage and total number of regulatory $CD4^+Foxp3^+$ T cells in wild type mice, but not in Galectin-3-deficient mice (86). Thus, our findings suggest that host-derived Galectin-3 could lead to an increase in the number and percentage of Treg cells, which promotes the formation of an immunosuppressive tumour network and can be one of the main facilitative mechanisms important for tumour metastasis. A high number of Treg cells has been directly correlated to cancer progression (100). In our experimental model of B16-F1 murine melanoma, it appears that the most important role of Treg cells is suppression of NK cell function. A previous study demonstrated that a high number of Treg cells was inversely correlated to the frequency and function of NK cells (100). For example, co-cultivation of human allogeneic Treg cells with resting NK cells led to markedly reduced NK cell natural cytotoxicity, cytokine production and expression of NKG2D activating receptors *in vitro* (101).

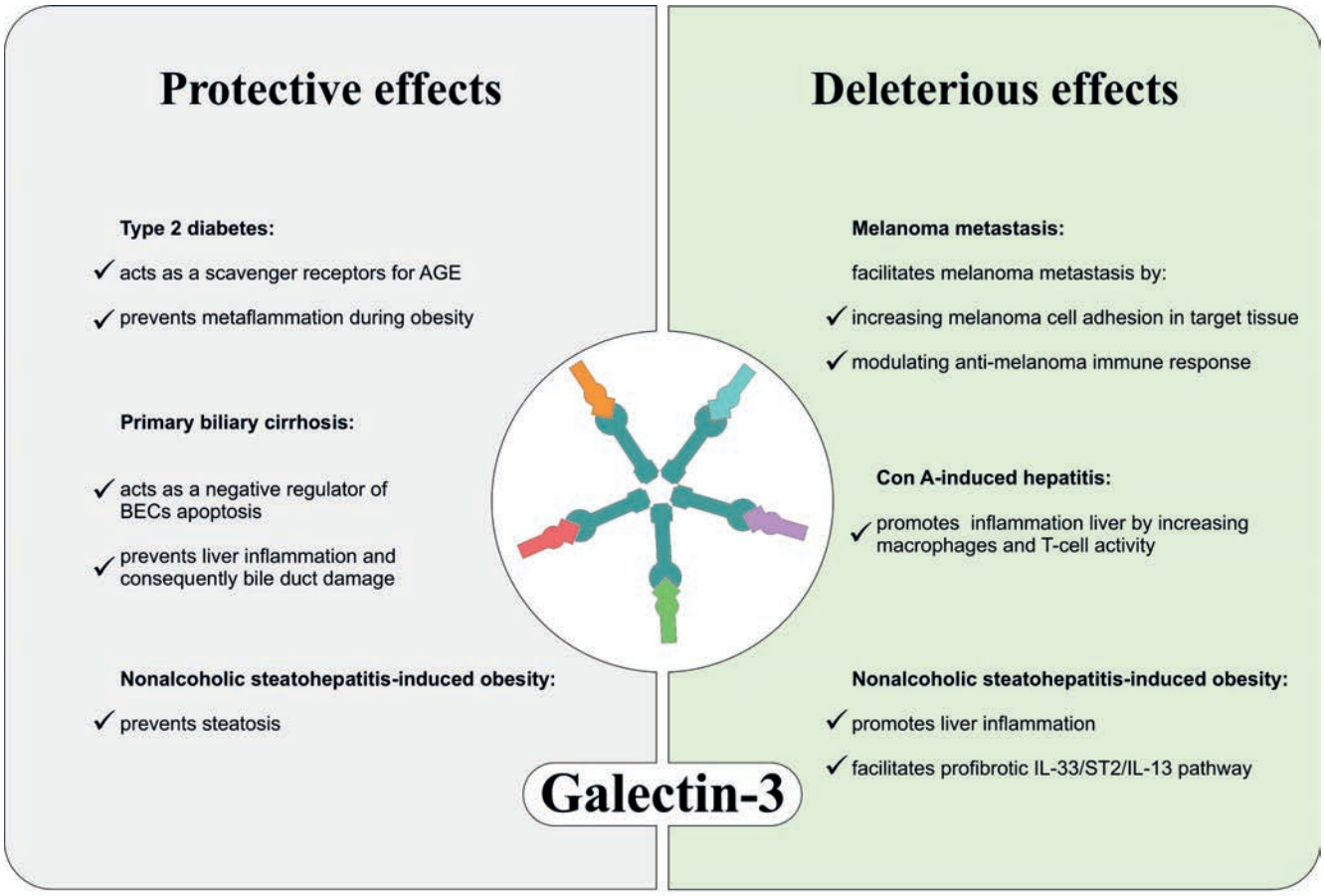

Figure 2. The effects of Galectin-3 in inflammation, autoimmunity and melanoma metastasis.

While IL-17 exhibited both protumour and antitumour roles (102), IFN-γ exerted potent antitumour immunity against melanoma and various other cancers (103). It has been reported that IFN-γ has direct antiproliferative and proapoptotic effects on tumour cells in animal models (104, 105) and that these effects prevent B16 experimental metastasis by directly inhibiting cell growth (106). We observed higher serum levels of IFN-γ and IL-17 in tumour-bearing hosts in Galectin-3 deficiency, which was not accompanied by differences in the number of $CD4^+$ and $CD8^+$ T cells in the spleen. This does not exclude the possibility that the number of tumour-specific Th1 cells and cytotoxic $CD8^+$ T cells is different (86). Reported data suggest that Galectin-3 suppresses IFN-γ production by antigen-specific $CD8^+$ T cells *in vitro* (95). Further, dendritic cells lacking the Galectin-3 gene have been shown to increase both T-cell numbers and cytokine production in helminthic infections and promote an effective Th17 immune response (107, 108). Altogether, it appears that Galectin-3 is involved in the tuning of acquired immunity against tumours by determining the cytokine milieu in the tumour microenvironment.

In summary, our data determined several findings supporting Galectin-3 as a potential facilitator of melanoma lung metastasis. First, host-derived Galectin-3 is a critical player in tumour cell adhesion to the metastatic target and thus contributes to the initial adhesion and survival of circulating metastatic cells. Second, Galectin-3 shapes the immune response against B16-F1 melanoma cells by suppressing effector NK cells and enhancing expansion of $CD4^+Foxp3^+$ T cells. Thus, our findings clearly confirm the results obtained in other studies suggesting that Galectin-3 has a wide range of pivotal protumourigenic functions that are associated with tumour growth and metastasis, implying that it would be an ideal therapeutic target to prevent tumour progression.

EXPERT OPINION

The cell- and tissue-specific profiles of Galectin-3 expression differ under physiological and pathological conditions (14, 109, 110); they can undergo a variety of changes under the stress conditions encountered in tumour microenvironments, inflammation or fibrosis. Divergent specificities and affinities of Galectin-3 for various glycoproteins appear to contribute to the multiplicity of its activities.

Galectin-3 is a highly versatile protein and a potent modulator of pivotal cellular processes. The role of Galectin-3 in various pathological conditions is complex, with diverse and sometimes opposing functions. We report

herein that Galectin-3 exhibits deleterious roles in the promotion of tumour progression and liver inflammation after Con A injection and the aggravation of T cell-mediated autoimmune diseases such as type 1 diabetes and experimental autoimmune encephalomyelitis. However, it appears that Galectin-3 has a protective function in conditions such as type 2 diabetes and primary biliary cirrhosis. In addition, the paradoxical role of Galectin-3 in nonalcoholic steatohepatitis during obesity is reflected in the fact that it contributes to inflammation and fibrosis in the liver, but reduces steatosis (as illustrated in Figure 2). The functional dichotomy in various pathological conditions suggests that the effects of Galectin-3 depend on cellular and tissue localization, or the availability, glycosylation status or expression level of its ligands.

The key question is: How does Galectin-3 achieve its target specificity to become a meaningful effective molecule? The identification and characterization of the nature and structural diversity of Galectin-3 ligand(s) (e.g., glycoconjugate or protein) responsible for target selectivity (111) will be the basis for not only developing rational anti-inflammatory and antitumoural approaches but also for taking advantage of the protective functions of Galectin-3 in metabolic abnormalities or primary biliary cirrhosis. Specifically, the major aim for therapeutic manipulation is singling out the pathological aspect in target tissue while avoiding harm at the physiologic site. Ideally, Galectin-3 therapy should be strictly spatially confined.

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REDOX STATUS IN PATIENTS WITH FEMORAL NECK FRACTURES

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ABSTRACT

The femur transfers the body weight from the pelvic bone to the shinbone. Femur fractures are a significant cause of morbidity and mortality among the group of locomotor apparatus injuries, especially in the elderly population. Considering that oxidative stress occurs as a result of increased production of free radicals that damage cell function and cause numerous pathological conditions and diseases, the aim of this study was to investigate oxidative stress parameters in older patients with femoral neck fractures. This clinical study included 70 patients, of which 35 had femoral neck fractures (26 males and 9 females), while the other half of the patients formed the matched control group. Markers of oxidative stress (NO_2^-, TBARS, H_2O_2 and O_2^-) and antioxidative enzymes (SOD, CAT, and GSH) were measured. Results showed that the levels of O_2^- increased, while levels of NO_2^-, H_2O_2 and all the antioxidative enzymes decreased in patients with femoral neck fractures. These findings indicate that fractures cause oxidative stress, probably because of the reduced activity of osteoblasts and the increased activity of osteoclasts.

Keywords: *Oxidative stress, Antioxidant enzymes, Fracture, Femoral neck*

SAŽETAK

Femur je parna kost i ima ulogu da prenese težinu tela sa karlične kosti na golenjaču. Prelomi femura predstavljaju značajan uzrok morbiditeta i mortaliteta u grupi povreda lokomotornog aparata, posebno kod starije populacije. S obzirom da je poznato da oksidacioni stres nastaje kao posledica prekomerne produkcije slobodnih radikala koji oštećuju ćelijsku funkciju i dovode do nastanka mnogih patoloških stanja i bolesti, cilj ovog istraživanja bio je da se ispitaju parametri oksidacionog stresa kod starijih pacijenata sa preloma vrata butne kosti. Ova prospektivna klinička studija je obuhvatila 70 ispitanika. Prelom vrata butne kosti imalo je 35 pacijenata (26 - muškaraca; 9 - žena), dok je druga polovina pacijenata istog pola, starosti i karakteristika predstavljala kontrolnu grupu. Markeri oksidacionog stresa (NO_2^-, TBARS, H_2O_2 i O_2^-) i antioksidacioni enzimi (SOD, CAT, GSH) su određivani spektrofotometrijski. Rezultati su pokazali da je nivo O_2^- povećan, dok su nivoi NO_2^-, H_2O_2 i svi antioksidacioni enzimi smanjeni kod pacijenata sa preloma vrata butne kosti. Ovi rezultati pokazuju da prelom uzrokuje nastanak oksidacionog stresa, najverovatnije zbog smanjene aktivnosti osteoblasta i povećane aktivnosti osteoklasta.

Ključne reči: *Oksidacioni stres, Antioksidativna zaštita, Prelom, Vrat butne kosti*

ABBREVIATIONS

CAT - Catalase
MDA – Malondialdehyde
NO - Nitric oxide
ROS - Reactive oxygen species
SOD - Superoxide dismutase

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INTRODUCTION

The femur is the longest, strongest and largest bone in the human body. It transfers the body weight from the pelvic bone to the shinbone (1). Femur fractures are a significant cause of morbidity and mortality among the locomotor apparatus injuries, especially in the elderly population (2). Although any part of this bone can break, the femoral neck or hip is the most common part to fracture (3). The number of femoral fractures is increasing, and this trend will continue. In particular, higher incidences of hip fractures are recorded in the Scandinavian countries, the United States and Western Europe, while the rate is much lower in the Far East and Africa (4, 5). The factors contributing to this epidemic include, the length of time since the implantation of a prosthesis, loose stems and an aging population. Because of these factors, a large number of hip arthroplasties are performed each year (6). In young patients, femur fractures occur very rarely, and when they do, they occur due to major trauma (traffic accidents, fall from heights). Femur fractures occur more frequently in the elderly (over sixty-five years), where trauma plays a less significant role but factors such as low mineral density due to osteoporosis, osteopenia, or muscle atrophy play important roles (7). The main signs of fracture are pain and functional impotence. The patient cannot actively raise either the injured limb or their heel from the ground (8).

Considering that oxidative stress occurs as a result of the increased production of free radicals that damage cell function and cause many pathological conditions and diseases, it is not surprising that in recent years, its role has been increasingly examined in a variety of fractures. Data from existing literature has shown that reactive oxygen species (ROS) in physiological conditions contribute to the destruction of calcified tissue (9). Some studies have found that increased production of free radicals is associated with reduced bone density (10). Additionally, the increased activity of osteoclasts and decreased activity of osteoblasts may contribute to the imbalance between pro-oxidants and antioxidants in patients with various broken bones (11). In a study, Yeler and coworkers have shown that the production of free radicals is highest immediately after the fracture and continues for several months during the period of healing (12).

Despite extensive research, the impact of free radicals and antioxidant enzymes on fractures and whether the ROS production exceeds the antioxidant enzyme activity has not been fully understood. Based on the above data, the aim of this study was to investigate oxidative stress parameters in older patients with femoral neck fractures.

MATERIALS AND METHODS

Subjects

The prevalence study included 70 patients, of whom 35 patients had femoral neck fractures (26 males and 9 females), while the other half of the patients were matched with regard to sex, age and other characteristics and designated as the control group. The average age of the patients with femoral neck fractures is 66 years (women - 65.9 years; males - 66.1 years). All patients with fractures were admitted to the orthopaedic ward of the Clinical Center in Kragujevac during the period from February 2015 to May 2015.

All subjects were over 60 years old, and all patients from experimental group showed indications that required surgical intervention. The exclusion criteria included a lack of thigh/lower leg, a stump unsuitable for prosthesis (a stump with trophic changes due to islands, ulcer, fistula, a painful neuroma, deformities stump, extensive scarring, or extreme muscle atrophy), damage to the spinal cord or peripheral nerve injury (quadriplegia, paraplegia and hemiplegia) with or without loss of control of urination and defecation, and tertiary stages of malignant diseases.

All patients were familiarized with the study's protocol and their written consent was obtained. The study was approved by the Ethical committee of the Clinical Center of Kragujevac, Kragujevac.

The study protocol

This was a prospective clinical study conducted from February 2015 to June 2015.

The venous blood samples from subjects in the control group were obtained only once. Blood samples were taken from the patients in the experimental group during the first 12 hours after a femoral neck fracture. All blood samples were collected from the antecubital veins into Vacutainer test tubes containing sodium citrate anticoagulant. Blood was centrifuged to separate plasma and red blood cells (RBCs). Isolated RBCs were washed three times with three volumes of ice-cold 0.9 mmol/L NaCl, and haemolysates containing approximately 50 g Hb/L were used in the determination of antioxidant enzymes.

Biochemical assays

The following markers were measured from plasma: superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), index of lipid peroxidation (measured as TBARS), and nitric oxide (NO) in the form of nitrite (NO_2^-). The activities of antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH)) were determined in the haemolysates.

Biochemical analyses of oxidative stress parameters and antioxidant enzymes were conducted in the Laboratory of Cardiovascular Physiology at the Faculty of Medical Sciences in Kragujevac. The spectrophotometric measurements were conducted using the Shimadzu UV-1800 instrument, North America.

Measurement of superoxide anion radical (O_2^-) concentration

Determination of the superoxide anion radical ($O_2^{\bullet-}$) concentration is based on the reaction of $O_2^{\bullet-}$ with nitro blue tetrazolium (NBT), which results in nitro blue forma-

zan (13). The maximum absorption wavelength used for these measurements was λmax=550 nm.

Determination of the hydrogen peroxide (H_2O_2) concentration

Determination of the hydrogen peroxide (H_2O_2) concentration is based on oxidation of phenol red using hydrogen peroxide, a reaction that is catalysed by the enzyme horseradish peroxidase (HRPO) (14). This reaction results in the formation of a compound that has a maximum absorption at wavelength λmax= 610 nm.

Determination of the index of lipid peroxidation (TBARS)

The levels of lipid peroxidation were determined indirectly by measuring the products of lipid peroxidation reactions with thiobarbituric acid (Thiobarbituric Acid Reactive Substances - TBARS). This method is based on the determination of the levels of one of the lipid peroxides malondialdehyde (MDA) based on its reaction with thiobarbituric acid (TBA) (15). Distilled water was used as the blank probe. Measurements was obtained at a wavelength of λ=530 nm.

Determination of nitrite (NO_2^-) levels

Nitric oxide (•NO) decomposes rapidly to form stable nitrite/nitrate metabolic products. The method for the detection of plasma nitrite levels is based on the Griess reaction. Nitrite (NO_2^-) levels were determined as an index of NO production, which reacts with the Griess reagent to form a purple diazo-complex (16). Nitrites were measured at a wavelength of 550 nm.

Determination of superoxide dismutase (SOD) activity

The determination of superoxide dismutase (SOD) activity is based on the epinephrine method. A mixture of 100 µL lysate and 1 mL carbonate buffer was prepared, and 100 µL of epinephrine was then added to the mixture. The detection was performed at a wavelength of 470 nm. This method belongs to the 'negative type' group of methods, because it monitors the decrease in auto-oxidation speed in an alkaline medium, which is dependent on O_2^- levels (17).

Determination of catalase (CAT) activity

The determination of catalase (CAT) activity in the sonificate is based on the methods described by Beutler (18, 19). The lysates were diluted with distilled water (1:7 v/v) and treated with chloroform-ethanol (0.6:1 v/v) to remove haemoglobin. Then, 50 µL catalase buffer, 100 µL sample, and 1 mL 10 mM H_2O_2 were added to the samples. The results were detected at a wavelength of 360 nm. Distilled water was used as the blank probe (20).

Determination of reduced glutathione (GSH) levels

The reduced glutathione (GSH) concentration is determined spectrophotometrically using the Beutler method (21). The absorbance (A) is measured at a maximum absorption wavelength of λ max = 420 nm.

Statistical Analysis

Statistical analysis was conducted using the statistical package SPSS 20.0 for Windows. The results are expressed as means ± standard deviation of the mean (SD). The data distribution was analysed using the Shapiro-Wilk and Kolmogorov-Smirnov tests, and depending on the results, the appropriate parametric or nonparametric test was used. The alpha level for significance was set to $p < 0.05$.

RESULTS

O_2^- levels

The values of O_2^- levels were significantly higher in the experimental group compared to the control group ($p < 0.05$) (Figure 1).

H_2O_2 values

The values of H_2O_2 levels were significantly lower in patients with femoral neck fractures compared with healthy subjects ($p < 0.01$) (Figure 2).

TBARS values

The values of TBARS were not statistically different in the experimental group compared with the control group ($p>0.05$) (Figure 3).

NO_2^- levels

The values of NO_2^- levels were significantly lower in patients with femoral neck fractures compared to healthy patients ($p < 0.01$) (Figure 4).

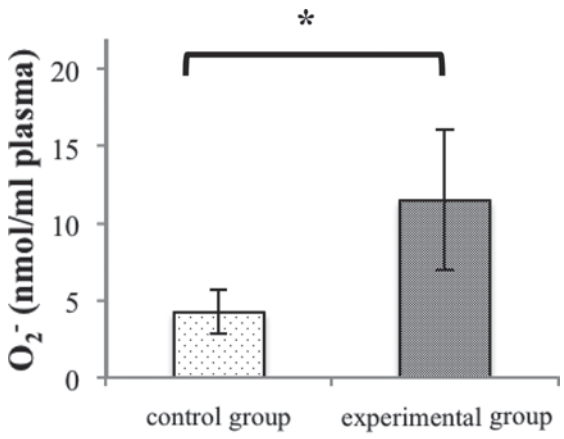

Figure 1. The values of superoxide anion radical levels in the control and experimental groups. The values are represented as X ± SD; X - mean, SD - standard deviation; *p<0.05, **p<0.01.

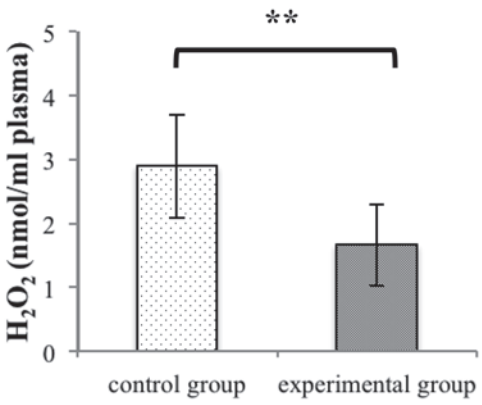

Figure 2. The values of hydrogen peroxide levels in the control and experimental groups. The values are represented as X ± SD; X - mean, SD - standard deviation; *p<0.05, **p<0.01.

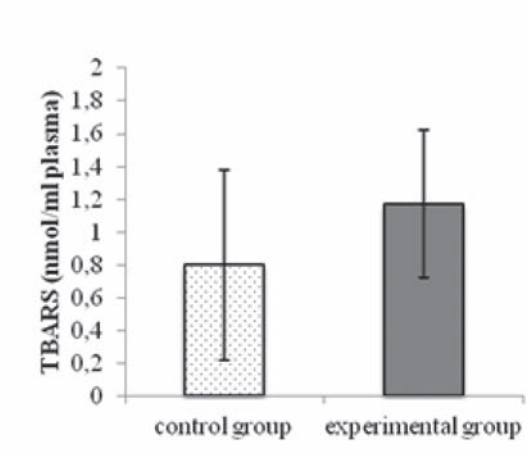

Figure 3. The values of lipid peroxidation (measured as TBARS) in the control and experimental groups. The values are represented as X ± SD; X - mean, SD - standard deviation; *p<0.05, **p<0.01.

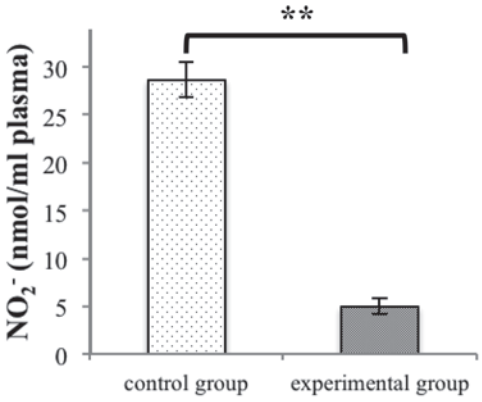

Figure 4. The values of nitric oxide levels in the control and experimental groups. The values are represented as X ± SD; X - mean, SD - standard deviation; *p<0.05, **p<0.01.

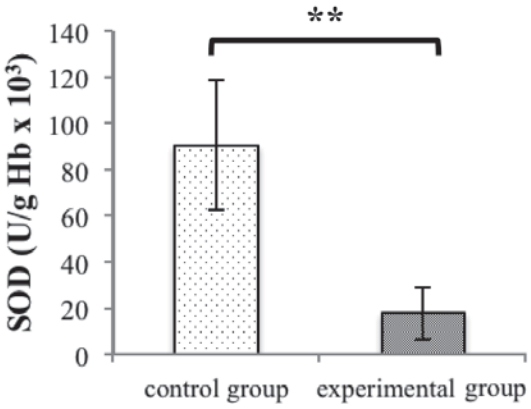

Figure 5. The values of superoxide dismutase levels in the control and experimental groups. The values are represented as X ± SD; X - mean, SD - standard deviation; *p<0.05, **p<0.01.

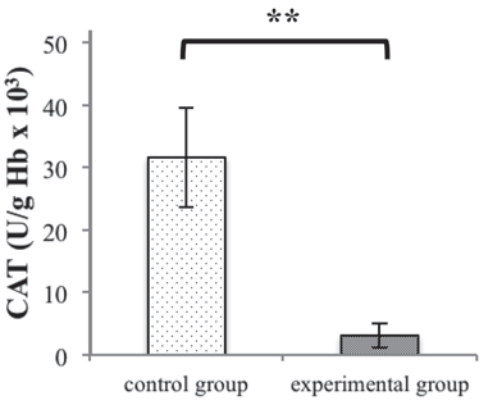

Figure 6. The values of catalase levels in control and experimental groups. The values are represented as X ± SD; X - mean, SD - standard deviation; *p<0.05, **p<0.01.

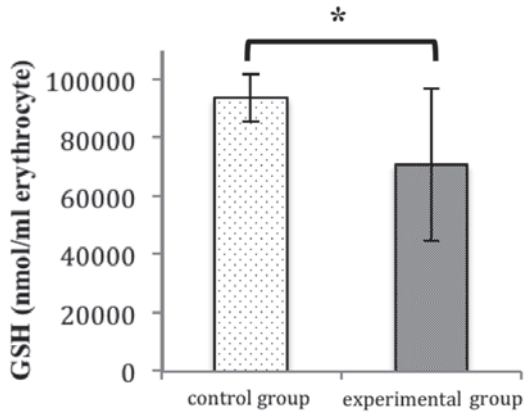

Figure 7. The values of reduced glutathione levels in the control and experimental groups. The values are represented as X ± SD; X - mean, SD - standard deviation; *p<0.05, **p<0.01.

Values of antioxidative enzymes CAT, SOD and GSH

The values of all measured antioxidative enzymes were statistically lower in the experimental group compared to the control group ($p < 0.01$) (Figure 5, 6, 7).

DISCUSSION

Fracture of the femoral neck (hip) is a typical fracture in elderly patients. Despite all the surgical achievements in recent years, hip fractures cause high rates of complications and thus increase disability, morbidity and mortality. One-third of the patients require a higher level of long-term care; furthermore, the death rate in the hospitals is approximately 10%, and mortality during the first year of fracture is approximately 27% (22). As stated above, the risk of fractures is correlated with the age of the patient because any loss of osteoblasts or increase of osteoclasts leads to osteoporosis, lower bone density, decrease in bone mass and deterioration of bone, and thus results in an increased risk for fractures (9).

Considering these factors, the aim of this study was to investigate the value of the oxidative stress parameters and antioxidant enzymes in elderly patients with femoral neck fractures. We must note that many scientists believe that the damage caused by free radicals is the main factor that leads to aging of cells and tissues (23, 24). In fact, it is believed that free radicals damage cellular macromolecules, including proteins, lipids, and DNA, leading to aging and cell death. In addition, there is evidence to support that the aging of cells results in an imbalance between pro oxidants and antioxidants, and leads to the damage of cellular macromolecules (25). This theory explains why the values of oxidative stress parameters were increased in elderly patients.

In contrast to the several studies that have examined the impact of bone fractures in elderly patients on the immune system (26, 27), very few studies have tested their effects on redox status, which is discussed in this study.

During this study, we observed a statistically significant increase in the values of superoxide anion radicals in the experimental group compared to the control group (Figure 1). Opinions about the specific primary mechanisms that cause high concentrations of free radicals are divided. Some researchers believe that high concentrations of superoxide anion radicals is probably the result of the activities of numerous phagocytes including monocytes, macrophages and neutrophils, caused due to the fracture of the femur (28). Others believe that an increase in O_2^- levels occurs as a result of bone resorption by osteoclasts (29). When bone fracture occurs, a remarkable yield of free radicals is generated by the damaged tissues. However, controlled production of free radicals by normally functioning osteoclasts can accelerate the destruction of calcified tissues and assist in bone remodelling. Enhanced osteoclastic activity observed in bone disorders and fractures may be responsible for increased production of reactive oxygen species (ROS) in the form of superoxide anion radicals (30). Moreover, the inhibition of the activity of antioxidant enzymes, such as superoxide dismutase, was associated with increased O_2^- production by the osteoclasts (30, 31). In the present study we found a significant decrease in the activity of SOD, which may be one of the potential reasons for the diminished decomposition of O_2^- and, therefore, its increased release.

Unlike the O_2^- levels, the levels of hydrogen peroxide are lower in patients with femoral neck fractures compared to those who do not have a fracture (Figure 2). These results are puzzling, because the activity of both the enzymes involved in the degradation of H_2O_2 (SOD and CAT) was also reduced. In the literature, there are no other studies that examine H_2O_2 levels in similar conditions, which provides us with a limited explanation of these results.

Because ROS have extremely short half-lives, they are difficult to measure directly. Instead, what can be measured are several products of the damage due to oxidative stress, as in the TBARS assay (32). TBARS assay values are usually reported in malondialdehyde (MDA) equivalents, a compound that is a result of the decomposition of polyunsaturated fatty acid lipid peroxides. The TBARS assay is a well-recognized, established method for quantifying these lipid peroxides, although it has been criticized for its reactivity with compounds other than MDA (32). Although there has been an increase in TBARS values in patients with fractures, this increase was not statistically significant (Figure 3). Wang and colleagues also showed an increase in this parameter in elderly patients with femoral neck fractures (2). In their study, they investigated the value of TBARS in younger patients with femur fractures and have obtained results that show a statistically significant increase unlike those observed in elderly patients. The difference in average age between the patients in our study (66 years old) and their study (86 years old), is approximately 20 years, and could be one of the reasons why our results did not show a statistically significant increase in TBARS values.

The concentration of nitric oxide in physiological conditions is low (33). In our study, the measurement of NO_2^- levels was lower in the experimental group (Figure 4), which may be due to the interaction of NO_2^- with several free radicals (34). Sandukji and colleagues suggest that the excessive production of free radicals occurs three days after the fractures, and one of the reasons for the observed decline may be because the NO_2^- levels were measured too soon (31).

However, it can also be expected that fracture causes changes in the antioxidative enzymatic system. Therefore, we investigated the activity of three major antioxidative enzymes, CAT, SOD and GSH, to create a complete picture of the redox state in patients with femoral neck fractures.

Superoxide dismutase is the most abundant antioxidant enzyme and its main role is to catalyse the neutralization of superoxide anions (35). A study that was carried out in France in the early 1990's recorded a significant decrease in the levels of this enzyme in older patients (36), which is consistent with the theory that connects the free radi-

cals and the process of aging (23). In our study, there was a drastic decline in the SOD concentrations in the experimental group compared to the control group (Figure 5). Many researchers believe that the reason for the low concentrations of this enzyme in patients with femoral neck fractures is the excessive production of superoxide anion radicals (37, 38). Specifically, osteoclasts lead to an increased production of O_2, which is catalysed by SOD, and cause a reduction in the levels of the enzyme antioxidant defence system (34).

Moreover, CAT decomposes H_2O_2 to H_2O and O_2 (29). Our results showed that there was a statistically significant decline of this enzyme in the experimental group (Figure 6). Although the literature mentions a possible decline of antioxidant enzymes during femur fractures (33), there are no studies that specifically examine the values of parameters such as the CAT and GSH levels. Glutathione peroxidase, like CAT, protects cells from hydrogen peroxide. Additionally, our results suggest that the fracture of the femur leads to a drop in GSH levels (Figure 7). However, this is not as drastic as the decrease in CAT levels. One possible explanation for the present imbalance between pro-oxidants and antioxidants in patients with hip fracture is the combined effect of the reduced activity of osteoblasts and the increased activity of osteoclasts. Decreased activity of all the measured antioxidants is difficult to explain, keeping in mind the lack of data in the existing literature. However, one of the potential mechanisms for these results could be a loss of caspase-2 activity during bone fracture, which leads to a reduction in the expression of antioxidant genes (40).

Considering all these results of the present study together may help to better understand the molecular mechanisms involved in femoral neck (hip) fractures. These findings could be of clinical interest and enable the implementation of antioxidant supplements as adjuvant therapy in these patients.

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EFFECTS OF PLANT LECTINS ON HUMAN ERYTHROCYTE AGGLUTINATION

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EFEKTI BILJNIH LEKTINA NA AGLUTINACIJU ERITROCITA KOD LJUDI

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ABSTRACT

Plant lectins are carbohydrate binding proteins or phytohaemagglutinins present in most plants, especially seeds and tubers, which include cereals, potatoes and beans. Lectins have great significance in the diet because of their involvement in gastrointestinal difficulties and erythrocyte agglutination. Blood agglutination activity against A, B, AB and O groups was shown after exposing blood to extracts obtained from 55% of tested plants, while in 45% of plants, agglutination was absent. The results of our study have shown that in humans, 40% of plant extracts exhibited activity against A, 40% of plant extracts exhibited activity against B, and 50% of plant extracts exhibited activity against AB and O groups in humans. The concentration of plant lectins depends on the part of the plant. Lectins from the seeds of certain plants cause the greatest percentage of erythrocyte agglutination, while the lowest agglutination was caused by plant bulbs and leaves. However, lectins derived from all plant species of the family Fabaceae agglutinated erythrocytes of all blood types to some extent.

Keywords: *lectins, agglutination, blood groups*

SAŽETAK

Biljni lektini ili fitohemaglutinini su proteini koji vezuju ugljene hidrate prisutini u mnogim biljkama, posebno u semenkama i gomoljima žitarica krompira i mohunarki. Lektini imaju veliki značaj u ishrani zbog njihovog delovanja na gastrointestinalni trakt i aglutinaciju eritrocita. Aglutinacija krvnih grupa (A, B, AB i O) se pojavila kod 55% uzoraka na testirane biljke, dok je kod 45% uzoraka aglutinacija izostala. Rezultati naše studije su pokazali da 40% biljnih ekstrakata lektina deluje na aglutinaciju krvne grupe A, 40% ekstrakta deluje na aglutinaciju krvne grupe B, a po 50% ekstrakta biljaka utiče na aglutinaciju krvnih grupa AB i O. Koncentracija lektina zavisi od dela biljke. Lektini iz semenki određenih vrsta su pokazali najveći uticaj na stepen aglutinacije eritrocita, dok je najmanji stepen aglutinacije prilikom korišćenja lukovica i listova. Primećeno je da lektini iz svih biljaka porodice Fabaceae utiču na aglutinaciju eritrocita kod svih krvnih grupa.

Ključne reči: *lektini, aglutinacija, krvne grupe*

INTRODUCTION

Lectins are carbohydrate binding proteins present in most plants and in some animals. Lectins do not cause any antigenic stimulation within the immune system, but they have the basic capacity to bind analogously to an antibody (1). The specific capacity of lectins to bind with the cell surface mainly depends on the monosaccharides or simple oligosaccharides, which, when present, inhibit lectin associated reactions. They are involved in cellular interactions (2-3) and the phenomenon of biological recognition such as the binding of microorganisms to target tissues, protein sorting, control of morphogenesis, cell differentiation, fertilization, leukocyte adhesion, metastasis and inhibition of natural killer cell activity against healthy cells (4).

These proteins are widely distributed in living organisms such as algae, animals, microorganisms, fungi and plants (5-7). Plant lectins have mostly been found in seeds and in almost all types of vegetative tissues, including fruits, bulbs, leaves, stems and roots (8-11). Plant lectins from the Fabaceae family that are not effectively degraded by digestive enzymes and that have

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an affinity for the surface epithelial cells of the gastrointestinal tract may be toxic (12). On the other hand, some lectins are slightly absorbed in the gastrointestinal tract and are relatively non-toxic in moderate concentrations (13). Lectins bind to glycosyl groups on the membranes of cells lining the digestive tract; this lectin binding is used as a potential tool for the specific targeting of drugs and for bioadhesive applications. Areas of epithelial cells and even whole zones are necrotized, which can be observed in biopsies of the mammal (14) and insect (15) intestines. However, when cells are treated with lectins *in vitro*, even in high doses, necrosis is not observed, although many other responses have been noted including mutagenesis (16), formation of vacuoles (17) and inhibition of exocytosis.

Over the past few decades, it has been reported that many lectins are toxic and inflammatory. Lectins are resistant to both heat and digestion. Some lectins are highly resistant to gastric acid and proteolytic enzymes (18). According to some studies, some foods containing lectins pass through the intestinal wall, which can result in the deposition of lectins in distant organs (19). Lectins are very active if consumed in fresh food, although activity does not decrease with heat treatment. Haemagglutination activity has been found in processed wheat germ agglutinin, peanuts and dry cereal (20), tomato lectin (12), and navy bean lectin (21), and lectins have been recovered intact in stool (20-21). Wheat gliadin, which causes celiac disease, contains a lectin like substance that binds to the human intestinal mucosa (22). Nephropathy might be caused or aggravated by wheat lectins (23). Wheat lectins are known to induce hyperplasia and hypertrophy of the small intestine and cause changes in body weight and in intestinal function in experimental rats (24). At high dietary levels, lectins cause severe damage to the structure of the small intestine (25-26) and lead to hypersensitivity of the immune system (27).

Recent studies have shown different lectin reactions toward the ABO blood type (28), while other studies show that lectins preferential for a particular ABO type are not found in food and that lectins with ABO specificity are more frequently found in non-food plants or animals (29-30). Despite these criticisms, there seems to be a correlation between diet and blood type. Therefore, when food containing lectin proteins that are incompatible with one's blood type antigen are eaten, the lectins begin to agglutinate blood cells.

MATERIALS AND METHODS

Blood collection from human subjects

The blood samples for this analysis were obtained by venipuncture from healthy individuals of both sexes who have blood types A, B, AB and O (n=24).

Table 1. Percentage of agglutination ratio between blood types

	A	B	AB	O	total
Negative	12	12	10	10	45 %
Positive	8	8	10	10	55 %
%	40	40	50	50	

Preparation of extracts

A total of 20 different plant species which are found in the human diet were purchased. The plant materials were first thoroughly washed with tap water and then rinsed with distilled water. Different plant parts that were being used for isolating lectins were collected from the available sources and taxonomically identified. Portions (1 g) of the edible portions of each food were placed in a warring blender. Two millilitres of 0.9 % NaCl was added and the contents were homogenized for 1 to 3 minutes. The resulting suspension was filtered through cheesecloth, and the filtrate was then centrifuged at 10000 rpm for 10 minutes to obtain a clear extract. The supernatant was separated and stored.

Erythrocyte agglutination

The samples were analysed using an "Olympus CH2" microscope, while digital pictures were taken using an "Olympus DP12 Digital Microscope". A drop of blood of a specific blood type was mixed with a drop of extract and then observed using a microscope (100x magnification). A sample containing a drop of extract and a drop of 0.9 % NaCl was examined as a control.

RESULTS

The percentage ratio of agglutination and the total number of plant species extracts with and without agglutination ability are presented in Table 1. The results showed that lectins from 55% of the plant species studied caused the agglutination of a particular blood group, while lectins from 45% of plant species showed no agglutination. From the 55% of plant species where lectins were capable of agglutination, 40% agglutinated blood types A and B, while the remaining 50% agglutinated blood types AB and O.

The studied plant species and plant parts as well as presence or absence of agglutination is presented in Table 2. It was found that the following nine plant species do not cause agglutination (45%): grape, tomato, banana, walnut, carrot, pumpkin, lemon, garlic and onion. The lectins from beetroot caused blood type O agglutination, while those from apple caused blood type AB agglutination. Furthermore, the lectins from cucumber cased blood type AB and O agglutination. Cashew, melon, strawberry, soy, bean, pea, eggplant and potato caused agglutination in all cases.

Plant species whose seeds were used for the extraction of lectins, such as soy, bean and pea (with the exception of

Table 2. Blood type agglutination and plant species

PLANT NAME	VERNACULAR NAME	FAMILY	PART USED	BLOOD TYPE AGGLUTINATION			
				A	B	AB	O
1. *Allium cepa*	Onion	Amaryllidaceae	bulbs	-	-	-	-
2. *Allium sativum*	Garlic juice	Amaryllidaceae/ Liliaceae	bulbs	-	-	-	-
3. *Anacardium occidentale*	Cashew	Anacardiaceae	fruit	+	+	+	+
4. *Beta vulgaris*	Sugar Beet	Chenopodiaceae	leaf	-	-	-	+
5. *Citrullus vulgaris*	Watermelon	Cucurbitaceae	fruit	+	+	+	+
6. *Citrus limon*	Lemon	Rutaceae	fruit	-	-	-	-
7. *Cucumis sativus*	Cucumber	Cucurbitaceae	fruit	-	-	+	+
8. *Cucurbita pepo*	Pumpkin	Cucurbitaceae	seed	-	-	-	-
9. *Daucus carota*	Carrot	Apiaceae	fruit	-	-	-	-
10. *Fragaria vesca*	Strawberry	Rosaceae	fruit	+	+	+	+
11. *Glycine max*	Soyabean	Fabaceae	seed	+	+	+	+
12. *Juglans regia*	Walnut	Juglandaceae	kernel	-	-	-	-
13. *Malus domestica*	Apple	Rosaceae	fruit	-	-	+	-
14. *Musa paradisiaca*	Banana	Musaceae	fruit	-	-	-	-
15. *Phaseolus vulgaris*	Black kidney bean	Fabaceae	seed	+	+	+	+
16. *Pisum sativum*	Pea	Fabaceae	seed	+	+	+	+
17. *Solanum lycopersium*	Tomato	Solanaceae	fruit pulp	-	-	-	-
18. *Solanum melongena*	Eggplant	Solanaceae	fruit and seed	+	+	+	+
19. *Solanum tuberosum*	Potato	Solanaceae	bulb	+	+	+	+
20. *Vitis vinifera*	Green grapes	Vitaceae	fruit pulp	-	-	-	-

pumpkin), caused agglutination of erythrocytes of all blood types, while the plants whose fruit were used for the extraction of lectins showed different ability to cause agglutination. Plants such as watermelon, cashew, strawberry and eggplant agglutinated erythrocytes of all blood types, while lemon, carrot, banana, tomato and grape did not cause agglutination of erythrocytes. The bulbs used for the extraction of lectins (garlic and onion) did not cause agglutination of erythrocytes, and the lectins from the leaf of beetroot agglutinated blood type O, as shown in Figure 1.

The plants from the Fabaceae and Anacardiaceae families agglutinated red blood cells in all four blood types

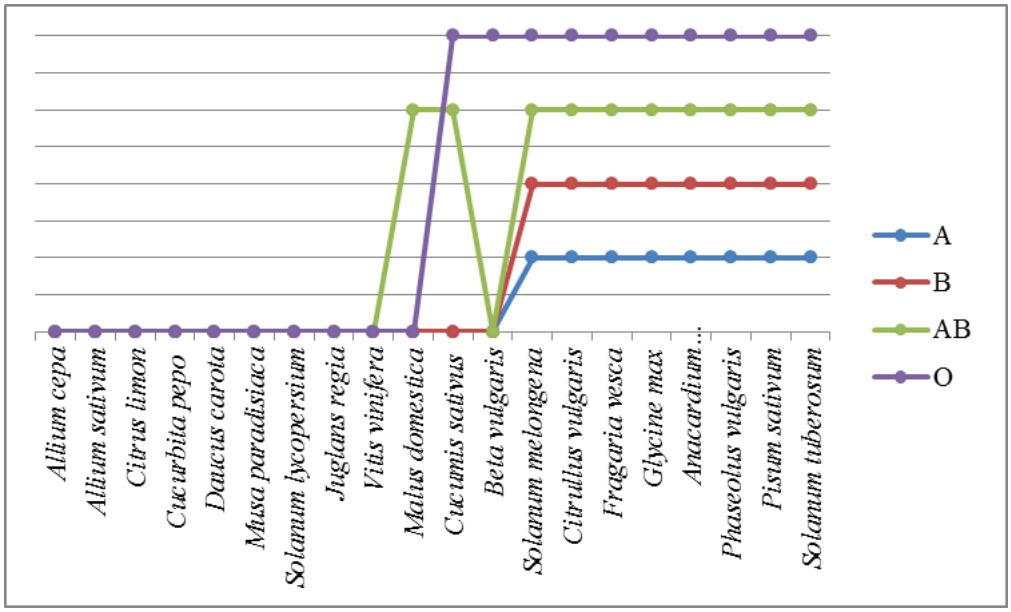

Figure 1. Plant species and agglutination in different blood types

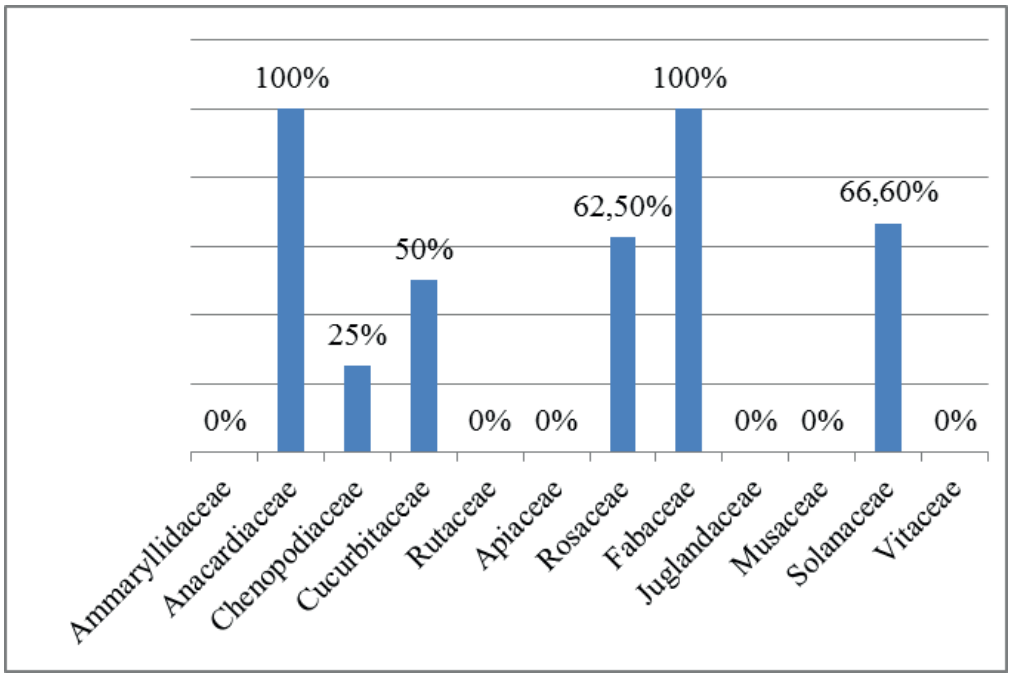

Figure 2. Correlation between percentage of agglutination and plant family

(100%), while the plants from the Amaryllidaceae, Rutaceae, Apiaceae, Juglandaceae, Musaceae and Viaceae families had no effect on erythrocyte agglutination (0%), as shown in Figure 2.

DISCUSSION

Some of the dietary lectins are heat-stable and react with the gastrointestinal tract, causing subclinical effects in humans, especially when used in large quantities (31). Lectins in excess can cause gastrointestinal damage, type-2 IgG immune responses, mild allergies and haemagglutination. It is possible that lectins may not be responsible for short term toxicity caused by the consummation of a raw meal (32). Therefore, lectins play a key role in the daily life of people based on their geographic existence, genetic constitution and adaptability to specific plant products. The development of a lectin tolerance is the result of active immune mechanisms and both the development and maintenance of a lectin tolerance. Nutrition may be the source of antigens that aid in increasing the lectin tolerance of the immune system as well as in providing factors, including nutrients; they themselves might modulate immune maturation and responses and provide factors that influence intestinal microbiota (33). Allergenic proteins present in food may contain components that induce food intolerance or allergy (34). Research related to lectins in food and their interaction with the gastrointestinal tract reveal that there is a direct correlation between the evolution of blood types and food intake types (35). The development of blood groups is based on many factors such as genetic constitution, geographical and environmental factors and the adaptation to various food types from the early days of growth during evolution. Anthropologists recommended that the order of blood group development be based on the food habits of humans (28). This concept states that the first blood group evolved on earth was O: it belonged to the ancestors who hunted, made their own weapons and ate meat. The second blood group that evolved was blood group A, who comprised pure vegetarians. Blood type B was the third blood type developed, which emerged as a result of migration and further climate change. People with this blood type were habituated to a diet that included meat and plants as well as dairy products. The final blood type to evolve was type AB (36).

In this study, it was established that a high percentage of plants (55%) used in the human diet have the ability to cause agglutination of erythrocytes. Lectins from 40% of the plants tested had the ability to agglutinate blood group A, 40% had the ability to agglutinate blood group B, 50% had the ability to agglutinate blood group AB, and 50% had the ability to agglutinate blood group O. Nine plants did not show any lectin activities against any blood groups tested in our study. Some authors claim that the absence of lectin activity in these plants against blood groups has great evolutionary importance in the diversified tolerance development to these foods in the internal lining of the gastrointestinal tract from the mouth to the anus in humans. Studies have shown that lectins from the family Fabaceae usually cause agglutination. Three plant species, *Pisum sativum*, *Phaseolus vulgaris*, and *Glycine max* caused agglutination of erythrocytes from all blood groups. Lectins are found in abundance in le-

gume seeds. *Phaseolus vulgaris* is an herbaceous annual plant grown worldwide for its edible beans, popular in both dry and green bean forms. The commercial production of beans is well distributed worldwide. Haemagglutinating activity in the processed *Phaseolus vulgaris* and *Pisum sativum* that we tested showed high lectin activity. It can be concluded that at least some lectins in food products will survive one or both degradative processes to interact with cells, secretions, and microflora of the digestive tract resulting in, as yet unknown, functional consequences. Extracts from *Cucumis sativus* (Cucurbitaceae) caused agglutination of the AB and O blood groups. Lectins from *Musa paradisiaca* (Musaceae) did not agglutinate human erythrocytes. Extracts from the fruit pulp of the plant *Vitis vinifera* (Vinaceae) did not agglutinate erythrocytes; however, lectins extracted from grape skin caused agglutination of all blood types, as shown in one previous research study (35). The different agglutination abilities of one plant species occur because lectins are present in various plant parts in different concentrations. It is evident that most lectins are present in plant seeds and fruit, while lower concentrations are found in plant roots, stems and leaves. We observed that lectins from seeds (soy, beans and peas) agglutinated all erythrocyte blood types, and lectins from fruit agglutinated different blood types.

The subject of the lectin pathways and effects on organisms is very broad and deserves more discussion. Although lectins are identified as potential toxins, there are some lectins that are beneficial to the body. Lectins provide a diverse and an increasing number of applications. For example, the ability of lectins to bind selectively to carbohydrate residues of glycoproteins makes these proteins useful as differentiating markers to study cancer cells (37) and to characterize differentiation among stem cells (38). Several lectins are reported to possess different biological activities that include anti-bacterial (39), anti-fungal (40), anti-HIV (41), and immunomodulative effects (42) as well as anti-proliferative and mitogenic stimulation for specific cell types. Lectins are also being studied as tools for drug delivery (43). With the numerous and increasing applications of lectins, it is imperative and relevant to identify novel plant sources of lectins.

CONCLUSIONS

The identification of the plant parts that have different concentrations of lectin is important from the aspect of human nutrition due to gastrointestinal problems caused by food containing a large amount of lectins and the ability of lectins to agglutinate human erythrocytes of different blood groups. A large number of plants used in food contain lectins and the largest concentration of lectin is usually found in the plant's seeds. The proper differentiation and knowledge of plant species is important from the aspect of nutrition, and all present selection for a blood type diet.

Authors' contributions

Nađa Zubčević, Bachelor of Biology, Biochemistry and Physiology field designed experiments as well as collected and prepared blood samples and plant specimens. Dr. Sci. Damir Suljević and Muhamed Fočak, MSc. of Biochemistry and Physiology, conducted the analysis to determine the presence or absence of agglutination after mixing blood with the water extract of plants. Dr. Sci. Dunja Rukavina performed research in the laboratory, explored other authors' research and compared their data with our own.

Conflict of interest

We do not have any conflicts of interest to report.

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THE EFFECTS OF SUBCHRONIC METHIONINE OVERLOAD ADMINISTERED ALONE OR SIMULTANEOUSLY WITH L-CYSTEINE OR N-ACETYL-L-CYSTEINE ON BODY WEIGHT, HOMOCYSTEINE LEVELS AND BIOCHEMICAL PARAMETERS IN THE BLOOD OF MALE WISTAR RATS

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EFEKTI SUBHRONIČNOG OPTEREĆENJA METIONINOM SAMOSTALNO ILI U KOMBINACIJI SA L-CISTEINOM ILI N-ACETIL-L-CISTEINOM NA TELESNU MASU, VREDNOSTI UKUPNOG HOMOCISTEINA I BIOHEMIJSKE PARAMETRE U KRVI MUŽJAKA WISTAR PACOVA

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ABSTRACT

Hyperhomocysteinemia (HHC), both basal and after methionine load, may occur due to genetic disorders or deficiencies of nutrients that affect the remethylation or transsulphuration pathways during methionine metabolism. HHC is involved in the pathogenesis of many illnesses as a

SAŽETAK

Hiperhomocisteinemija (HHC), kako bazalna tako i nakon opterećenja metioninom, može nastati usled poremećaja genetskih ili nutricionih faktora u putevima remetilacije ili transsulfuracije tokom metabolizma metionina, uključena u patogenezi brojnih bolesti kako svojim prooksidantnim dejstvom,

ABBREVIATIONS

AHDL - direct high-density cholesterol;
ALB – albumin;
ALP - alkaline phosphatase;
ALT - alanine aminotransferase;
AMY – amylase;
AST - aspartate aminotransferase;
bw – body weight
BUN - blood urea nitrogen;
Ca – calcium;
Cl – chloride;
CHOL – cholesterol;
CREA – creatinine;
ECL -electrochemiluminescence immunoassay system;
GLUC – glucose;
GGT - gamma glutamyl transferase;
GSH – glutathione;
Hcy – homocysteine;
HHC – hyperhomocysteinemia;
IBCT - total iron binding capacity;
K – potassium;
L-cys - L-cysteine
LDL - low-density cholesterol;
MET - DL-methionine;
NAC - N-acetyl-L-cysteine;
Na – sodium;
RCRP - C-reactive protein;
ROS – reactive oxygen species;
TBI - total bilirubin;
TGL – triglyceride;
tHcy – total homocysteine;
TP - total protein;
URCA - uric acid

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result of its prooxidative effect and its impairment of antioxidative protection. The aim was to examine the effects of subchronic methionine overload on the body weight and standard biochemical parameters in rat serum and to examine whether simultaneous subchronic intraperotoneal administration of methionine alone or together with L-cysteine or N-acetyl-cysteine resulted in a change in the body weight and biochemical parameters in the rat serum. The research was conducted during a three-week period (male Wistar albino rats, n=36, body weight of approximately 160 g, age of 15-20 days), and the animals were divided into a control group and three experimental groups of 8-10 animals each: a) control group (0.9% sodium chloride 0.1-0.2 ml/day); b) methionine (0.8 mmol/kg/bw/day) (MET group); c) methionine (0.8 mmol/kg/bw/day) + L-cysteine (7 mg/kg/bw/day) (L-cys+MET group); and d) methionine (0.8 mmol/kg/bw/day) + N-acetyl-L-cysteine (50 mg/kg/bw/day) (NAC+MET group). In addition to the body weight monitoring, the levels of total homocysteine and the standard biochemical parameters in blood samples (plasma or serum) were determined. The results indicated that monitoring the homocysteine levels and standard biochemical parameters in blood could be used for analysis and could provide an excellent guideline for distinguishing between toxic and non-toxic doses of methionine intake, which may be meaningful for clinical applications.

Keywords: *methionine, L-cysteine, N-acetyl-L-cysteine, hyperhomocysteinemia, rat*

tako i oštećenjem antioksidativne zaštite. Cilj ovog rada bio da se ispitaju efekti subhroničnog opterećenja metioninom na promenu telesne mase i vrednosti standardnih biohemijskih parametara u serumu pacova i da li istovremena subhronična intraperotonealna primena metionina sa L-cisteinom ili N-acetil-L-cisteinom utiče na promenu telesne mase i vrednosti standardnih biohemijskih parametara u serumu pacova. Istraživanje je sprovedeno tokom perioda od 3 nedelje (mužjaci pacova soja Wistar albino n=36; tm oko160 g; starosti 15-20), pri čemu su životinje bile podeljene na jednu kontrolnu grupu i tri eksperimentalne grupe sa po 8-10 životinja u grupi, prema sledećoj shemi: a) kontrolna grupa (0,9% NaCl 0,1-0,2 ml/dan); b) metionin (0,8 mmol/kg/tm/dan) (MET grupa); metionin (0,8 mmol/kg/tm/dan) + L-cistein (7mg/kg/tm/dan) (L-cys+MET grupa); metionin (0,8 mmol/kg/tm/dan) + N-acetil-L-cistein (50 mg/kg/tm/dan) (NAC+MET grupa). Pored praćenja telesne mase životinja, u uzorcima krvi (plazma ili serum) određivane su vrednosti totalnog homocisteina i standardni biohemijski parametri. Na osnovu dobijenih rezultata zaključujemo da praćenje nivoa homocisteina i standardnih biohemijskih parametara u krvi predstavlja temelj u analizi i odličnu smernicu pri razlikovanju toksičnih od netoksičnih doza unetog metionina. Pored toga, rezultati ukazuju na antioksidacione i antiinfamatorne osobine N-acetilcisteina i L-cisteina što može imati važnu kliničku primenu.

Ključne reči: *metionin, L-cistein, N-acetil L-cistein, hiperhomocisteinemija, pacov*

INTRODUCTION

Homocysteine (Hcy) is a semi-essential amino acid that contains sulphur obtained from methionine. The necessary quantity of methionine in organisms is maintained by remethylation of homocysteine. The metabolism of methionine is the only known pathway of thiol amino acid production in organisms. Hyperhomocysteinemia (HHC), both basal and after methionine load, may occur due to genetic disorders or nutrition factors that affect the remethylation or transsulphuration pathways during methionine metabolism (1). The reactions in the methionine cycle also lead to formation of the tripeptide glutathione (GSH), with a considerable effect on the processes underlying the formation of free radicals and lipid peroxidation (2). The results of studies of chronic methionine overload suggest the presence of oxidative stress in the rat liver (3, 4). The hyperhomocysteinemia induced by methionine administration leads to increased production of free radicals and inflammation markers in macrophages of the mouse peritoneum (5). An *in vitro* and *in vivo* study on rats showed the alteration of oxidative stress parameters after the methionine administration (6), and hemosiderin accumulated in rat hepatocytes after a four-week methionine treatment (7). The occurrence of hyperhomocysteinemia was also reported in a population of patients with inflammatory bowel disease after an oral overload with methionine (8).

The literature data show that an increased level of homocysteine in blood is a risk factor for cardiovascular diseases, including ischemic cardiac disease and numerous vascular pathologies, chronic renal insufficiency and cerebrovascular disorders (9, 10). Many clinical studies showed that hyperhomocysteinemia is a risk factor for gastrointestinal diseases (11). The increased homocysteine level was shown in colon mucosa and plasma in patients with inflammatory bowel disease (12, 13).

Inflammatory bowel disease is a chronic, idiopathic inflammatory disease of the gastrointestinal tract, with examples including Crohn's disease and ulcerous colitis (14). The evolution of Crohn's disease might include intestinal and extra-intestinal complications, especially atherothrombotic events (15). Hyperhomocysteinemia has been recognized as one of numerous risk factors for the development of thrombotic conditions in patients with cardiovascular diseases (16). The metabolites of homocysteine and Hcy-related genes may be involved in the pathogenesis of ulcerous colitis (17) and coeliac disease (18). Hyperhomocysteinemia is related to inflammation and may be a risk factor for colorectal carcinoma, stomach carcinoma and carcinogenesis in patients with inflammatory bowel disease (19, 20, 21).

Therefore, hyperhomocysteinemia is probably involved in the pathogenesis of numerous diseases with both

its pro-oxidative effect and impairment of anti-oxidative protection. The superoxide dismutase, glutathione peroxidase and catalase enzymes are involved in protecting cells from the harmful effects of oxygen radicals (22, 23). The pathogenesis of numerous gastrointestinal diseases, such as peptic ulcer, gastrointestinal carcinoma and inflammatory bowel disease, partly results from oxidative stress (22). *Matte* et al. (24) noted the occurrence of oxidative stress in the liver of homocysteine-treated rats. In the conditions in which oxidative stress is a molecular cause of the disease, therapy with antioxidative agents, namely supplements with sulphur-containing amino acids (25), is increasingly mentioned in the literature as a possible solution.

N-acetyl-L-cysteine (NAC) is well known as an antioxidative agent that acts directly and/or by increasing intracellular GSH, especially in hepatic tissue. The administration of NAC has been reported to be helpful in many chronic clinical conditions, such as inflammatory bowel disease, obstructive lung disease and other lung diseases, systemic sclerosis, cystic fibrosis, humane immune deficiency syndrome, septic shock, diabetes and liver diseases (26, 27).

Methionine or α-amino γ-methylthio butyric acid, an essential amino acid, is a thiamine acid, as are cysteine and cystine. Methionine participates in protein synthesis, transmethylation reactions and the synthesis of the amino acids cysteine and cystine (28). Methionine is the final precursor of cysteine (one of the three amino acids that form GSH) (29).

The present knowledge about inflammatory bowel disease therapy has been provided by papers that show the protective, anti-inflammatory and antioxidative effects of N-acetyl-L-cysteine on experimentally induced colitis in rats (30, 31). The antioxidative capacity of N-acetyl-L-cysteine was also demonstrated in a model of chronic liver damage in rats (32). A study conducted in humans showed that the supplementation with N-acetyl-L-cysteine improved the oxidative status of patients after surgical treatments in the abdomen, which indicates the importance of the clinical application of this substance (33). Cysteine also has protective effects on the digestive tract, as shown in an experimental model of stomach ulcer in rats (34).

Due to the poor literature data, the aim of this paper was to investigate the effects of subchronic methionine overload on the body weight and standard biochemical parameters of rat serum. Additionally, the paper also examined whether simultaneous subchronic administration of methionine together with L-cysteine or N-acetyl-cysteine resulted in a change in the body weight or standard biochemical parameters in rat serum.

MATERIAL AND METHODS

Experimental animals

Male *Wistar albino* rats (n=36), with a body weight of approximately 160 g and age of 15-20 days at the beginning of the experiment (vivarium of Military Medical Academy in Belgrade), were used in the research on the subchronic administration of methionine. The rats were housed singly or in pairs in transparent Plexiglas cages with a wood-chip floor. Food and water were available *ad libitum*, and the ambient conditions were constant (temperature 21±2 °C; humidity 55±5 %; 12 h light-dark cycle with the light period beginning at 07:30 a.m.).

Experimental protocol

The research was conducted during a three-week period, for which the animals were divided into a control and three experimental groups, with 8-10 animals each. The animals in the experimental groups were administered an intraperitoneal daily dose of a specific substance, depending on the group, and the control group received the same quantity of 0.9% sodium chloride solution daily (35) according to the following protocol: a) control group (0.9% sodium chloride 0.1-0.2 ml/day) (n=10); b) methionine (0.8 mmol/kg/bw/day) (n=10) (MET group); c) methionine (0.8 mmol/kg/bw/day) + L-cysteine (7 mg/kg/bw/day) (n=8) (L-cys+MET group) (36); and d) methionine (0.8 mmol/kg/bw/day) + N-acetyl-L-cysteine (50 mg/kg/bw/day) (n=8) (NAC+MET group) (37). Prior to and during the research, all animals were permanently monitored, with tracking of the body weight of each animal in each group.

On the twenty-second day, the subchronic treatment with the appropriate amino acid(s) was complete; the animals were then sacrificed using a rat guillotine, and blood samples were taken. For this process, blood was collected through a glass funnel and placed in appropriate vacutainers (note: it is known that when this method is used, somewhat higher values of certain biochemical parameters are expected due to the severe mechanical trauma). After the collection, the samples remained at room temperature for 15 minutes and were then centrifuged (15 min x 3000 rpm) and analysed (plasma or serum). At the beginning of and after the experiment, the blood was analysed using the electrochemiluminescence method (ECL- electrochemiluminescence immunoassay system, ADVIA Centaur XP System, Siemens Healthcare GmbH, Erlangen, Germany); the range of reference values was Hcy< 15 μmol/l.

The serum biochemical parameters were measured using a spectrophotometric method and an indirect potentiometric method (sodium, potassium and chloride), while the C-reactive protein (CRP) level was determined by using the immunoturbidimetric method (PETIA). Commercial kits from Siemens Healthcare Diagnostics Ltd. (Frimley, Camberley UK) and an automatic analyser (Dimension Xpand, Siemens, Germany) were used.

Statistical analysis

For the statistical analysis of the experimental data, the parameters of descriptive statistics were used. For testing statistical significance after testing the normality of the

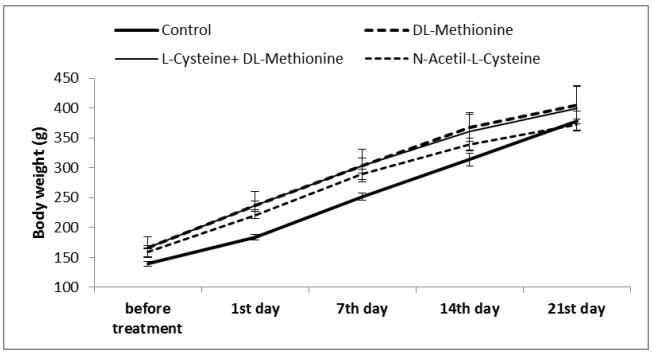

Figure 1. Changes of rat body weight in the control and experimental groups during the three-week experimental period of the intraperitoneal administration of tested substances (the results are shown as X±SE).

distribution, Student's T test (parameter test) was used for dependent and independent variables. Statistical calculations were performed using SPSS computer program (SPSS Inc. Chicago, SAD). P <0.005 was considered statistically significant.

RESULTS

Body weight (BW)

Body weight was the first parameter and was monitored during the entire experimental period (3 weeks). In the control group, which did not receive any of the examined substances, the body weight increased noticeably after each week of research, as in the experimental groups. The highest increase in body weight was found after the first and the third weeks of research. In the MET group, the increase in body weight was lower after the third week, while in the L-cys+MET group, a lower increase was found after both the second and third weeks of research. The same dynamics of this parameter was also found in the NAC+MET group (Fig. 1).

Table 1. Total homocysteine values (tHcy) in the blood of the examined rats (the results are presented as x±SE).

Groups	Total homocysteine (µmol/L)
Control	9.98±0.65
DL-Methionine	9.51±0.59
L-Cysteine + DL-Methionine	10.35±0.93
NAC + DL-Methionine	10.21±0.71

Table 2. Values from the statistical analysis for comparing total homocysteine levels in the blood for the various experimental groups. Values of p<0.05 were considered statistically significant.

Groups	Total homocysteine (µmol/L)
Control vs. DL-Methionine	0.549
Control A vs. L-Cysteine + DL-Methionine	0.741
Control vs. NAC + DL-Methionine	0.741
DL-Methionine vs. L-Cysteine + DL-Methionine	0.477
DL-Methionine vs. NAC + DL-Methionine	0.477

Total homocysteine (tHcy) in blood

The homocysteine levels in the group that received methionine alone (MET group) were lower than those in the groups that received L-cysteine + methionine (L-cys+MET) or N-acetylcysteine + methionine (NAC+MET). The values of this parameter were compared between the experimental and control groups, and differences in homocysteine levels were found but were not statistically significant (Tables 1 and 2)

Standard biochemical parameters

The glycaemia level was also monitored in this research. No statistically significant differences were found in the values of this parameter in any experimental groups compared to the control. In all experimental groups, a statistically significant decrease in the creatinine values was found, while the urea levels were significantly lower than in the control group. The comparison of experimental groups revealed a statistically significant difference in this parameter between the MET and L-cys+MET groups and also between the MET and NAC+MET groups. In contrast, compared to the control group, the NAC+MET group did not show any statistically significant differences in this parameter (Tables 3 and 4).

The lipid profile of animals was analysed in terms of the following parameters: cholesterol, triglycerides, LDL, and HDL. The cholesterol levels in the L-cys+MET group were considerably lower than those in the control group. The triglyceride levels were considerably lower in the L-cys+MET and MET groups, while the HDL levels were considerably lower in the MET group compared to the control. In contrast, the HDL levels were significantly higher in the L-cys+MET group than in the control group.

The electrolyte balance in the blood was monitored by recording the changes in the concentrations of Na, K, Cl and Ca. The sodium levels in the blood were significantly higher in all experimental groups than in the control, whereas there was no significant difference between the L-cys+MET and NAC+MET groups. The potassium levels were significantly lower in all groups compared to the control, but no difference between experimental groups was found. A statistically significant increase in the chloride and calcium levels was only found in the NAC+MET group compared to the control group, and a more significant increase was found when comparing the NAC+MET to the L-cys+MET group. The iron levels in the blood, the next parameter of interest, were only significantly lower in the NAC+MET group compared to the control.

Table 3. Values of standard biochemical parameters, shown as x ± SE.

PARAMETER/ GROUPS (x±SE)	CONTROL	DL-METHIONINE	L-CYSTEINE + DL-METHIONINE	N-ACETYL-L-CYSTEINE + DL-METHIONINE
GLUC	7.64 ± 0.12	6.66 ± 0.87	7.70 ± 0.21	7.61 ± 0.10
BUN	8.80 ± 0.64	6.24 ± 1.19	6.98 ± 0.28	7.60 ± 0.31
CREA	38.19 ± 1.25	29.71 ± 1.57	30.13 ± 1.19	32.25 ± 2.46
URCA	87.88 ± 4.37	47.50 ± 1.61	60.25 ± 4.09	59.75 ± 2.39
TBI	1.89 ± 0.09	2.50 ± 0.20	2.30 ± 0.21	2.14 ± 0.28
TP	63.63 ± 0.63	61.80 ± 0.57	66.50 ± 0.85	63.13 ± 0.77
ALB	13.56 ± 0.94	13.00 ± 0.30	13.13 ± 0.44	12.25 ± 0.37
CHOL	1.82 ± 0.03	1.72 ± 0.07	1.68 ± 0.05	1.64 ± 0.03
AHDL	1.59 ± 0.10	1.47 ± 0.04	1.63 ± 0.05	1.47 ± 0.03
TGL	0.71 ± 0.07	0.93 ± 0.05	1.05 ± 0.09	1.13 ± 0.11
Na	141.50 ± 0.29	145.60 ± 0.45	143.63 ± 0.63	143.00 ± 0.73
K	8.11 ± 0.16	6.76 ± 0.11	7.05 ± 0.22	7.54 ± 0.35
Cl	104.63 ± 0.24	104.70 ± 0.50	104.50 ± 0.27	105.25 ± 0.45
Ca	2.30 ± 0.12	2.06 ± 0.25	2.35 ± 0.02	2.47 ± 0.03
IRON	31.60 ± 1.79	28.02 ± 1.77	29.65 ± 1.89	31.59 ± 2.96
IBCT	112.44 ± 1.66	112.99 ± 2.62	114.25 ± 2.43	117.69 ± 3.08
AST	255.38 ± 13.63	202.20 ± 9.50	251.13 ± 18.88	241.25 ± 19.11
ALT	73.94 ± 8.20	55.40 ± 2.07	66.50 ± 3.76	67.25 ± 4.70
ALP	285.50 ± 12.50	253.60 ± 13.14	277.38 ± 14.74	298.50 ± 12.64
GGT	6.00 ± 0.35	6.00 ± 0.15	5.63 ± 0.18	5.50 ± 0.19
AMY	926.75 ± 77.34	1169.17 ± 48.97	1228.38 ± 50.10	1275.25 ± 40.02
RCRP	0.20 ± 0.03	0.80 ± 0.12	0.90 ± 0.13	0.90 ± 0.09

The liver and pancreas function was evaluated through the enzymes ALT, AST, γGT, ALP and AMY. The AST level was only significantly lower when comparing the MET group to the control, while the ALT level was significantly lower in the MET group compared to the L-cys+MET group. The ALP level was significantly lower in the MET group compared to the control and NAC+MET groups. The γGT levels did not significantly change in any of the groups, while the serum amylase levels were significantly higher in the MET and L-cys+MET groups compared to the control.

The CRP levels were significantly higher in all experimental groups compared to the control, but the differences were not significant for comparisons between experimental groups (Table 3). Table 4 shows the statistical values for the comparisons of the standard biochemical parameters between the different experimental groups.

DISCUSSION

The aim of the research was to evaluate the effects of subchronic methionine overload on body weight, the standard biochemical parameters in serum and plasma and, especially, the homocysteine level in rat blood. Additionally, the effects of simultaneous subchronic administration of methionine with L-cysteine or N-acetyl-L-cysteine on the body weight and the levels of standard biochemical parameters in the rat serum were examined.

Table 4. The statistical values for the comparisons of the standard biochemical parameters between the different experimental groups.

PARAMETER/ GROUPS	GLUC	BUN	CREA	URCA	TBI	TP	ALB	CHOL	AHDL	TGL	NA	K	CL	CA	IRON	IBCT	AST	ALT	ALP	GGT	AMY	RCRP
Control vs. DL-Methionine	.232	.008	.003	.000	.014	.038	.008	.179	.008	.015	.000	.000	.828	.854	.329	.712	.029	.051	.126	.255	.055	.001
Control vs. L-Cysteine + DL-Methionine	.538	.001	.000	.001	.021	.016	.036	.020	.500	.007	.008	.003	.649	.602	.501	.462	1.000	.854	.854	.639	.017	.000
Control vs. NAC + DL-Methionine	.951	.012	.031	.001	.110	.756	.003	.002	.009	.008	.108	.118	.183	.358	.903	.098	.520	.927	.297	.322	.011	.000
DL-Methionine vs. L-Cysteine + DL-Methionine	.228	.386	.861	.010	.261	.003	.781	.824	.045	.424	.024	.181	.708	.229	.756	.859	.051	.045	.197	.126	.302	.809
DL-Methionine vs. NAC + DL-Methionine	.334	.962	.450	.003	.166	.138	.136	.563	.563	.131	.009	.090	.465	.230	.398	.328	.168	.056	.021	.053	.071	.557
L-Cysteine + DL-Methionine vs. NAC + DL-Methionine	.526	.226	.598	.793	.708	.011	.145	.563	.031	.753	.520	.493	.154	.035	.431	.345	.529	.958	.345	.626	.462	.874

Methionine, which is an essential and thiol amino acid, is necessary for muscular contractions, haemoglobin synthesis, cholesterol and fat degradation (38). Hence, methionine participates in numerous biochemical processes in living organisms; its role in breaking down fat depots, synthesizing creatinine and increasing body weight is interesting (39). The intraperitoneal administration of methionine, i.e., its isomer DL-methionine, did not lead to a statistically significant increase in body weight that was proportional to time compared to the values in the control group. *Kluge* et al. examined the effects of DL-methionine on the growth of ducks during a 3-week period and concluded that the group of animals that received methionine in their food experienced an increase in body weight and growth without statistical significance and without improvement in physical performance, which is consistent with our results (40). However, recent experimental and clinical studies increasingly report that NAC is a potential therapeutic agent. *Elshorbagy* et al. examined the effect of sulphur amino acids (NAC, cysteine) on the prevention of obesity in rats. The animals received a methionine-restricted diet and were treated with the mentioned acids for 12 weeks; the results suggest that NAC and cysteine had significant effects with a decrease in body weight, primarily in the adipose tissue. Thus, the changes in body weight in the rats in our research can be explained by the presence of methionine during the entire experimental period, while NAC and L-cysteine probably blocked the capacity of methionine to prevent obesity, perhaps through the stearoyl-coenzyme A desaturase-1 enzyme (41, 42). However, further research is necessary to confirm this finding due to the considerable variations in the results of previous studies. These differences are primarily related to the variety in the experimental models (type of model, duration of experiment, dose, and form of the examined substance) and the differences in the sulphur amino acid administration procedures because the effects (e.g., NAC effects) significantly differ between intravenous and oral intake (43).

In addition to methionine and cysteine, homocysteine is a sulphur acid. Its modulatory effects are increasingly reported, and Hcy is considered a new risk factor, i.e., a damage marker. Numerous factors, including supplementation with sulphur amino acids, influence the homocysteine level in blood (44).

All compounds of this type have a free sulphur group and are capable of forming disulphides in plasma. Through this type of reaction with homocysteine, sulphur amino acids decrease the homocysteine concentration. However, in our study, the homocysteine level in blood only decreased in the group that received methionine alone. In contrast, when administered with L-cysteine or NAC, methionine did not decrease the Hcy level but instead increased it. In an experimental study, a 6-week methionine diet (1%) led to an increase in the homocysteine level in blood as a result of remethylation and transsulphuration processes (45), which conflicts with the findings in our study. However, chronic methionine overload is very toxic to various tissues and organs (46), thus increasing oxidative stress, thus stimulating adaptive reactions, i.e., compensatory responses. Such oxidative stress is correlated with the homocysteine level in blood; therefore, high homocysteine levels are expected when large doses of methionine are administered. This prediction indirectly agrees with our results because we used much less toxic doses in a shorter period than described in the mentioned study.

To further elucidate the subchronic effects of DL-methionine during this research, standard biochemical parameters were monitored (Table 1) and determined from blood samples (plasma or serum).

When ROS production is high, homeostasis is disturbed, leading to oxidative stress, which has a key role in the development of liver and other chronic diseases (5, 6). Oxidative stress generates liver damage by causing changes in lipids, proteins and DNK molecules and even more importantly, by modifying pathways that control normal biological functions. Moreover, the systemic oxidative stress that develops after liver disease may also lead to damage in other organs, such as the brain and kidneys. Consequently, homeostasis of the whole organism was monitored through biochemical parameters that are primarily associated with the biological processes in the liver, kidneys, pancreas and bowels. Due to the key role of oxidative stress in diseases of the liver and other organs, potential antioxidants, NAC and L-cysteine were also tested (27).

In our research, methionine alone or in combination with NAC and L-cysteine did not significantly influence the glycaemia levels. *Cole* et al. studied the effect of hyperglycaemia on the homocysteine and S-adenosyl-methionine (SAM) levels in the blood and plasma, and they showed that hyperglycaemia was correlated with the increased and decreased level of homocysteine (47).

These results are consistent with ours, due to the obvious decrease in Hcy levels and glycaemia in the DL-treated group. In the other groups, the increase in glycaemia was proportional to the increase in the Hcy level in the blood, probably as a result of lipid peroxidation after the oxidative stress. Both cysteine and NAC, together with other compounds that contain a cysteine group, participate in the regulation of the production of insulin and the maintenance of blood glycose levels, thus decreasing glyco-oxidation (48). Our study did not reveal statistically significant changes in this parameter after the administration of NAC and L-cysteine.

Impaired liver function is mostly shown by the change in "liver" parameters. Therefore, better knowledge of the changes in the pattern of serum parameters related to morphological and functional liver status might contribute to better treatment of diseases of the liver and other organs, thus making it easier to select optimal therapeutic modalities. In our study, the cholesterol and triglyceride levels were significantly lower than those in control group due to the effect of NAC and L-cysteine. This finding confirms the antioxidative capacity of these compounds because N-acetylcysteine is a glutathione prodrug that protects the liver from hepatic steatosis by

limiting the production of reactive oxygen species (6,7). Methionine removes metabolic waste products from the liver and can thus decrease the risk of hepatic and arterial steatosis. Methionine is important for the health of the liver and its detoxification and normal function. With the help of choline and inositol, methionine prevents fat accumulation in the liver. In our research, methionine also led to a decrease in hepatic lipid levels, although the decrease was not significant, possibly due to the shorter administration period compared to other studies (49). When compared to cysteine in another study, methionine inhibited the accumulation of fat to a higher degree; this result was also confirmed by our findings (50).

The administration of DL-methionine significantly reduced the parameters of hepatocellular and cholestatic liver damage, while the administration of NAC and L-cysteine caused a less significant decrease. In contrast, *Early* et al. investigated the effect of high concentrations of sulphur amino acids (DL-methionine, L-cystine) on the function and morphology of rat liver and concluded that an overload with sulphur amino acids during a short time resulted in liver atrophy and damage (51). Hence, the antioxidative properties of methionine, NAC and L-cysteine are probably dose dependent, i.e., only occur when lower doses are administered.

The degree of lipid peroxidation greatly depends on the degree of antioxidative protection in the organism at the moment when free radical production increases. When it occurs, lipid peroxidation changes the fluidity and permeability of the cell membrane, which results in electrolyte transport disorders, changes in protein content and modified functioning of organelles. *Roediger* compared the effects of two sulphur sources, inorganic NaHS and the amino acid L-methionine, on the redox status in rats to determine the mechanism underlying the development of ulcerous colitis to elucidate possible treatments. He concluded that L-methionine considerably decreased the beta-oxidation caused by NaHS in the colon and that exogenous methionine strongly influenced the quantity of SAM, which decreased the quantity of disulphide (52).

As a mechanism in the development of disease, a disruption of redox balance in favour of ROS occurs in many inflammatory conditions, primarily in the gastrointestinal tract (ulcerous colitis and Crohn's disease) (53). Proteins are oxidized in inflammatory diseases; thus, cell apoptosis occurs as a consequence of the cell exposure to a large quantity of ROS. The parameters of inflammation during the administration of three different amino acids were monitored in this paper, and no statistically significant change in CRP was found between these treatments and the control group. However, it is interesting that inflammatory parameters can be simultaneously monitored with homocysteine levels. In their study, *Panq* et al. reported that CRP directly participates in the occurrence and progression of atherosclerosis and that the homocysteine levels were correlated with the expression of CRP, whereby the administration of NAC decreased the expression of C-reactive protein. These data are interesting; they will certainly expand the perspectives in the domain of the prevention, diagnostics and therapy of inflammatory diseases (54).

The roles of iron in the metabolism of homocysteine have been described in the literature. Iron (Fe) catalyses the formation of Hcy from methionine, S-adenosylhomocysteine and cystathionine, thus increasing the circulating tHcy levels. Furthermore, free Fe catalyses the production of free oxygen radicals and the oxidation of small density lipoproteins, which is a known risk factor for vascular damage (55). The iron levels in blood were monitored in all groups. A significantly lower level of this parameter was found only in the NAC+MET group, while the tendency for increased iron levels was observed in the groups where the homocysteine levels were proportionally higher, which is a very important fact. Accordingly, *Lee* et al. noted that the increased values of iron in blood were related to mitochondrial dysfunction, which might result in increased ROS production; therefore, in the conditions of iron overload, N-acetylcysteine could be used as an antioxidant with the capacity to reduce ROS (56). This knowledge of the significance of Fe as a diagnostic and prognostic parameter, especially in conjunction with other biochemical parameters, is thus of great importance given that hyperhomocysteinemia is increasingly described in the literature as an independent risk factor for cardiovascular diseases.

CONCLUSIONS

The obtained results suggest that monitoring homocysteine levels and standard biochemical parameters in blood is crucial for analysis and provides an excellent guideline for distinguishing between toxic and non-toxic doses of methionine intake. Hence, homocysteine is a unique and reliable surrogate marker for evaluating the progression of metabolic disorders. In addition, supplementation with N-acetylcysteine and L-cysteine can have potential clinical applications, which needs to be confirmed by further clinical investigations.

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EEG ABNORMALITIES AS DIAGNOSTIC AND PROGNOSTIC FACTOR FOR ENCEPHALITIS

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EEG ABNORMALNOSTI KAO DIJAGNOSTIČKI I PROGNOSTIČKI FAKTOR ZA ENCEFALITIS

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ABSTRACT

The aim of the study is to examine whether EEG abnormalities in patients with encephalitis might be prognostic and diagnostic factors for final epilepsy outcome and/or be correlated with the severity of the disability.

The most frequent causes of encephalitis were HSV, WNV, INF V, MTB, St PN, St AU. There was a highly statistically significant positive correlation between the severity of the EEG abnormalities at the beginning of the disease (r = 0.410, p <0.01) and the ultimate outcome.

Electroencephalography in the early stages of encephalitis shows diagnostic and prognostic significance and, in combination with the overall severity of the clinical picture, could contribute to the diagnosis and assessment of outcomes and, ultimately, the correction of treatment and faster recovery of patients. This is particularly true for viral encephalitis.

Keywords: *encephalitis, EEG, consciousness disorder, seizure.*

SAŽETAK

Cilj naseg rada je da se utvrdi da li elektroencefalografi ja (EEG) kod pacijenata sa encefalitisom može biti prognosticki i dijagnosticki faktor u odnosu na krajnji ishod i da li korelira sa težinom bolesti.

Najčešći uzročnici encefalitisa bili su herpes simplex virus, West Nile virus, virus influence, Mycobacterium Tuberculosis, St. Pneumoniae, Staphylococcus aureus. Postoji visoko staatistički značajna pozitivnaa korelacija između težine EEG nalaza na početku bolesti (r=0.410, p<0.01) i kontrolnog EEG nalaza (EEG 2) i ishoda bolesti (r=0.391, p<0.01). Postoji visoko statistički značajna korelacija između EEG1 nalaza (r = 0.391, p<0.01) i EEG2 nalaza i neurološkog deficita (r = 0.477, p<0.01).

EEG u ranim stadijumima encefalitisa ima dijagnostički i prognostički značaj i u kombinaciji sa težinom kliničke slike može doprineti postavljanju dijagnoze i proceni ishoda bolesti i uz to korigovanju terapije i bržem oporavku pacijenata. Ovo se posebno odnosi na virusne encefalitise.

Ključne reči: *encefalitis, EEG, poremećaj svesti, epileptični napad.*

ABBREVIATIONS

AC - alpha coma;
BUE – bacteria of unknown aetiology;
CSF - cerebrospinal fluid;
EEG - electroencephalography;
FSA - focal slow activity;
GSA - generalized slow activity;
HSV - herpes simplex virus;
INF V - virus influence;
MTB - Mycobacterium Tuberculosis;
NF - nonspecific findings;
PCR - polymerase chain reaction;
St PN - St. Pneumoniae;
St AU - Staphylococcus aureus;
VUE - virus of unknown aetiology;
WNV - West Nile virus.

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INTRODUCTION

Encephalitis is defined as inflammation of the brain parenchyma (focal or diffuse) that is associated with signs of focal or diffuse neurological dysfunction (1). Encephalitis affects all ages and genders, although the greatest frequency of presentation is in children and in adults older than 65 years (2, 3).

Encephalitis can arise from infectious, immune-mediated or unknown aetiology. In most cases, the cause of encephalitis is unknown. The most common infectious agents are as follows: Herpes simplex virus (HSV), Mycobacterium tuberculosis (MTB), and the Varicella zoster virus. Less common causes of encephalitis include Streptococcus pneumoniae (ST Pn), Influenza A (INF V), and the Epstein-Barr virus (4). Other less common viruses carried by insects (arboviruses, West Nile virus (WNV)) have also become increasingly common in recent years in our country) (2).

The main clinical features of brain dysfunction in encephalitis are the following: fever, headache, seizures, lethargy, irritability, personality and/or behavioural changes, stiff neck, focal neurological signs, gastrointestinal symptoms, respiratory symptoms, rash, photophobia, or urinary symptoms (2, 4).

Rapid diagnosis and prompt treatment of encephalitis are crucial to the outcome (5). Diagnosis is based upon clinical, laboratory, neuroradiological and electroencephalographic (EEG) characteristics (6). EEG findings in patients with encephalitis have been shown to be correlated with the severity of disease and may have prognostic significance (7). A definitive diagnosis is made by lumbar puncture and the analysis of cerebro-spinal fluid (cytological, biochemical and microbiological). Etiological confirmation is achieved by culturing cerebro-spinal fluid (bacterial) or by the detection of the virus in the cerebrospinal fluid (CSF) of the central nervous system via PCR or specific antibodies. It is of less importance is to prove the presence of the virus outside the central nervous system (throat swabs, stool) or to demonstrate the presence of antibodies in the serum (5).

Encephalitis is a difficult to treat, life-threatening disease characterized by high mortality, and survivors risk multiple complications and consequences (epilepsy, behaviour disorder, disorder of memory and remembering, emotional instability, etc.) (8).

The aim of this study was to determine to what extent EEG recordings of brain function in patients with encephalitis could be used as prognostic tools for the final outcome (lethal or recovery), to what degree they could be prognostic tools for the degree of recovery, and to what degree they are correlated with the severity of disability and possible causal explanations for this disability.

PATIENTS AND METHODS

Our retrospective study included 46 patients with confirmed diagnoses of encephalitis or meningoencephalitis, with or without one or more epileptic seizures. The study was conducted at the Clinic for Infectious Diseases and at the Clinic of Neurology, Clinical Centre in Kragujevac and included all patients treated in the period from March 2012 to January 2015.

At initial patient intake, we evaluated their somatic statuses, neurological statuses and states of consciousness. Blood was drawn for laboratory analysis, and we performed a lumbar puncture and cytological examinations of the CSF. To establish the aetiological diagnosis and disclosure of a causal infection, we also conducted virology, bacteriology, and immune-serology based tests and used PCR.

Standard EEG and video EEG monitoring were performed for all patients in the electroencephalography ward at the Clinic of Neurology. The EEG was recorded on two occasions during hospitalization. The first recording was made within the first three days of hospitalization whenever possible, or as early as possible in critically ill patients, no later than seven days from the start of disease. The recordings were compared with each other and with the general clinical picture of the patient. The second recording was made at the end of hospitalization (at discharge of the patient). Over the next 3-6 months, patients were followed in our infectology and neurology outpatient clinic to determine their somatic, neurological and mental statuses. Over the same period, we also conducted one other control EEG recording.

Disturbed states of consciousness were graded in three levels: low disturbance (somnolence), medium-heavy disturbance (sopor) and heavy disturbance (coma).

Neurological deficits were graded in three levels: low (weakness in the extremities fixation), medium-heavy (manifest weakness) and severe (paralysis of the extremities). A fourth category was composed of patients without neurological deficits.

Pathological EEG findings were classified into 3 categories: Generalized slow activity (GSA), as an indicator of generalized brain damage, focal slow activity (FSA) as an indicator of focal brain damage and Alpha coma (AC) as an indicator of severe brain damage. We also found a group of patients with nonspecific EEG abnormalities (NF) and normal EEGs.

At the final follow-up for disease outcome, we classified patients into 3 categories: partial recovery, full recovery or lethal outcome.

The recordings were compared with previous findings (EEG findings of all three) as well as with the current situation of the patient to determine if the initial EEG correlated to the ultimate severity (EEG time point 1 and EEG time point 2) and whether there were correlations with the final outcome (all three EEG findings).

All data were analysed using descriptive and analytical statistics. Chi-squared tests were used for categorical variables and Student's t-tests were used for continuous variables. We used correlations for EEG findings, outcomes and neurological deficits. SPSS (version 20.0) was used for statistical analyses. Statistical significance was set at $p<0.05$.

RESULTS

We included 46 patients (25 males and 21 females) with a mean age 42.8 years.

The most common encephalitis symptom was a disturbed state of consciousness -97.8% (somnolence 22, sopor 11, coma 12 patients). Other frequent symptoms were fever (95%) and headache (59%).

Twenty-two patients (48%) experienced one or more provoked epileptic attacks during hospitalization (Figure 1), while 5 patients (11%) experienced repeated attacks during the monitoring period and were diagnosed with symptomatic epilepsy.

We observed severe neurologic deficits in 24% of patients, medium-heavy deficits in 11% and no or low-grade disturbances in 65% of patients.

EEG findings during the disease and the control EEG findings are presented in Table 1.

The most frequent causes of encephalitis were HSV (PCR from CSF to HSV-1) WNV (serology from serum and CSF), INF V and viral encephalitis of unknown origin (Table 2).

We found isolated bacteria in 35% of patients: MTB (PCR from CSF and Lowenstain +), St PN (CSF culture and haemocultures), and St AU (CSF culture and haemocultures). Five patients were diagnosed with bacterial meningitis of unknown origin, with the initial lumbar puncture after the start of antibiotic therapy.

We isolated a Candida species (Candida IgM and IgG + in CSF) in one patient.

Lethal outcomes were observed in 13.8% of patients with viral encephalitis, partial recovery in 34.5% and complete recovery in 51.8% of patients. Lethal outcomes were observed in 37.5% of patients, with all others making a full recovery (62.5%).

There was a highly statistically significant positive correlation between the severity of the EEG findings at beginning of the disease (EEG 1) and the disease outcome or degree of recovery (r = 0.410, p<0.01). There was also a highly statistically significant positive correlation between the control EEG reading (EEG 2) and the disease outcome (r = 0.391, p <0.01), but there was no statistically significant correlation between the findings from EEG 3 and disease outcome (r = + 0.131, p = 0.446).

There was a highly significant correlation between the EEG time point 1 findings and the degrees of neurological deficit (r = 0.391, p <0.01) and between EEG time point 2 findings and the degrees of neurological deficits (r = 0.477, p <0.01). There were no statistically significant correlations between EEG 3 readings and neurological deficits (r = 0.224, p = 0.189).

There was a highly statistically significant positive correlation between the degree of neurological deficit and the disease outcome (r = 0.736, p <0.01).

A total of 69% of patients (20 patients) with viral encephalitis showed abnormal findings on their EEG. That there was a highly statistically significant positive correlation between the severity of abnormalities at EEG time point 1 and the disease outcome (r = + 0.515. p<0.01) and between EEG time point 2 and disease outcome (r = 0.562, p<0.01), indicating the potential value of EEG monitoring for prognosis. There was no statistically significant correlation between the abnormalities observed at EEG 3 and the disease outcome (r = 0.204, p=0.328). There was a highly statistically significant positive correlation between the severity of the neurological deficits and the disease outcome (r = 0.597, p<0.01). Other patients (31%) showed nonspecific or normal findings at EEG 1 and EEG 2.

For patients with bacterial encephalitis, EEG 1 and EEG 2 findings were abnormal in 43.8% of patients, and there were no correlations with the severity or with recovery. There were no statistically significant correlations among the severity of abnormalities at EEG 1, EEG 2 or EEG 3 and disease outcome. (r = + 0.375, p=0.153; r =

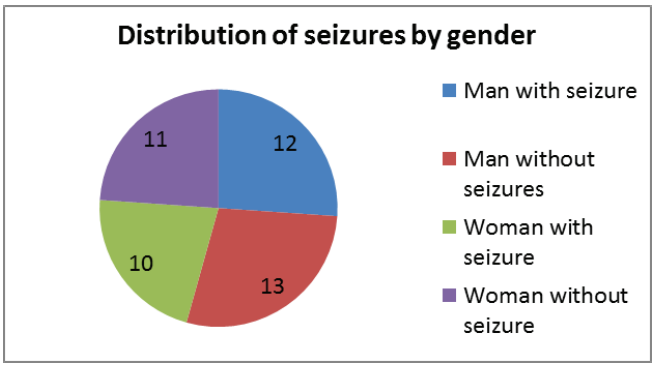

Figure 1. Distribution of seizures by gender

Table 1. Number of patients with EEG findings during disease

EEG findings	EEG 1	EEG 2	EEG 3
AC	2	1	0
GSA	16	10	0
FSA	12	9	0
NF	12	20	8
Normal EEG	4	5	29
Without EEG	0	1	9

Table 2. Causes of encephalitis.

Causes		male	female	Number of patients	%
Viruse	HSV	7	11	18	39.1
	WNV	2	1	3	6.5
	INF V	2	2	4	8.7
	VUE	3	1	4	8.7
Bacteria	MTB	3	1	4	8.7
	St PN	2	2	4	8.7
	St AU	2	1	3	6.5
	BUE	4	1	5	10.9
Fungus	Candida.	0	1	1	2.2

0.253, p=0.362; r = -0.167, p=0.645). There was a highly statistically significant positive correlation between the severity of neurological deficits and the disease outcome (r = 0.946, p<0.01).

There were no statistically significant differences in the outcome of the disease depending upon the cause (viral vs. bacterial) (p=0.546). There were no statistically significant differences in neurological deficits depending upon the cause (viral vs. bacterial) (p=0.728). None of the three EEG recording showed statistically significant correlations with the cause (viral vs. bacterial) (p1=0.203, p2=0.342, p3 =0.346).

DISCUSSION

HSV encephalitis is the most common type of sporadic encephalitis in the world and in our country (39% of patients) (4). In our sample of patients, three patients showed evidence of WNV, which is a relatively rare and new agent in the region. In 2013, the first published cases of encephalitis caused by the virus in our country were reported; 44 patients with WNV encephalitis were admitted during the summer of 2012 (2). There are no precise data on the incidence of encephalitis caused by INF in Serbia except in the form of case reports from patients who had neurological complications in the form of encephalopathy caused by this virus (9). Mycobacterium tuberculosis is a relatively rare cause of encephalitis (1-5% worldwide) (4, 10); however, in our study, the frequency of this bacterium was 8.7%, which was as common as other bacterial pathogens (St Pn, St Au). The cause of encephalitis in more than one-third of cases is typically unknown (4), whereas in our study it was 19.6%.

In our total population of patients with encephalitis, abnormal EEG findings were predictive of a severe clinical picture and poorer prognosis as early as the first and as late as the control EEG findings. There was a significant correlation between the severity of the EEG findings and the degree of recovery. It has previously been suggested in HSV encephalitis that the weight of the initial EEG findings may be a useful prognostic factor for disease outcome (11).

All patients with viral encephalitis and a lethal outcome showed EEG abnormalities that correlated with the severity and could serve as a diagnostic, as well as prognostic tool, as seen in other studies (11). Survivors of viral encephalitis usually emerge with cognitive changes and damage to their executive functions (12). In most patients with partial recovery, we observed EEG abnormalities that reflected the seriousness of their clinical picture as early as the EEG 1 and EEG 2 time points (recorded during the early stages of the disease). Abnormalities at EEG 1 and EEG 2 in patients with a viral infection correlated with the severity and the final outcome. EEG 3 was typically normal or showed nonspecific abnormalities, and it did not correlate with recovery or severity. In our study, it showed little practical significance.

Patients with bacterial encephalitis with lethal outcomes consistently showed abnormal, pathological findings on their EEG, but none of the three EEG recordings were specific for the severity or prognosis of the disease. One possible explanation is that the time interval over which we worked was inadequate to capture these effects. The window between the first EEG, which was recorded very early, and the second EEG, may have been too long given that the full clinical picture develops more slowly in bacterial infection compared to viral infection, and there is a prompt and favourable response to causal therapy. The third EEG in most cases was nonspecific and corresponded to recovery, with no statistically significant correlations and with no significance as a prognostic tool.

Of the total 46 patients, death occurred in 10 patients (22%). Other studies have shown differing rates of mortality of 10-18% (13, 14). Mortality in bacterial and viral encephalitis is typically similar, but in our study, a higher mortality rate was observed in bacterial encephalitis, which was not unexpected because these were patients of older age, had more comorbidities, and developed more severe complications, all of which are common in other studies (14). The frequency of provoked epileptic seizures and symptomatic epilepsy correlated with the severity of disability (11).

In 36 patients, there was a favourable outcome. The majority made a full recovery (56%), and 22% made a partial recovery. One study demonstrated an inverse relationship between patients with complete and partial recovery (36% vs. 56%). There was a significant positive correlation between the severity of the complete clinical picture and the final outcome of both viral and bacterial encephalitis. A more severe clinical picture was associated with a worse prognosis, which was expected (14).

CONCLUSION

Electroencephalography in the early stages of encephalitis shows diagnostic and prognostic significance, and, in combination with severity of the clinical picture, could contribute to the diagnosis and assessment of outcomes and therefore the earlier correction of treatment and faster recovery of patients. This is particularly true for viral encephalitis, as electroencephalography was not specific in bacterial encephalitis. The diagnostic usefulness of EEG in bacterial encephalitis requires further verification in a larger sample. In most retrospective studies, the sample size are small, and our research should be extended to a larger sample.

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GALECTIN-3 DELETION ENHANCES VISCERAL ADIPOSE TISSUE INFLAMMATION AND DYSREGULATES GLUCOSE METABOLISM IN MICE ON A HIGH-FAT DIET

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DELECIJA GALEKTINA-3 POSPEŠUJE INFLAMACIJU U VISCERALNOM MASNOM TKIVU I NARUŠAVA HOMEOSTAZU GLUKOZE U MIŠEVA NA ISHRANI BOGATOJ MASTIMA

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ABSTRACT

Obesity and type 2 diabetes mellitus (T2DM) constitute major health problems worldwide. Increased visceral adiposity enhances the risk of insulin resistance and type 2 diabetes. The mechanisms involved in obesity-associated chronic inflammation in metabolic tissues (metaflammation) that lead to insulin resistance and dysregulated glucose metabolism are incompletely defined. Galectin-3 (Gal-3), a β-galactoside-binding lectin, modulates immune/inflammatory responses and specifically binds to metabolic danger molecules. To dissect the role of Gal-3 in obesity and diabetes, Gal-3-deficient (LGALS3$^{-/-}$) and wild-type (WT) C57Bl/6 male mice were placed on a high-fat diet (HFD, 60% kcal fat) or a standard chow diet (10% kcal fat) for 6 months and metabolic, histological and immunophenotypical analyses of the visceral adipose tissue were performed. HFD-fed LGALS3$^{-/-}$ mice had higher body weights and more body weight gain, visceral adipose tissue (VAT), hyperglycaemia, hyperinsulinemia, insulin resistance and hyperlipidemia than diet-matched WT mice. Compared to WT mice, the enlarged VAT in obese LGALS3$^{-/-}$ mice contained larger adipocytes. Additionally, we demonstrate enhanced inflammation in the VAT of LGALS3$^{-/-}$ mice compared with diet-matched WT mice. The VAT of LGALS3$^{-/-}$ mice fed a HFD contained more numerous dendritic cells and proinflammatory F4/80^{+}CD11c^{+}CD11b^{+} and F4/80^{high} macrophages. In contrast to WT mice, the numbers of CXCR3^{+} and CD8^{+} T cells were increased in the VAT of Gal-3-deficient mice after 6 months of high-fat feeding. We provide evidence that Gal-3 ablation results in enhanced HFD-induced adiposity, inflammation in the adipose tissue, insulin resistance and hyperglycaemia. Thus, Gal-3 represents an important regulator of obesity-associated immunometabolic alterations.

Keywords: *Galectin-3, obesity, hyperglycaemia, insulin resistance, metaflammation*

SAŽETAK

Gojaznost i tip 2 diabetes mellitusa (T2DM) predstavljaju veliki svetski zdravstveni problem. Uvećanje visceralnog masnog tkiva u gojaznosti je povezano sa većim rizikom za nastanak insulinske rezistencije i T2DM. Molekularni mehanizmi povezani sa hroničnom inflamacijom u metabolički aktivnim tkivima (metaflamacijom) u gojaznosti koji leže u osnovi insulinske rezistencije i narušene homeostaze glukoze nisu do kraja razjašnjeni. Galektin-3 je multifunkcionalni lektin sa značajnom ulogom u imunoregulaciji i metaflamaciji. Sa ciljem da se ispita uloga galektina-3 u nastanku gojaznosti i T2DM, galektin-3 deficijentni miševi (LGALS3$^{-/-}$) i miševi divljeg soja (WT) stavljeni su na ishranu sa visokim sadržajem masti (60% kcal od masti) ili standardnu ishranu (10% kcal od masti) u trajanju od 6 meseci nakon čega su ispitivani metabolički parametri, morfologija visceralnog masnog tkiva i fenotipske karakteristike infiltrišućih ćelija. LGALS3$^{-/-}$ miševi na ishrani sa visokim sadržajem masti imali su veću telesnu težinu, veću količinu visceralnog masnog tkiva, hiperglikemiju, hiperinsulinemiju, izraženiju insulinsku rezistenciju i hiperlipidemiju u poređenju sa WT miševima na istom režimu ishrane. Adipociti iz uvećanog visceralnog masnog tkiva LGALS3$^{-/-}$ miševa imali su veći dijametar u poređenju sa adipocitima u WT miševa. Izražena inflamacija u visceralnom masnom tkivu LGALS3$^{-/-}$ miševa na ishrani sa visokim sadržajem masti bila je praćena većom zastupljenošću dendrtičnih ćelija, proinflamatornih F4/80^{+}CD11c^{+}CD11b^{+} i F4/80^{high} makrofaga, CD3^{+}CXCR3^{+} i CD8^{+} T limfocita u poređenju sa WT miševima na istom režimu ishrane. Dobijeni rezultati ukazuju na značajnu protektivnu ulogu galektina-3 u nastanku gojaznosti, insulinske rezistencije i T2DM.

Ključne reči: *Galektin-3, gojaznost, hiperglikemija, insulinska rezistencija, metaflamacija*

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INTRODUCTION

Obesity and type 2 diabetes mellitus (T2DM) constitute a major public health problem worldwide, particularly in developing countries (1). Obesity, mainly abdominal adiposity, is linked to various metabolic alterations that increase the risk for T2DM (2). T2DM is a metabolic disorder characterized by insulin resistance (IR) followed by pancreatic β-cell dysfunction (3). Pancreatic β-cells normally compensate for obesity-associated reduced insulin sensitivity by secreting more insulin to maintain glucose homeostasis. Hyperinsulinemia occurs early in the disease progression and before the β-cell function becomes impaired, leading to late-stage (insulin-dependent) T2DM (4).

Obesity-induced diabetes is associated with low-grade inflammation in the visceral adipose tissue (VAT). Inflammation in the VAT plays an important role in metabolic abnormalities such as IR (5). Antigen-presenting cells, including dendritic cells (DCs) and macrophages, are increased in the VAT in obesity, where they potentiate both innate and adaptive immune/inflammatory responses. Numerous studies have shown the increased infiltration of the visceral adipose tissue by macrophages during obesity (6,7). Macrophages may be derived from blood monocytes and are classified as classically (M1) and alternatively (M2) activated macrophages (8). Recent studies have shown that macrophages are key cells that mediate obesity-induced metabolic abnormalities. In obesity, a shift from alternatively activated M2 to classically activated M1 macrophages, characterized by elevated F4/80, CD11b and CD11c expression, has been demonstrated (9,10). Moreover, the ablation of CD11c-positive cells normalizes insulin sensitivity in obese insulin-resistant animals (11). Adipocytes damaged by high lipid intake produce signals that promote a proinflammatory Type 1 immune response. The number of T cells is increased in the enlarged VAT during obesity in response to adipose tissue-specific factors (12). Adipose tissue-associated regulatory T cells (Tregs) protect against (13), while the recruitment of Type 1 CD4 and $CD8^+$ T lymphocytes precedes, metabolic alterations in obesity (14,15). The chemokine receptor CXCR3 is highly expressed in activated T cells and is involved in the regulation of T cell trafficking and maturation. Additionally, it has been shown that CXCR3-positive cells play an important role in the modulation of obesity-induced visceral adipose tissue inflammation and systemic insulin resistance (16).

Galectin-3 (Gal-3) is a β-galactoside-binding lectin that is expressed in different tissues and cells and plays an important role in obesity, T2DM and inflammation (17,18,19). Gal-3 regulates inflammation and adipogenesis, and this lectin is expressed in adipocytes and infiltrating immune cells in the adipose tissue (20). Additionally, Gal-3 regulates adipocyte cell proliferation and differentiation (21) and has a variety of regulatory roles in the innate and adaptive immune response, depending on the disease conditions (18,22,23). However, the role of Gal-3 in type 2 diabetes remains incompletely understood. The Gal-3 levels are increased in the sera of obese subjects and negatively correlate with the levels of glycosylated haemoglobin (24). In contrast with these data, Okhura et al. reported that low serum Gal-3 levels are associated with insulin resistance and T2DM (25). However, controversial results have been reported regarding the effects of Gal-3 ablation in experimental models of diabetes. It has been shown that Gal-3-deficient mice were relatively resistant to diabetogenesis in streptozotocin-induced diabetes (26). On the other hand, Pejnovic et al. reported that obese Gal-3-deficient mice had enhanced adiposity, hyperglycaemia, hyperinsulinemia, IR and systemic inflammation in comparison with their diet-matched wild-type controls (18). Moreover, in a study by Peng et al. (27), Gal-3-null mice fed a HFD had increased adiposity and dysregulated glucose metabolism. In addition, the same authors found that young Gal-3-deficient mice fed a standard diet exhibit altered glucose homeostasis, thus suggesting the modulation of glucose metabolism and possibly β-cell function by Galectin-3 (Gal-3) independently of obesity and inflammation.

To investigate the role of Gal-3 in high-fat diet-induced obesity, we used Gal-3-deficient mice on a C57Bl/6 background. We examined inflammation in the visceral adipose tissue and metabolic abnormalities following long-term HFD exposure in Gal-3-deficient and wild type (WT) mice. We report here that Gal-3 deletion enhanced high-fat diet (HFD)-induced obesity and visceral adiposity, amplified inflammation in the visceral adipose tissue and led to dysregulated glucose metabolism characterized by hyperglycaemia and insulin resistance.

MATERIALS AND METHODS

Experimental mice and study design

Gal-3-deficient mice on a C57BL/6 background and their littermate controls were obtained from the University of California Davis (Davis, CA; by courtesy of D.K. Hsu and F.T. Liu) and accommodated in our animal facilities under standard laboratory conditions in a temperature-controlled environment with a 12 h light/darkness cycle. Male 2-month-old wild type (WT) and Gal-3-deficient ($LGALS3^{-/-}$) mice were fed either normal chow or a 60% fat/kcal diet (Mucedola, Italy) *ad libitum*. After 6 months, the mice were sacrificed and blood samples and visceral adipose tissue were collected for analyses. All animal procedures were approved by the ethical committee of the Faculty of Medical Sciences, University of Kragujevac (Permit Number 01-2759/2).

Body weight and glucose metabolism analyses

Body weights and fasting blood glucose levels were measured once per month and after 6 months on a standard or

HFD. The mice were fasted for 4 h, and their glucose levels (mmol/L) were determined using the Accu-Chek Performa glucometer (Roche Diagnostics, Mannheim, Germany). The serum concentrations of total cholesterol and triacylglycerol were measured using the Olympus AU600 Chemistry Immuno Analyzer (Olympus, Tokyo, Japan) and the fasting insulin was measured using an Insulin ELISA kit (Alpco, Salem, NH, USA). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the following formula: HOMA-IR = [(Glucose mmol/L x Insulin mU/L)] / 22.5.

Histological analysis of visceral adipose tissue

Visceral adipose tissue (VAT), including the epididymal, mesenteric and renal fat pads, was dissected and weighed as visceral fat content. After weighing, part of the epididymal fat was fixed in 10% buffered formalin. The mean adipocyte size of the visceral fat pad was determined by computer-assisted image analysis of paraffin-embedded adipose tissue sections (5 **µm) stained with** haematoxylin and eosin (H&E). The adipocyte size was measured from a total of 50 cells per mouse in three separate fields using a light microscope (BX51; Olympus) equipped with a digital camera and *ImageJ* software. Analyses were performed in a blinded fashion by two independent observers. The data are expressed as the mean adipocyte diameter (µm) for each tissue in each animal.

Isolation of visceral adipose tissue stromal ascular fraction cells

Total visceral adipose tissue (VAT) was subjected to the isolation of stromal vascular fraction (SVF) cells. Collagenase digestion (1 mg/ml collagenase type II and 2% BSA (Sigma-Aldrich, St. Louis, MO)) was used to separate the SVF from the adipocytes of the VAT, as previously described (18). SVF was used for flow cytometric analysis, as described below.

Flow cytometric analyses

Adipose tissue SVF cells were stained with the following fluorescence-tagged monoclonal antibodies: anti-mouse CD45, CD3, CD4, CXCR3, CD8, CD11b, Lineage cocktail (BD Biosciences, San Jose, CA), Sca-1, NK 1.1, F4/80 and CD11c (BioLegend, San Diego, CA) or isotype-matched controls (BD Biosciences). The cells were analysed using a FACSCalibur flow cytometer (BD Biosciences) and FlowJo software (Tree Star).

Statistical analyses

Statistical analysis was performed using SPSS 13.0. The data are presented as the means ± SEM. Statistical significance was determined by an independent-sample Student's t test and, where appropriate, a Mann-Whitney U test. Statistical significance was assumed at $p<0.05$.

RESULTS

Galectin-3 ablation accelerated HFD-induced obesity and obesity-related metabolic alterations

After 6 months, HFD increased the body weight and weight gain in both genotypes of mice, and these parameters were higher in HFD-fed LGALS3$^{-/-}$ mice than in diet-matched WT mice (Fig. 1A). HFD feeding increased the visceral fat mass in both genotypes of mice, which was more pronounced in HFD-fed LGALS3$^{-/-}$ mice than in diet-matched WT mice (Fig. 1B). The serum total cholesterol and triglyceride levels were significantly higher in obese LGALS3$^{-/-}$ mice than in WT mice (Fig. 1C).

Galectin-3 deletion modulates HFD-induced adipocyte morphology

In view of the fact that Gal-3 is expressed in the adipose tissue and regulates adipocyte cell proliferation and differentiation, we analysed adipose tissue morphology and adipocyte size. The HFD significantly increased the adipocyte size in both genotypes of mice compared with mice on a standard diet. A significantly higher amount of total visceral adipose tissue was accompanied by larger adipocytes in LGALS3$^{-/-}$ mice on a HFD than in diet-matched WT animals (Fig. 1D).

Gal-3 ablation accelerated HFD-induced hyperglycaemia, hyperinsulinemia and obesity-associated insulin resistance

In addition to accelerated HFD-induced obesity, the fasting blood glucose, insulin and HOMA-IR were significantly higher in obese LGALS3$^{-/-}$ mice than in WT mice (Fig. 2). Additionally, the fasting blood glucose levels, insulin and insulin resistance were significantly higher in LGALS3$^{-/-}$ mice than in WT mice on a standard diet (Fig. 2).

Increased dendritic cells and proinflammatory macrophages in the VAT in LGALS3$^{-/-}$ mice on a HFD

Because obesity-induced diabetes is strongly associated with adipose tissue inflammation, we first analysed the innate immune cells in the visceral adipose tissue in both genotypes of mice after 6 months on a HFD. DCs and macrophages are known to be increased in the visceral adipose tissue during obesity. The VAT from HFD-fed LGALS3$^{-/-}$ mice contained higher numbers of total CD11c^{+} ($p=0.034$) and CD11c^{+}F4/80^{-} ($p=0.043$) DCs than the VAT from HFD-fed WT mice (Fig. 3A). In addition, the subsets of mature proinflammatory F4/80^{hi} ($p=0.034$) and triple-pos-

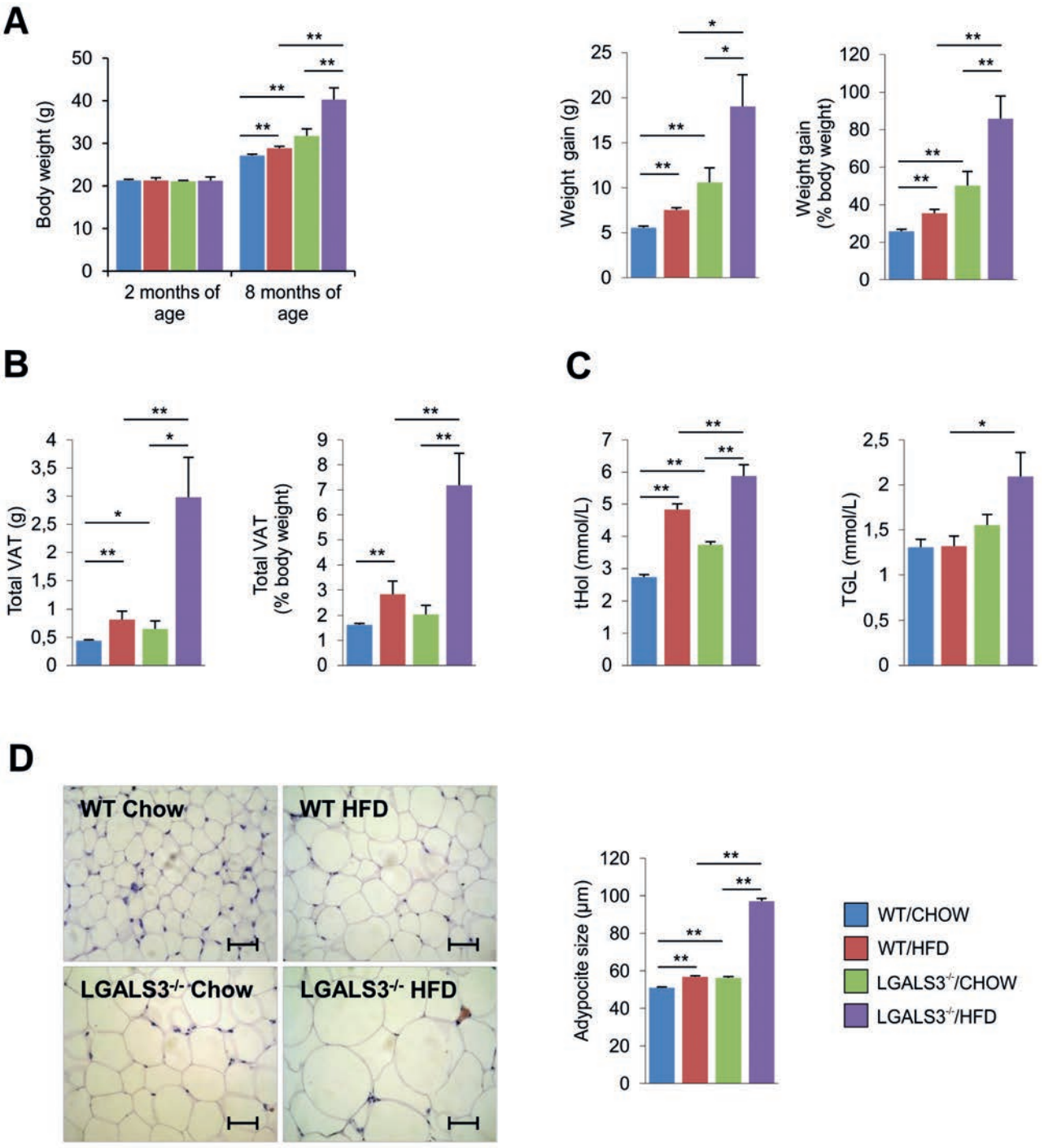

Figure 1. Galectin-3 ablation accelerated HFD-induced obesity and adipocyte hypertrophy

Male 2-month-old wild type (WT) and Gal-3-deficient (LGALS3$^{-/-}$) mice were fed either standard chow or a HFD *ad libitum* for 6 months. Metabolic parameters after 6 months on the HFD or standard diet are shown. (A) Body weight and weight gain were higher in HFD-fed LGALS3$^{-/-}$ mice than in diet-matched WT mice. (B) Total VAT and visceral fat mass (% body weight) were significantly higher in LGALS3$^{-/-}$ mice fed a HFD than in diet-matched WT animals. (C) Serum lipid levels were significantly higher in obese LGALS3$^{-/-}$ mice than in WT mice. (D) Representative images depicting the larger adipocyte size in LGALS3$^{-/-}$ mice on a HFD than in diet-matched WT animals (original magnification 40x, scale bar = 50 µm). The results are shown as the means ± SEM (n=5-6 mice/group), *P<0.05, **P<0.01.

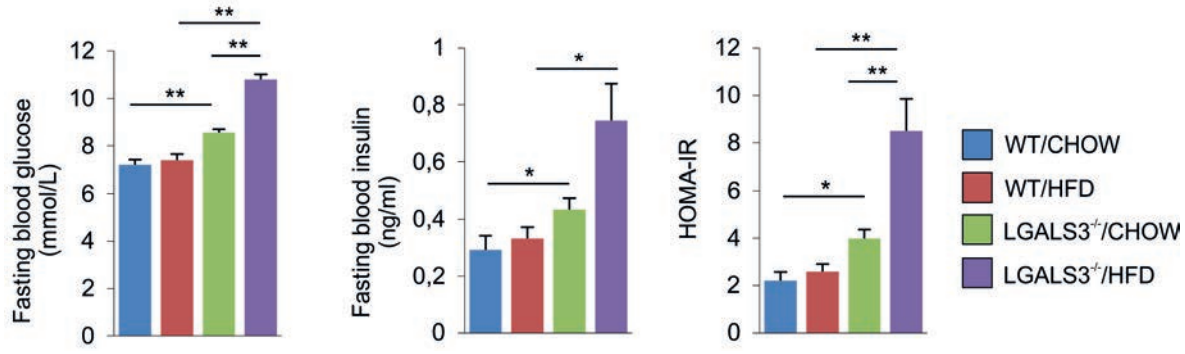

Figure 2. Galectin-3 ablation accelerated HFD-induced hyperglycaemia, insulinemia and insulin resistance
The fasting blood glucose, insulin and HOMA-IR were significantly higher in LGALS3$^{-/-}$ mice than in WT mice after 6 months on a HFD. The results are shown as the means ± SEM (n=5-6 mice/group), *P<0.05, **P<0.01.

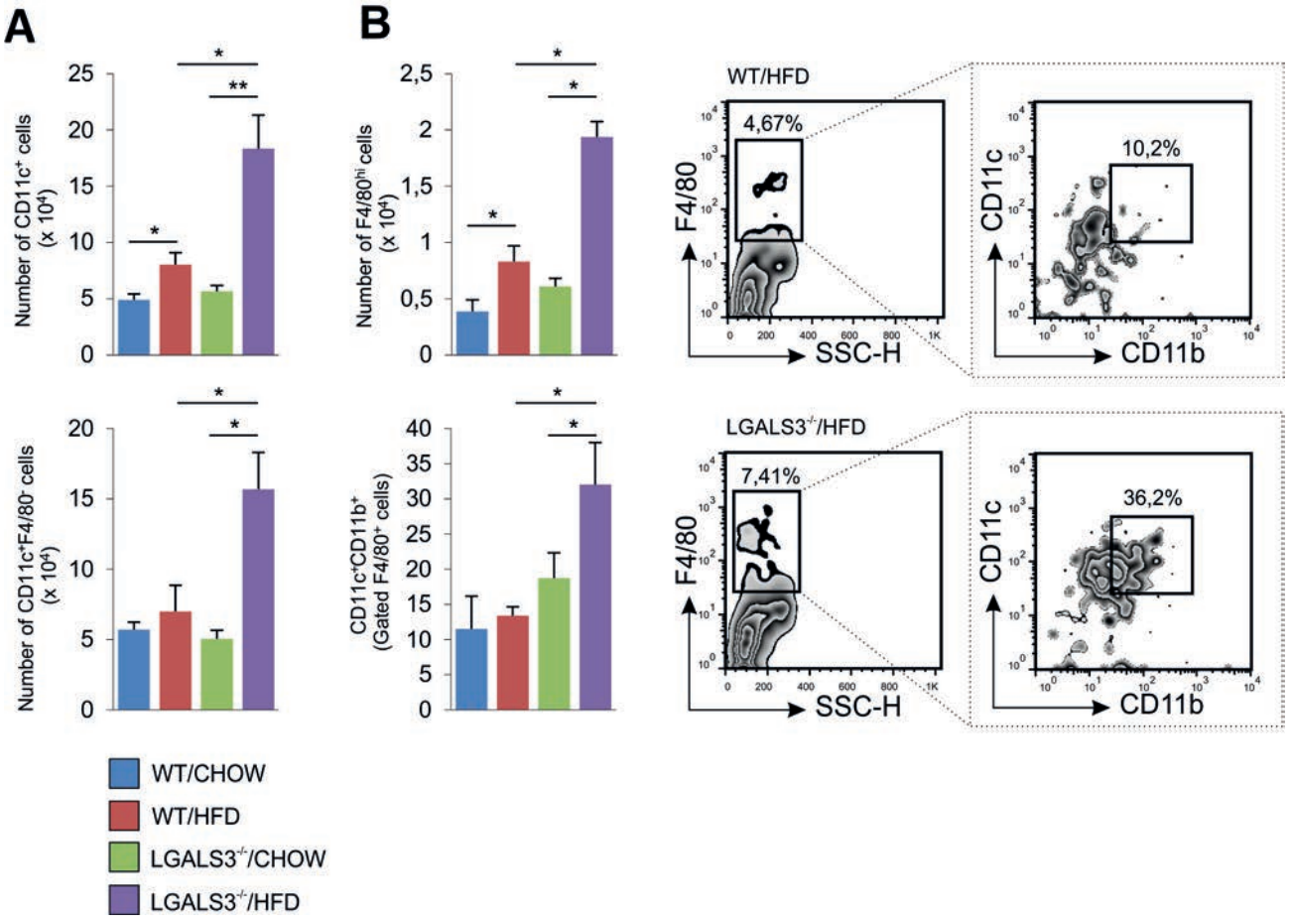

Figure 3. Increased dendritic cells and proinflammatory macrophages in the visceral adipose tissue of LGALS3$^{-/-}$ mice on a HFD
Representative images and flow cytometric analysis of VAT stromal vascular fraction cells from WT and LGALS3$^{-/-}$ mice after 6 months on a HFD or standard diet. (A) Dendritic cell numbers were increased significantly in the VAT in obese LGALS3$^{-/-}$ mice. (B) Proinflammatory macrophages were significantly increased in LGALS3$^{-/-}$ mice fed a HFD compared with diet-matched WT mice. Representative FACS plots are shown. The results are shown as the means ± SEM (n=5-6 mice/group), *P<0.05, **P<0.01.

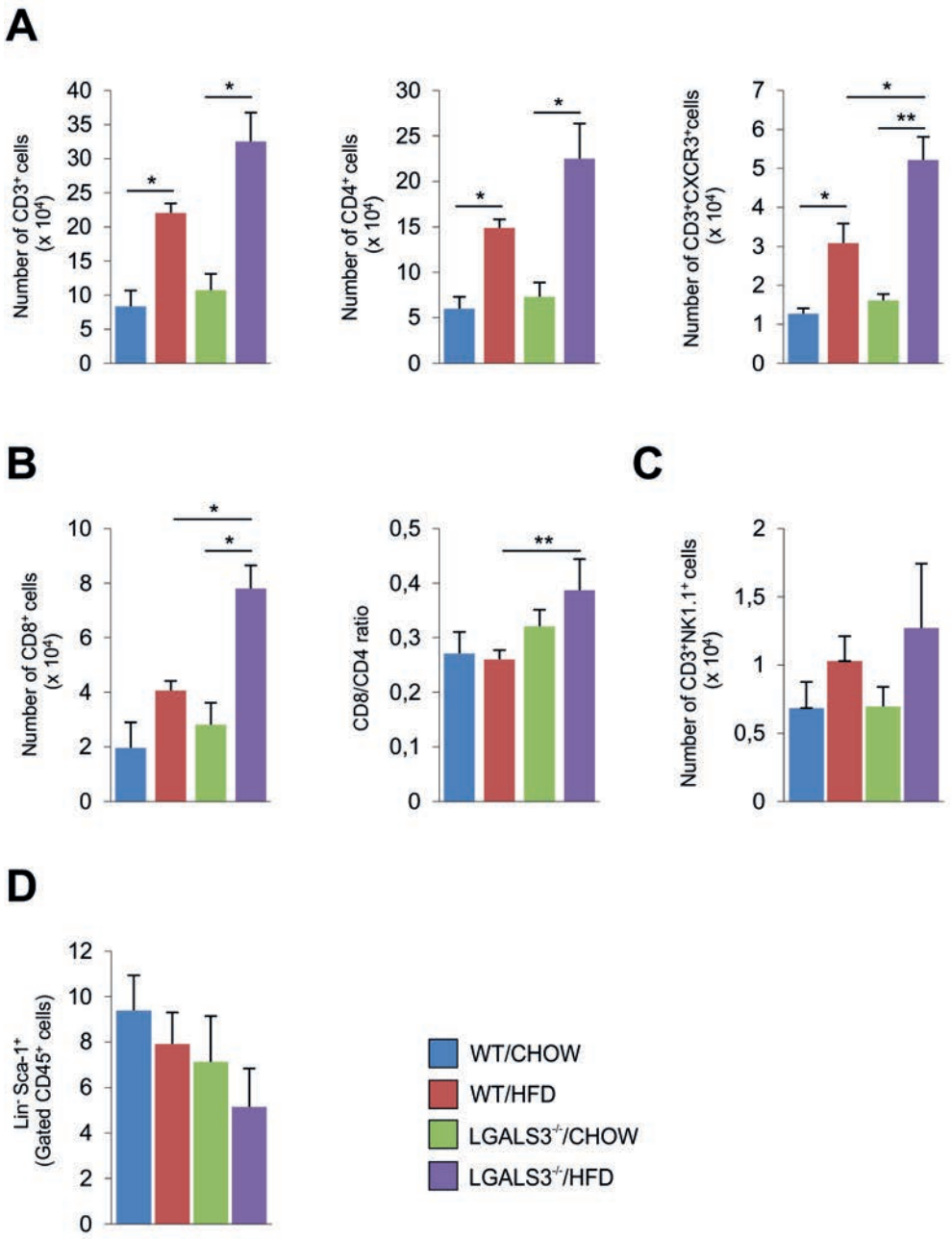

Figure 4. Increased CXCR3+ and CD8+ T lymphocytes in the visceral adipose tissue of LGALS3$^{-/-}$ mice on a HFD
Flow cytometric analysis of VAT stromal vascular fraction cells from WT and LGALS3$^{-/-}$ mice after 6 months on a HFD or standard diet. (A) CD3^{+}, CD4^{+} and CD3^{+}CXCR3^{+} T lymphocytes in the VAT. (B) The number of CD8^{+} T lymphocytes and the CD8/CD4 ratio were higher in Gal-3-deficient mice on a HFD than in diet-matched WT mice. (C) NKT lymphocytes in the VAT (D) Lin^{-} Sca-1^{+} innate lymphoid cells in the VAT. The results are shown as the means ± SEM (n=5-6 mice/group), *P<0.05, **P<0.01.

itive F4/80^{+}CD11b^{+}CD11c^{+} macrophages (p=0.034) were significantly higher in the VAT of obese LGALS3$^{-/-}$ mice than in WT mice (Fig. 3B).

Increased CXCR3+ and CD8+ T cells in the VAT in LGALS3$^{-/-}$ mice on a HFD.

Increased Type 1 T cells and cytotoxic CD8^{+} T cells in the VAT are hallmarks of obesity-induced diabetes and are strongly associated with adipose tissue inflammation. Therefore, we performed phenotypic analyses of T lymphocytes in the visceral adipose tissue in both genotypes of mice after 6 months on a HFD.

The HFD significantly increased the number of CD3^{+} and CD4^{+} T lymphocytes in both genotypes of mice compared with mice on a standard diet. There was no significant difference in the number of CD3^{+} and CD4^{+} T cells between the genotypes on a HFD (Fig. 4A). The HFD sig-

nificantly increased the number of $CD3^+CXCR3^+$ T cells in both genotypes of mice compared with mice fed a standard diet. However, the number of $CD3^+CXCR3^+$ cells ($p=0.031$) was higher in the VAT from $LGALS3^{-/-}$ mice than in the VAT from WT mice, both on a HFD (Fig. 4A). Moreover, the number of $CD8^+$ ($p=0.034$) T lymphocytes and the CD8-to-CD4 ratio (Fig. 4B) were higher in $LGALS3^{-/-}$ mice fed a HFD than in diet-matched WT mice ($p=0.014$). There was no difference in the number of NKT cells (Fig. 4C) or the percentage of $CD45^+Lin^-Sca\text{-}1^+$ innate lymphoid cells between the two genotypes of mice on both diets (Fig. 4D).

DISCUSSION

In this report, we demonstrate increased obesity, visceral adipose tissue inflammation and dysregulated glucose metabolism in $LGALS3^{-/-}$ mice on a long-term high-fat diet. These effects appear to be mediated by both the metabolic and the immunoregulatory effects of Gal-3. After 6 months on a HFD, the body weight, weight gain and amount of total visceral tissue were significantly higher in $LGALS3^{-/-}$ mice than in diet-matched WT mice (Fig. 1A and 1B). HFD feeding in Gal-3-deficient mice resulted in increased weight and visceral fat mass followed by significantly higher serum total cholesterol and triglyceride levels (Fig. 1C). Additionally, the fasting blood glucose and insulin levels, as well as the HOMA-IR, were significantly higher in obese and lean $LGALS3^{-/-}$ mice than in WT mice (Fig. 2).

There is increasing evidence that Gal-3 plays an important role in obesity and T2DM (18). However, controversial results have been reported regarding the effects of Gal-3 in obese patients and experimental models. Our results are in agreement with the study reported by Okhura et al., which demonstrated that low serum Gal-3 levels are associated with insulin resistance in T2DM patients (25). It has been recently demonstrated that obese $LGALS3^{-/-}$ mice have increased fasting blood glucose and insulin levels compared with diet-matched WT animals (18). Additionally, Pejnovic et al. (18) reported significantly increased IFN-γ-producing Type 1 T/NKT cells and pro-inflammatory M1 macrophages and reduced T regulatory cells and alternatively activated M2 macrophages in the VAT of $LGALS3^{-/-}$ mice fed a HFD compared with diet-matched WT animals. Pang et al. (27) reported that Gal-3-deficient mice fed a HFD for 12 weeks develop increased adiposity and systemic inflammation. In addition, the same authors showed that despite the increased adiposity in Gal-3-deficient mice, there was no significant difference in the size of the adipocytes (27). In this study, we demonstrated that LGALS3-/- mice fed a HFD for 6 months developed visceral adiposity, hyperglycaemia and IR. Increased adiposity in Gal-3-deficient mice on a HFD was associated with adipocyte hypertrophy (Fig. 1D).

Inflammation in the VAT during obesity plays a central role in metabolic abnormalities such as IR. We demonstrate that a long-term HFD induced innate and adaptive immune cell infiltration in the VAT (Fig. 3 and 4). It has been reported that DCs, as professional antigen-presenting cells, have an important role in obesity-induced VAT inflammation (28). The obesity-associated increase of $CD11c^+$ cells in the adipose tissue suggests that DCs play a role in macrophage recruitment and activation (29). The VAT from HFD-fed $LGALS3^{-/-}$ mice contained higher numbers of total $CD11c^+$ and $CD11c^+F4/80^-$ DCs than HFD-fed WT animals (Fig. 3A). Additionally, the expression of CD11c is one of the key characteristics of proinflammatory (M1) macrophages in addition to specific markers such as F4/80 and CD11b. Triple-positive $F4/80^+CD11b^+CD11c^+$ macrophages (30) were more numerous in the VAT of $LGALS3^{-/-}$ mice fed a HFD (Fig. 3B) and were recently described as a proinflammatory macrophage subset (10). This macrophage population is increased in the adipose tissue in obese vs. lean mice (10). In addition, the subset of proinflammatory $F4/80^{hi}$ macrophages (30) was significantly higher in obese $LGALS3^{-/-}$ mice than in WT mice (Fig. 3B). This result is in agreement with the reported data of increased proinflammatory $F4/80^+CD11c^+CD206^+$ macrophage numbers in the VAT of $LGALS3^{-/-}$ after 11 weeks on a HFD (18). Type 1 T cells have a major role in obesity-associated chronic inflammation (31). The HFD increased the number of $CD3^+$ lymphocytes in the VAT of both genotypes of mice compared with chow-fed mice (Fig. 4A). CXCR3, a chemokine receptor that is highly expressed on activated T cells, is involved in T cell trafficking and activation (32). $CD3^+$ cells expressing CXCR3 were higher in the VAT from $LGALS3^{-/-}$ mice than in WT mice, both on a HFD (Fig. 4A). Despite the overlapping expression of chemokine receptors, CXCR3-positive cells represent Th1 lymphocytes. Th1 lymphocytes expressing CXCR3 produce more Th1-type cytokines such as IFN-γ and enhance the activity of CD8+ T cell effectors *in vitro* (33). Obesity is associated with increased $CD8^+$ cells in the VAT (33). In our study, the number of $CD8^+$ cells and the CD8/CD4 ratio (Fig. 4B) in the VAT was increased in $LGALS3^{-/-}$ mice fed a HFD compared with diet-matched WT mice, suggesting the role of cytotoxic $CD8^+$ cells in VAT inflammation and related metabolic abnormalities.

In summary, we provide evidence that Gal-3 deletion enhanced long-term HFD-induced adiposity, visceral adipose tissue inflammation, insulin resistance and hyperglycaemia. These data contribute to better understanding of the role Gal-3 in obesity, metabolic inflammation and T2DM.

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THE PREVALENCE OF ALCOHOL CONSUMPTION BY ADOLESCENTS IN SERBIA AND ITS CORRELATION WITH SOCIODEMOGRAPHIC FACTORS – A NATIONAL SURVEY

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RASPROSTRANJENOST KONZUMIRANJA ALKOHOLA KOD ADOLESCENATA U SRBIJI I POVEZANOST SA SOCIODEMOGRAFSKIM FAKTORIMA- NACIONALNO ISTRAŽIVANJE

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ABSTRACT

The aim of this study was to determine the prevalence of alcohol consumption among adolescents in Serbia and its association with sociodemographic characteristics. This paper is based on data from a national health survey of the population of Serbia in 2013 (no data for Kosovo and Metohija), conducted by the Ministry of Health of the Republic of Serbia. For the purposes of this study, data on households and individuals over 15 years of age were used; thus, the final sample for analysis included 858 patients (aged 15 to 19 years). Researchers used demographic characteristics (age, gender, type of home, region) and socioeconomic characteristics (income per household member, the index of well-being, self-assessment of health, cigarette smoking, tendency towards psychological and physical violence) as the independent variables. A χ2 test was applied to test the differences in the frequencies of categorical variables. The correlations between alcohol consumption, as the dependent variable, and the independent variables (mentioned above) were tested by logistic regression. All results less than or equal to 5% probability ($p \leq 0.05$) were considered statistically significant. The prevalence of alcohol consumption among adolescents in Serbia is 51.6%. Alcohol consumption is significantly associated with sex, type of home and the index of well-being ($p < 0.05$). The prevalence of alcohol consumption is higher in males (57.1%), in adolescents who come from urban areas (59.3%) and in adolescents who, according to the index of well-being, belong to the wealthiest financial category (23.9%).

Keywords: *alcohol, prevalence, adolescents, Serbia*

SAŽETAK

Cilj rada je utvrđivanje prevalence konzumiranja alkohola kod adolescenata u Srbiji i povezanost sa sociodemografskim karakteristikama. Rad je zasnovan na podacima nacionalnog istraživanja zdravlja stanovništva Srbije u 2013. godini (bez podataka za Kosovo i Metohiju), koje je sprovelo Ministarstvo zdravlja Republike Srbije. Za potrebe ovog rada korišćeni su podaci o domaćinstvima i stanovništvu starosti 15 i više godina, tako da je u konačni uzorak za analizu ušlo 858 ispitanika (starosti 15 do 19 godina). Od nezavisnih varijabli u istraživanju su korišćena demografska obeležja (starost, pol, tip naselja, region) i socijalno-ekonomske odlike (prihodi po članu domaćinstva, indeks blagostanja, samoprocena zdravlja, konzumiranje cigareta, sklonost ka psihičkom i fizičkom nasilju). Za ispitivanje razlika u učestalosti kategorijskih varijabli primenjen je χ2 test. Povezanost konzumiranja alkohola, kao zavisne varijable, i nezavisnih varijabli (gore pomenutih) ispitivana je logističkom regresijom. Statistički značajnim smatrali su se svi rezultati gde je verovatnoća jednaka ili manja od 5% ($p \leq 0.05$). Prevalenca konzumiranja alkohola među adolescentima u Srbiji iznosi 51,6%. Konzumiranje alkohola značajno je povezano sa polom, tipom naselja i indeksom blagostanja ($p<0.05$). Prevalenca konzumiranja alkohola veća je kod muškaraca (57,1%), kod adolescenata koji potiču iz gradske sredine (59,3%) i kod adolescenata koji prema indeksu blagostanja pripadaju kategoriji najbogatijih (23,9%).

Ključne reči: *alkohol, prevalenca, adolescenti, Srbija*

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INTRODUCTION

Adolescence is defined as a period of transition from childhood to adulthood that is characterized by efforts to meet the expectations of one's culture, as well as the requirements of physical, mental, emotional and social development. This is a period of development that has its own characteristics in biological, psychological and social terms (1).

Insecurity, instability of mood, lack of spontaneity, egocentricity, rebelliousness, conflicts with authority, fear of failure, and a desire to be successful are features that characterize the personalities and behaviours of adolescents (2).

The period of adolescence is described as a time of experimentation with risky behaviours, which has an adaptive purpose as young people gain experience and skills (3). However, adolescents are immature and unprepared and are thus prone to engaging with all that their environments provide and more frequently resorting to various problematic behaviours, of which some do not always have positive and adaptive functions (4).

There are common initial motives for consuming alcohol in adolescent populations: the desire to satisfy curiosity; the need to belong to a group; and the desire to have new experiences, avoid boredom, escape from problems, and reduce social fears and uncertainty (5).

THE AIM OF THE WORK

The aim of this work was to determine the prevalence of alcohol consumption among adolescents in Serbia and its association with sociodemographic characteristics.

METHODS

Data source and type of study

This paper is based on data from a national health survey of the population of Serbia in 2013 (no data for Kosovo and Metohija). This is the third national survey of health of the population, conducted by the Ministry of Health of the Republic of Serbia. The first such survey was conducted in 2000, and the second was conducted in 2006. The survey was conducted in accordance with the methodology and instruments of the European Health Survey - Second Wave (EHIS-wave 2). The health survey of the Serbian population was carried out through interviews, anthropometric measurements and blood pressure measurements. We used a nationally representative random sample: a stratified two-stage sample with a known probability of selection of sample units at every stage of sampling. The sample was selected to provide a statistically reliable estimate of the large number of indicators of the health of the population at the national level.

The mechanisms used to obtain a random sample of households and respondents represent a combination of two sampling techniques: stratification and multi-stage sampling. For the purposes of this study, data on households and individuals over 15 years of age were used; thus, the final sample for analysis included 858 patients (aged 15 to 19 years).

Variables

Researchers used demographic characteristics (age, gender, type of home, region) and socio-economic characteristics (income per household member, the index of well-being, self-assessment of health, cigarette smoking, tendency towards psychological and physical violence) as the independent variables.

Statistical data analysis

All data of interest were analysed by adequate mathematical-statistical methods appropriate for the data type. A $\chi 2$ test was applied to test the differences in the frequencies of categorical variables. The correlations between alcohol consumption, as the dependent variable, and the independent variables (mentioned above) were tested by logistic regression.

All results less than or equal to 5% probability ($p \leq 0.05$) were considered statistically significant. Statistical analysis was performed using a commercial, standard software package SPSS, version 19.0 (The Statistical Package for Social Sciences software; SPSS Inc., version 19.0, Chicago, IL).

RESULTS

The prevalence of alcohol consumption among adolescents in Serbia is 51.6%. One in ten adolescents (10.1%) claimed to consume alcoholic drinks once or twice a week, while 24% of adolescents said that they drink one to three times a month. When asked how many days they drink from Monday to Thursday, the highest percentage of adolescents claimed to drink alcohol one day out of four (16.9%), while one in three adolescents in our study (35.1%) claimed to drink one day from Friday to Sunday. The largest percentage of adolescents (14.6%) drinks six or more alcoholic drinks on one occasion once a month (e.g., at parties, with food, during evenings spent with friends, alone at home). In terms of the type of alcoholic beverage, commonly consumed beverages include liquor, beer and wine. Analysis of the influence of sociodemographic factors on the prevalence of alcohol consumption among adolescents in Serbia (shown in Table 1) showed that alcohol consumption is significantly associated with sex, type of home and the index of well-being ($p < 0.05$). The prevalence of alcohol consumption was higher in males (57.1%), in adolescents who come from urban areas (59.3%) and in adolescents who, according to the index of well-being, belong to the wealthiest financial category (23.9%). It was also observed that adolescents who smoke cigarettes more often drink alcohol (75.1%) and that ado-

Table 1. The prevalence of alcohol consumption among adolescents in Serbia in relation to sociodemographic characteristics

Variables		Alcohol consumption n (%)	Not alcohol consumtion n (%)	p*
Gender	Male	233 (57.1)	169 (44.2)	p<0.001
	Female	175 (42.9)	213 (55.8)	
Type of settlement	Urban	242 (59.3)	185 (48.4)	p<0.001
	Rural	166 (40.7)	197 (51.6)	
Region	Belgrade	84 (20.6)	69 (18.1)	p>0.05
	Vojvodina	114 (27.9)	88 (23.0)	
	Šumadija and Western Serbia	109 (26.7)	116 (30.4)	
	South-East Serbia	101 (24.8)	109 (28.5)	
Income per household	Do 9.000	110 (27.0)	136 (35.6)	p>0.05
	9.001 – 14.000	95 (23.3)	78 (20.4)	
	14.001 – 20.000	70 (17.2)	59 (15.4)	
	20.001 – 29.000	36 (8.8)	21 (5.5)	
	Preko 29.000	15 (3.7)	13 (3.4)	
	refuses to answer	82 (20.1)	75 (19.6)	
Well-being index	Poorest	60 (14.7)	80 (20.9)	p<0.05
	Poorer	73 (17.9)	81 (21.2)	
	Middle	81 (19,9)	82 (21.5)	
	Richer	97 (23.7)	78 (20.4)	
	Richest	98 (23.9)	61 (16.0)	
Self-assessed health	Very good	274 (67.2)	257 (67.3)	p>0.05
	Good	121 (29.7)	112 (29.3)	
	Fair	11 (2.7)	10 (2.6)	
	Bad	1 (0.2)	3 (0.8)	
	Very bad	1 (0.2)	0	
The tendency to psychological violence		50 (76.9)	15 (23.1)	p<0.001
The tendency to physical violence		46 (50.7)	11 (19.3)	p<0.001
The consumption of cigarettes		145 (75.1)	48 (24.9)	p<0.001

* χ^2 (chi-squared)

lescents who consume alcohol are more likely to have experienced psychological (76.9%) and physical abuse (50.7%). These results are statistically significant ($p < 0.05$).

To examine the impact of certain predictors on alcohol consumption, binary logistic regression was applied. A significant contribution to the model was made by three independent variables: gender, financial status and tobacco consumption. The strongest predictor was tobacco smoking, with a probability coefficient of 3.99. In fact, individuals who smoke were almost 4 times more likely to consume alcohol than non-smokers. Males were 1.74 times more likely to consume alcohol than females. Respondents who belong to the wealthiest financial category more often consume alcohol (OR = 1.18) (Table 2).

DISCUSSION

Alcohol is a psychoactive substance that is commonly used and abused by adolescents worldwide (6). During adolescence, alcohol consumption is of great importance because of the frequency and extent of its use and its impact on the health of adolescents (7). Studies of alcohol consumption among European adolescents (ESPAD) indicate that the use of alcohol among young people in almost all European countries is on the rise (8), despite the fact that much research shows that high school students are well informed about the health risks arising from the consumption of alcohol (9). Our results show a lower incidence of alcohol consumption among adolescents in Serbia (51.6%) than among adolescents in many other European countries, such as Romania and Sweden (71-74%); Montenegro, Norway, Albania and Iceland (65-43%) (10); Italy (63.3%) (11); Germany (52.3%) (12); and the US (71%) (13). However, our results are higher than those recorded in the 2006 study of the health of the Serbian population (48.7%) (14). Alcohol consumption by young people is a national problem in many countries, primarily due to the harmful effects of alcohol consumption on the social, physical and neurological development of adolescents. Nearly two-thirds of Australian adolescents consume alcohol (15), as well as 50% of adolescents in Brazil

Table 2. Logistic regression analyses

Variables	B	Exp(B)	95% C.I.for EXP(B)		p
			Lower	Upper	
Sex (1)	0.55	1.730	1.278	2.341	0.000
Region	-0.07	.933	.809	1.076	0.339
settlement type	-0.11	.898	.614	1.313	0.578
financial situation	0,17	1,181	1.028	1.356	0.019
income	0,00	1.000	1,000	1,000	0.648
self-assessment of health	-0.03	,972	.746	1.267	0.836
Smoking	1.38	3.992	2.736	5.825	0.000
psychological abuse	0.00	1.000	1.000	1.000	0.669
physical abuse	0.00	1.000	1.000	1.000	0.797

(16). Alcohol consumption among young people is a major public health problem in the U.S. More than 27% of 12 to 20 year-olds drink alcohol throughout the year, averaging almost 5 drinks per session. Excess alcohol consumption increases the risk of acute and chronic alcohol-related problems, including risky sexual behaviour, injury and driving while intoxicated. Those who begin drinking alcohol as juveniles are more likely to develop symptoms of alcohol abuse and dependence as an adult than their peers who abstain from alcohol use (17). In our study, alcohol consumption in adolescents was significantly associated with sex, type of home and the well-being index, which corresponds to the results recorded in most other countries. It has been noted that young men consume alcohol more than women in Canada (18), Croatia (19), Italy (11), Iceland, Latvia and Sweden (10), and Germany (12), which corresponds to our results, while research in the United States (13) shows that women are more likely consume alcohol than men. Additionally, it was found that adolescents of higher socio-economic status (11) frequently consume alcoholic beverages, which is confirmed by our national survey. Other studies (Great Britain) have shown that the use of alcohol among adolescents is more frequent in households with higher incomes (20). There are predisposing factors to alcohol consumption in Spanish adolescents: frequently going out for fun in the evenings, high proportions of friends who drink or get drunk, early onset of alcohol use, low perceived risk of drinking, truancy, illegal drug use, and the amount of money spent on personal needs (21). Higher household income was associated with a greater risk of alcohol use (22). In our study, adolescents in urban areas more frequently consume alcohol; however, other studies have shown that young people from rural areas frequently consume alcohol. Apparently, in this case, adolescents with rural residences have fewer alternatives for inclusion in interesting leisure activities than adolescents who live in cities. This may be one reason for the more problematic patterns of consumption in this environment (23).

Adolescents who consume alcohol are more prone to other health risk behaviours, such as riding with a driver who has been drinking, engaging in risky sexual activity, cigarette smoking, and using illicit drugs, and are more likely to become the victims of violence or suicide, which has been shown in the results of many studies (24,11,25). Our research also supports previous facts as we observed that adolescents who consume alcohol are more often smokers and are more susceptible to mental (psychological harassment, insults) and physical violence (fights). Even a single episode of heavy drinking in adolescence significantly increases the risk of morbidity and mortality caused by negligent driving, various incidents, accidents and risky sexual activity (26). The results of certain studies show that alcohol is a leading cause of murder, suicide and accidents resulting in death in adolescence; more adolescents lose their lives due to the consequences of alcohol than due to the use of all other psychoactive substances combined (27). A large number of adolescents (70% of boys and 53% of girls) who have used alcohol more than five times in their lives state that they have experienced at least one problem associated with the use of alcohol (e.g., problems with the law, health problems, absence from school or going to school under the influence of alcohol, etc.) (28).

Primary socialization and education occurs in families and is the most important influence on adolescent health behaviour; parents model social and health behaviour patterns. The attitudes of parents, who can directly approve or indirectly support the abuse of alcohol, have a direct relevance for the formation of attitudes of adolescents, especially if there is a case of alcoholism in the family (29). It is therefore necessary to focus prevention programs on creating a harmonious relationship in the family and on preventing marital conflicts and child abuse and neglect.

CONCLUSION

The results of this research indicate that there is an increased prevalence of alcohol consumption among adolescents in Serbia. It is therefore very important to carry out preventive measures to prevent and control the risks of alcohol abuse by young people. These measures should include individuals, as well as families, schools, health institutions and society as a whole. Preventive activities should be conducted in an organized way, beginning in early childhood, at all levels of society.

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THE EFFECT OF THE ANTIOXIDANT DRUG U-74389G ON URIC ACID LEVELS DURING ISCHEMIA REPERFUSION INJURY IN RATS

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EFEKTI ANTIOKSIDACIONOG LEKA U-74389G NA VREDNOSTI MOKRACNE KISELINE TOKOM ISHEMIJSKO REPERFUZIONE POVREDE KOD PACOVA

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ABSTRACT

This experimental study examined the effect of the antioxidant drug U-74389G in a rat model using a renal ischaemia-reperfusion (IR) protocol. The effects of the molecule were studied biochemically by assessing mean serum uric acid levels (SUA). In total, 40 rats (mean weight = 231.875 g) were used in the study. SUA levels were measured at 60 min of reperfusion for groups A and C and at 120 min of reperfusion for groups B and D. The drug U-74389G was administered only in groups C and D. U-74389G administration non-significantly increased the SUA levels by 15.43%±9.10% (p=0.096) at the representative endpoint of 1.5 h. The reperfusion time non-significantly decreased the SUA levels by 13.61%±9.18% (p=0.126). However, the interaction of U-74389G administration and reperfusion time non-significantly increased the SUA levels by 4.78%±5.64% (p= 0.387). Whether it interacted with the reperfusion time, U-74389G administration non-significantly increased SUA levels. It seems that U-74389G cannot reverse injury to IR tubular epithelial cells within 2 hours.

Keywords: *ischaemia, U-74389G, uric acid, reperfusion*

SAŽETAK

U ovoj eksperimentalnoj studiji ispitivani su efekti antioksidacionog leka U-74389G tokom ishemije i reperfuzije bubrega na modelu pacova. Efekti ispitivanog molekula su izučavani biohemijski, merenjem srednje vrednosti nivoa mokraćne kiseline u serumu. U studiji je korišćeno 40 pacova (prosečna telesna masa= 231,875g). Nivoi mokraćne kiseline u serumu su mereni za grupe A i C u šezdesetom minutu, a za grupe B i D u stodvadesetom minutu reperfuzije. U-74389G je primenjivan samo u grupi C i D. Administracija U-74389G nije dovela do statistički značajnog povećanja niova mokraćne kiseline u krajnjoj tački u devedesetom minutu 15,43%±9,10% (p=0,096). Reperfuzija nije dovela do statistički značajnog smanjenja nivoa mokraćne kiseline u serumu 13,61%±9,18% (p=0,126). Bez obzira na reperfuziono vreme administracija U-74389G nije statistički značajno povisila nivo mokraćne kiseline u serumu. Izgleda da u toku dva sata U-74389G ne može popraviti povredu tubularnih epitelijalnih ćelija nastalu ishemijom i reperfuzijom.

Ključne reči: *ishemija, U-74389G, mokraćna kiselina, reperfuzija*

ABBREVIATIONS

IR - ischaemia-reperfusion
SUA - serum uric acid
SD - standard deviation
L - lazaroid
GLM - generalized linear models
AIS - acute ischaemic stroke

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INTRODUCTION

Tissue ischaemia-reperfusion (IR) injury can induce permanent or transient damage with serious implications to adjacent organs and systems. The use of U-74389G in IR has been a challenge for many years. However, although progress has been significant, several practical questions have not been clarified. They include:

a) how potent U-74389G should be;
b) when it should be administered; and
c) the optimal dose at which U-74389G should be administered.

The promising effect of U-74389G in tissue protection has been noted in several IR studies. U-74389G, also known as 21-[4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl]-pregna-1,4,9(11)-triene-3,20-dione maleate salt, is an antioxidant that prevents both arachidonic acid-induced and iron-dependent lipid peroxidation (1). Lazaroids, a novel series of glucocorticoid compounds such as 21-aminosteroids, promote free radical scavenging. U-74389G is one of the 132 similar lazaroid compounds. It has a molecular weight of 726.90406 g/mol and a selective action on vascular endothelium with vitamin E-like properties. It also exhibits neuroprotection and membrane-stabilizing properties. It protected against IR injury in heart, liver and kidney models. These membrane-associated antioxidants are particularly effective in preventing permeability changes in monolayers of brain microvascular endothelial cells (2). A meta-analysis of 15 published studies, including red blood cell counts, haemoglobin and mean corpuscular haemoglobin levels, platelet count, plateletcrit, platelet distribution width, glucose, total protein, alkaline phosphatase, creatine phosphokinase, sodium, chloride, calcium, phosphorus and magnesium levels, examined in the same experimental setting, provided a total numeric evaluation of the U-74389G anabolic efficacy of approximately +2.14%±7.18% (p-value = 0.227) at the same endpoints (3, 4). Several publications addressed trials of other similar antioxidant molecules to which the studied molecule U-74389G belongs.

The aim of this experimental study was to evaluate the effect of U-74389G in a rat model of renal IR using mean serum uric acid levels (SUA).

MATERIALS AND METHODS

Animal preparation

This basic experimental study was licensed by Veterinary Address of East Attiki Prefecture under 3693/12-11- 2010 & 14/10-1-2012. All consumables, equipment and substances used were grants of the Experimental Research Centre of ELPEN Pharmaceuticals Co. Inc. S.A. at Pikermi, Attiki. Accepted standards of humane animal care were adopted for albino female Wistar rats. Seven days of normal pre-experimental housing allowed for an *ad libitum* diet in the laboratory. In total, 40 female albino Wistar rats (16 -18 weeks old) were used (mean weight ± standard deviation (SD): 232±37 g), with a minimum weight of 165 g and a maximum weight of 320 g. Rats' weights could be a confounding factor; e.g., more obese rats have higher SUA levels. This was also investigated. Post-experimental awakening of animals was not permitted, even if euthanasia was needed. Rats were randomly sorted into four experimental groups with 10 animals in each group using the following IR protocols: ischemia for 45 min followed by reperfusion for 60 min (group A); ischaemia for 45 min followed by reperfusion for 120 min (group B); ischaemia for 45 min followed by immediate U-74389G intravenous (IV) administration and reperfusion for 60 min (group C); and ischaemia for 45 min followed by immediate U-74389G IV administration and reperfusion for 120 min (group D). The dose of U-74389G was 10 mg/kg body mass. The protocol and doses were determined via the following experiments with favourable outcomes. Flessas I et al. found (5) that the role of U-74389G was protective in many emergency clinical situations of intestinal IR. Bimpis A et al. showed limited brain damage after (6) U-74389G administration. Tsaroucha AK et al. (7) showed attenuated liver damage after U-74389G administration. Andreadou I et al. showed that the small intestine (8) was protected after U-74389G administration.

The detailed prenarcotic and general anaesthesiologic techniques were described previously (3, 4). A continuous intra-experimental oxygen supply, electrocardiogram and acidometry were maintained. Ischaemia was caused by laparotomic clamping of the inferior aorta over the renal arteries with forceps for 45 min. Reperfusion was induced by removing the clamp and re-establishing the patency of the inferior aorta. After the exclusion of a blood flow, the IR protocol was applied as described above for each experimental group. U-74389G was administered at the time of reperfusion through the catheterized inferior vena cava. The SUA levels were determined at the 60th min of reperfusion (for the A and C groups) and at the 120th min of reperfusion (for the B and D groups).

Control groups

Twenty control rats (252±39 g) underwent ischaemia for 45 min followed by reperfusion.

Group A

Reperfusion lasted for 60 min (n=10 control rats, 243±46 g), and SUA levels were 1.03±0.176 mg/dl (Table 1).

Group B

Reperfusion lasted for 120 min (n=10 control rats, 262±31 g), and SUA levels were 0.95±0.295 mg/dl (Table 1).

Groups receiving the lazaroid (L) drug U-74389G

The 20 rats (211+17 g) receiving L experienced ischaemia for 45 min followed by reperfusion after 10 mg of U-74389G /kg body weight was IV administered.

Group C

Reperfusion lasted for 60 min (n=10 L rats, 212±17 g), and SUA levels were 1.27±0.457 mg/dl (Table 1).

Group D

Reperfusion lasted for 120 min (n=10 L rats, 210±18 g), and SUA levels were 1.05±0.201 mg/dl (Table 1).

Table 1: Weight and mean serum uric acid levels and SD of the groups.

Groups	Variable	Mean	SD
A	Weight	243 g	46 g
A	Uric acid	1.03 mg/dl	0.176 mg/dl
B	Weight	262 g	31 g
B	Uric acid	0.95 mg/dl	0.295 mg/dl
C	Weight	212.5 g	18 g
C	Uric acid	1.27 mg/dl	0.457 mg/dl
D	Weight	210 g	18 g
D	Uric acid	1.05 mg/dl	0.201 mg/dl

Standard deviation: SD

STATISTICAL ANALYSIS

Every weight and SUA level group was compared with each other using paired t-tests. Significant differences among SUA levels were investigated. A generalized linear model (GLM) was applied with SUA levels as the dependent variable. The 3 independent variables were: the presence/absence of U-74389G, the reperfusion time, and both variables in combination. Using rat weight as an independent variable in the GLM analysis, a non-significant relationship was detected (p=0.4431), so further investigation was not needed.

RESULTS

The application of GLM resulted in the following findings: U-74389G administration non-significantly increased SUA levels by 0.17 mg/dl [-0.026 mg/dl - 0.366 mg/dl] (p= 0.088). This finding was in accordance with a paired t-test (p = 0.103). The reperfusion time non-significantly decreased SUA levels by 0.15 mg/dl [-0.348 mg/dl - 0.048 mg/dl] (p= 0.134), also in accordance with the results of a paired t-test (p= 0.118). However, U-74389G administration and reperfusion time non-significantly increased SUA levels by 0.052 mg/dl [-0.069 mg/dl - 0.174 mg/dl] (p= 0.387). Tables 2 and 3 summarize the changes in the influence of U-74389G in connection with reperfusion time.

Table 2: The increasing influence of U-74389G associated with reperfusion time.

Alteration	95% c. in.	Reperfusion time	t-test	GLM
+0.24 mg/dl	-0.085 mg/dl -0.565 mg/dl	1 h	0.184	0.138
+0.17 mg/dl	-0.026 mg/dl -0.366 mg/dl	1.5 h	0.103	0.088
+0.1 mg/dl	-0.137 mg/dl -0.337 mg/dl	2 h	0.401	0.388
-0.15 mg/dl	-0.348 mg/dl -0.048 mg/dl	reperfusion time	0.118	0.134
+0.052 mg/dl	-0.069 mg/dl -0.174 mg/dl	interaction		0.387

confidence interval: c. in;

Table 3: The (%) alteration influence of U-74389G associated with reperfusion time.

Alteration	±SD	Reperfusion time	p-values
+20.86%	±14.44%	1 h	0.161
+15.43%	±9.10%	1.5 h	0.096
+10%	±12.11%	2 h	0.394
-13.61%	±9.18%	reperfusion time	0.126
+4.78%	±5.64%	interaction	0.387

DISCUSSION

SUA is considered to be a reliable index of renal function. Its production is influenced by ischaemia and by a certain mode. Chiquete E et al. showed (9) that SUA is a potent antioxidant, and its serum concentration increases rapidly after acute ischaemic stroke (AIS). They associated the magnitude of cerebral infarction with the mean SUA concentration and favourable outcomes (p = 0.004) in patients with AIS upon hospital arrival. Logallo N et al. positively correlated (10) the adjusted SUA level with clinical improvement (p = 0.02) and favourable stroke outcome (p = 0.04) in patients with tissue plasminogen activator thrombolysis upon admission. Seifert J et al. found (11) SUA levels on day 7 and ribose ingestion after 14 days. Hellsten-Westing Y et al. found (12) that SUA is taken up by the strenuous muscle, which is the main source of plasma hypoxanthine in the blood. SUA is also taken up by the liver, where most of it is converted to uric acid. Lazzarino G et al. found (13) significantly increased levels of SUA after cerebral IR in rats.

Although SUA predicts AIS and leads to gout, how renal SUA excretion is influenced by U-74389G is unknown. Moreover, only reperfusion time resulted in a non-significant decline of SUA levels, reflecting a non-significant increase in renal SUA excretion. All the other endpoints implicated by U-74389G administration exhibited discouraging but non-significant results. U-74389G increased SUA levels, reflecting a decrease in renal SUA excretion. This may stand for a generalized lack of amelioration of renal function by U-74389G administration. U-74389G

cannot reverse the injury to IR tubular epithelial cells. If the injury is severe, death by apoptosis and necrosis (acute tubular necrosis) occur, with the functional impairment of water and electrolyte homeostasis and reduced excretion of metabolic waste products, including SUA. A longer study duration or a higher U-74389G dosage may reverse apoptosis and necrosis of tubular epithelial cells. The body mass, as mentioned above, had no impact on protocol; the most pronounced mass difference between the B and D groups (p-value=0.0004) reflected a non-significant difference at their respective SUA levels (p-value=0.4013).

CONCLUSION

Whether it interacted with reperfusion time, U-74389G administration non-significantly increased SUA levels. U-74389G cannot reverse injury to IR tubular epithelial cells within 2 hours. Perhaps either a longer study time or a higher U-74389G dosage may reverse tubular apoptosis and prevent acute tubular necrosis.

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RENOVASCULAR HYPERTENSION: CLINICAL FEATURES, DIFFERENTIAL DIAGNOSES AND BASIC PRINCIPLES OF TREATMENT

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RENOVASKULARNA HIPERTENZIJA: KLINIČKE KARAKTERISTIKE, DIFERENCIJALNA DIJAGNOZA I OSNOVNI PRINCIPI LEČENJA

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ABSTRACT

Renovascular hypertension is caused by renal artery stenosis. Its prevalence in populations of hypertensive patients is 1-8%, and in populations of patients with resistant hypertension, it is up to 20%. The two main causes of stenosis are atherosclerosis and fibromuscular dysplasia of the renal artery. The main clinical consequences of renal artery stenosis include renovascular hypertension, ischemic nephropathy and "flash" acute pulmonary oedema. Unilateral stenosis of the renal artery causes angiotensin II-dependent hypertension, and bilateral stenosis of the renal arteries produces volume-dependent hypertension. Renovascular aetiology of hypertension should be questioned in patients with resistant hypertension, hypertension with a murmur identified upon auscultation of the renal arteries, and a noticeable side-to-side difference in kidney size. Non-invasive diagnostic tests include the determination of concentrations of peripheral vein plasma renin activity, the captopril test, captopril scintigraphy, colour Doppler ultrasonography, computed tomography angiography, and nuclear resonance angiography. Renovasography represents the gold standard for the diagnosis of renovascular hypertension. The indications for revascularization of the renal artery include haemodynamically significant renal artery stenosis (with a systolic pressure gradient at the site of stenosis of - $\Delta P \geq 20$ mmHg, along with the ratio of the pressure in the distal part of the renal artery (Pd) and aortic pressure (Pa) less than 0.9 (Pd/Pa < 0.9)), resistant hypertension, loss of renal function after administration of ACE inhibitors or angiotensin receptor II blockers, and recurrent flash pulmonary oedema associated with bilateral renal artery stenosis. The contraindications for renal artery revascularization include a longitudinal diameter of the affected kidney that is less than 8.0 cm, the resistance index measured from the segmental arteries peak blood flow (RI) > 0.8, chronic kidney disease (GFR <30 ml/min/1.73 m^2) and negative captopril scintigraphy (lack of lateralization).

Keywords: *renovascular hypertension, plasma renin activity, captopril test, resistance index*

SAŽETAK

Renovaskularna hipertenzija nastaje zbog stenoze renalne arterije. Njena prevalencija u populaciji bolesnika sa hipertenzijom iznosi 1-8%, a u populaciji bolesnika sa rezistentnom hipertenzijom i do 20%. Dva glavna uzroka stenoze su ateroskleroza i fibromuskularna displazija renalnih arterija. Glavne kliničke posledice stenoze renalne arterije su: renovaskularna hipertenzija, ishemijska nefropatija I "flash" akutni edem pluća. Stenoza jedne renalne arterije izaziva hipertenziju zavisnu od angiotenzina 2, a stenoza obe renalne arterije za posledicu ima hipertenziju zavisnu od volumena. Na renovaskularnu hipertenziju treba posumnjati kod bolesnika sa: rezistentnom hipertenzijom, hipertenzijom sa nalazom šuma pri auskultaciji arterija bubrega I sa razlikom u veličini bubrega. U neinvazivne dijagnostičke testove spadaju određivanje koncentracije plazma-reninske aktivnosti u uzorku krvi iz periferne vene, kaptoprilski test, kaptoprilska scintigrafija, kolor dopler ultrasonografija, kompjuterizovana tomografsfska angiografija i nuklearna rezonantna angiografija. Zlatni standard za dijagnostikovanje renovaskularne hipertenzije je renovazografija. U indikacije za revaskularizaciju renalne arterije spadaju: hemodinamski značajna stenoza renalne arterije (gradijent sistolnog pritiska na mestu stenoze - $\Delta P \geq 20$ mmHg i odnos pritiska u distalnom delu renalne arterije (Pd) i aorte (Pa) manji od 0.9 (Pd/Pa < 0.9)), rezistentna hipertenzija, gubitak funkcije bubrega posle primene blokatora konvertaze angiotenzina 1 ili blokatore receptora za angiotenzin 2, rekurentni "flash pulmonary oedema", povezan sa obostranom stenozom renalne arterije. U kontraindikacije za revaskularizaciju renalne arterije spadaju: uzdužni dijametar zahvaćenog bubrega manji od 8.0 cm, indeks rezistencije izmeren iz krive protoka krvi kroz segmentne arterije - RI > 0.8, hronična bolest bubrega (JGF < 30 ml/min/1.73m^2) i negativna kaptoprilska scintigrafija (odsustvo lateralizacije).

Ključne reči: *renovaskularna hipertenzija, plazma-reninska aktivnost, kaptoprilski test, indeks rezistencije*

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INTRODUCTION

Renovascular hypertension is defined as hypertension caused by renal artery stenosis (RAS). In the hypertensive patient populations (blood pressure >140/90 mmHg), renovascular hypertension prevalence is 1-8%. In the population of patients with resistant hypertension (defined as increased blood pressure despite the use of three antihypertensive drugs at the optimal dosage, including a diuretic), the prevalence of renovascular hypertension is higher, 2.5-20% (1-4).

Aetiopathogenesis of renovascular hypertension

The aetiology of renal artery stenosis

Renal artery stenosis may manifested as either unilateral or bilateral. The two most common causes of renal artery stenosis are atherosclerosis and fibromuscular dysplasia (5, 6). Atherosclerosis of the renal artery (ARAS) is the cause of stenosis in 90% of cases, and it usually involves the ostium and proximal third of the renal artery trunk. It usually affects patients over 65 years of age with known cardiovascular risk factors (obesity, hypertension, hyperlipidaemia, hyperglycaemia) and ranges from 30% among patients with coronary artery disease to 50% among the elderly and those with diffuse atherosclerotic vascular diseases (5). The one-year progression rate of renal artery stenosis caused by atherosclerosis is 0.5% (5, 6). Clinical features of renal artery stenosis include renovascular hypertension, ischemic nephropathy, and recurrent flash pulmonary oedema (bilateral atherosclerotic renal artery disease) (5, 6). In the USA, 12 - 14% of new patients entering dialysis programs have ARAS (a significant cause of end-stage chronic kidney disease) (5, 6).

Fibromuscular dysplasia (FMD) is a non-atherosclerotic, non-inflammatory disease, most commonly affecting the renal and internal carotid arteries (7, 8). It implicates renal artery stenosis in 5-10% of cases and affects the distal part of the renal artery. Any layer of the renal artery wall may be affected: the intima, media or adventitia (7, 8). The most common form is fibromuscular dysplasia of the media (fibroplasia of the media occurs in 70-95% of cases), which has been shown by pathognomonic angiography to cause slight stenosis along a vessel with intervening areas of dilatation (small aneurysms) creating a "string of beads" appearance (7, 8). It occurs most often in women 15-50 years of age. In most cases, the disease is asymptomatic, exhibits latency for several years, and is difficult to clinically recognize and diagnose. Clinical appearance often involves renovascular hypertension and irreversible kidney damage, such as ischemic nephropathy (the severity of the clinical outlook depends on the degree of stenosis and the type of fibromuscular dysplasia) (7, 8).

Pathogenesis of the development of renovascular hypertension and Pickering syndrome

Pathogenesis of renovascular hypertension

Unilateral stenosis (stenosis of one renal artery) induces increased renin release and activation of the renin-angiotensin-aldosterone system (RAAS). As the condition progresses, angiotensin II is increasingly produced and released, causing the development of angiotensin II-dependent hypertension (9). Perfusion of the other, unaffected, kidney is increased due to increased renal perfusion pressure, and this results in RAAS inhibition and increased excretion of sodium (natriuresis is dependent on pressure) (9).

In patients with bilateral stenosis (stenosis of both renal arteries), or in patients with only one active kidney that is effected by stenosis, renal perfusion pressure is decreased, causing increased renin release, increased RAAS activity, increased production of angiotensin II and aldosterone, and reduced excretion of sodium and water. Due to retention of sodium and water, blood volume in arterial circulation is increased, leading to the development of volume-dependent hypertension (9).

Pathogenesis of ischemic nephropathy

Renal artery stenosis causes kidney tissue hypoxia, increased local production of renin and angiotensin II, increased production of free oxygen radicals (reactive oxygen species (ROS)), platelet-derived growth factor-β (PDGF-β) and transforming growth factor β (TGF-β). These mediators cause glomerulosclerosis and tubulointerstitial injury, which results in a decrease of glomerular filtration rate (GFR) and the development of chronic kidney disease (ischemic nephropathy). In the last decade, ischemic nephropathy was recognized as an important cause of end-stage chronic kidney disease (9, 10).

Pathogenesis of sudden acute pulmonary oedema (flash pulmonary oedema)

Flash pulmonary oedema (FPO) or Pickering syndrome is defined as an unexpected and sudden form of acute heart failure (acute pulmonary oedema) in patients with bilateral renal artery stenosis (11). In patients with renovascular hypertension and unilateral RAS, prevalence of a FPO is 3.5%, compared to 14.3% in patients with renovascular hypertension and bilateral RAS (11). In patients with bilateral RAS, the activity of RAAS and the sympathetic nervous system is enhanced and hypertension is volume-dependent (hypervolemia) (11).

Hypervolemia with other predisposing factors, such as left ventricular hypertrophy, impaired left ventricular diastolic function, or increased systemic vascular resistance, lead to a sudden increase in end-diastolic pressure of the left ventricle (EDPLV), which is transferred to the left atrium and pulmonary capillaries. Increased hydrostatic pressure in the capillaries of the lungs and increased permeability of the alveolar-capillary membrane (allowing for transport of angiotensin II (Ang II), endothelin 1 (ET-1), catecholamines, and nitrous oxide (NO)) result in an unexpected development of FPO (11).

Clinical features of renovascular hypertension

Renovascular hypertension should be suspected in patients with moderate (diastolic blood pressure ≥ 105 mmHg) or severe hypertension (diastolic blood pressure ≥ 120

Table 1. The probability of the existence of renovascular hypertension

Probability	Clinical features
Low (RAS: < 1.0%)	Borderline or moderate hypertension (diastolic blood pressure ≥ 105 mmHg), without clinical signs
Moderate (RAS: 15-30%)	Severe hypertension (diastolic blood pressure ≥ 120 mmHg Resistant hypertension (use of ≥ 3 antihypertensive drugs) The sudden appearance of hypertension in people younger than 30 years (fibromuscular dysplasia) or persons over 50 years old (atherosclerosis). Hypertension with the finding of a murmur at auscultation of the renal arteries Moderate hypertension (diastolic blood pressure ≥ 105 mmHg) in patients with atherosclerotic disease (CAD, PAD)
High (RAS: 30-40%)	Severe hypertension (diastolic blood pressure ≥ 120 mmHg) with progressive renal impairment Accelerating hypertension (an increase of SAP > 15 mmHg for six months) or malignant hypertension (retinopathy grade III and IV) Hypertension with an increase in serum creatinine concentration after administration of ACE I or ARB [↑ serum creatinine 0.5-1.0 mg/dL (44.2-88.4 ìmol/L)] Moderate (diastolic blood pressure ≥ 105 mmHg) or severe hypertension (diastolic blood pressure ≥ 120 mmHg) with difference in the longitudinal diameter of the right and left kidney >1.5-2.0 cm

RAS - renal artery stenosis, **CAD** - coronary artery disease, **PAD** - peripheral arterial disease, **ACEI** - angiotensin-converting enzyme inhibitor, **ARB** - angiotensin II receptor blockers

mmHg), resistant hypertension (increased BP despite the use of three antihypertensive drugs including a diuretic), a murmur found by renal artery auscultation, when the difference in the longitudinal diameter between the right and left kidney is > 1.5 - 2.0 cm, and an increase in serum creatinine concentration after administration of angiotensin converting enzyme inhibitors (ACEI) or angiotensin receptor II blockers (ARB) (increase in serum creatinine of 0.5-1.0 mg/dL (44.2-88.4 ìmol/L)) after ACEI/ARB administration, (Figure 1) (12).

Diagnosis of renovascular hypertension

The diagnosis of renovascular hypertension is performed by detecting and demonstrating the haemodynamic significance of RAS. Tests for the diagnosis of renovascular hypertension can be divided into three groups. The first group includes functional tests, proof and assessment of the haemodynamic significance of stenosis, and assessment of renin-angiotensin system activation. The functional tests include direct measurement of plasma renin concentration and plasma renin activity (PRA) from a peripheral vein, the captopril test (measurement of plasma renin activity from a peripheral vein after administration of captopril), and measuring the concentration of renin from a renal vein sample and determining the side-by-side ratio of plasma renin activity (lateralization estimation). For assessment of individual renal function, radio isotopic techniques are used (scintigraphy and captopril renal scintigraphy). The second group consists of tests that assess the morphology of the renal artery. Non-invasive versions of these tests include: colour Doppler of the renal arteries, computed tomography angiography (CTA), and nuclear magnetic resonance angiography (NMRA), while invasive tests include angiography with contrast medium, or renovasography. A third group of tests judge the benefit of RAS revascularization (using colour Doppler ultrasonography and lateralization tests) (6, 9, 12).

Peripheral blood plasma-renin activity measurements

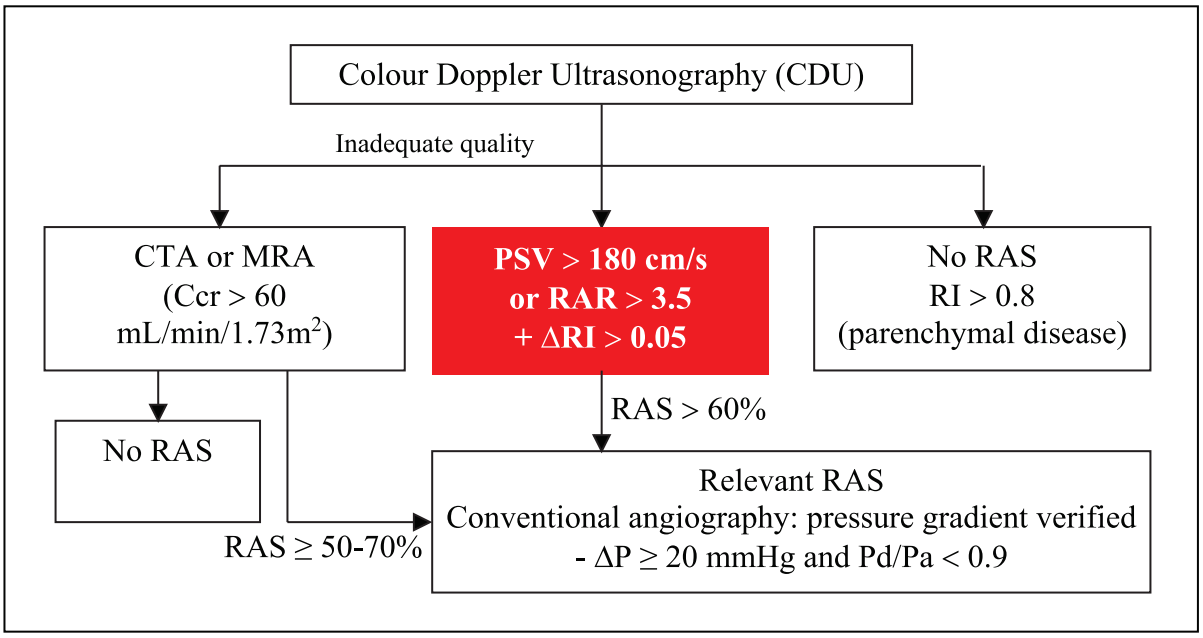
Peripheral blood plasma-renin activity (PRA) measurement and the captopril test play an important role in the diagnosis of renovascular hypertension (6, 9, 12). Low levels of plasma renin activity (PRA < 0.65 ng/mL/h), in association with hypokalaemia (K^+ < 3.5 mmol/L), indicate primary aldosteronism. When moderate levels of plasma renin (PRA = 0.65-3.2 ng/mL/h) are observed, additional tests for the detection of renovascular hypertension are required (such as the captopril test, colour Doppler of the renal artery, captopril scintigraphy, CT angiography, and NMR angiography). High plasma renin activity (considered to be PRA ≥ 3.2 ng/mL/h) strongly suggests existence of renovascular hypertension and requires direct renovasography (6, 9, 12).

The captopril test is the most sensitive test for the detection of RAS. Before the test is administered, adequate preparation of patients is required. Patients must have a normal salt intake. Additionally, three weeks prior to the test the use of several classes of drugs must be discontinued (i.e., drugs that affect the PRA such as angiotensin converting enzyme inhibitors, angiotensin receptor II blockers, beta blockers, and direct renin blockers). Thirty minutes prior to the blood sampling, the patient should rest and be relaxed. Alpha-1-blockers (doxazosin) have no influence on the concentration or the PRA. To determine the initial PRA, a blood sample is taken at 20, 25 and 30 minutes before the test. After that, a 25 mg captopril tablet is given to the patient and blood pressure is recorded after 15, 30, 45 and 60 minutes. After 60 minutes, a blood sample is taken for determination of stimulated PRA. The test is positive if PRA measures at ≥ 12 ng/mL/h, or if the absolute increase of PRA is ≥ 10 ng/mL/h (6, 9, 12).

Renal vein plasma renin activity

A good response after revascularization is indicated if the PRA ratio of the renal vein sample taken from a kidney affected by RAS to one without RAS is > 1.5.

Figure 2. Flow chart of renal artery stenosis assessment (*duplex sonography*)



RAS - renal artery stenosis, **CTA** - computed tomography angiography, **MRA** - magnetic resonance angiography, **Ccr** - endogenous creatinine clearance, **PSV** - peak systolic velocity, **RAR** - renal aortic ratio, **DRI** - resistance index difference, side to side, **Pd** - pressure in distal part of renal artery, **Pa** - aortic pressure.
Modified by reference [5].

Lateralization of plasma renin activity indicates haemodynamically significant stenosis. Generally, the higher the degree of lateralization, the greater the likelihood of optimal blood pressure control after revascularization of RAS (6, 9, 12).

Colour Doppler of renal arteries

Colour Doppler ultrasonography is a cheap, safe (no ionizing radiation) and non-invasive diagnostic procedure for detection and assessment of the severity of RAS (evaluating haemodynamic significance), and it is used to select patients who are good candidates for successful revascularization (12 -15). Colour Doppler ultrasonography of the renal arteries should be performed in all patients with a moderate to high probability for renovascular hypertension in which the PRA is ≥ 1.6 ng/mL/h (6, 9, 12).

Flow curve is a biphasic, low resistance colour Doppler technique with systolic and diastolic components. From the curve of blood flow through the renal artery, Doppler parameters can be calculated directly from blood flow through the main trunk and indirectly from intrarenal blood flow through the blood vessels (12 -15). The direct criteria for renovascular hypertension include peak systolic blood flow velocity (PSV) > 180 cm/s, end-diastolic flow velocity (EDFV) > 50 cm/s, reno-aortic ratio (RAR) > 3.5 and reno-renal ratio (RRR) > 4.0, (Figure 2) (12 -15). The indirect criteria for renovascular hypertension include loss of early systolic peak (ESP), resistance index (RI) < 0.45, acceleration time (AT) > 70 ms, acceleration (Acc) < 300 cm/s^2, and the difference in the resistance index (DRI) > 0.05 or 5% (Figure 2) (5, 12-15). In renal transplant patient groups, the presence of RAS is indicated by a PSV >2 00 cm/s, intrarenal artery blood flow curve AT > 100 ms, and a PSV ratio between the renal artery and kidney transplant external iliac artery of > 1.8 (16).

In cases where colour Doppler ultrasonography is not feasible or its findings are incomplete, either a CTA or NMRA is indicated. These two diagnostic methods require contrast mediums (such as the ionic contrast agent, gadolinium) and are not indicated in the group of patients with a reduced glomerular filtration rate (less than 30 mL/min/1.73 m^2), due to hazard of contrast nephropathy (CN) and nephrogenic systemic fibrosis (NSF) (17, 18).

The gold standard in RAS diagnostics remains a conventional intra-arterial digital subtraction angiograph (DSA). It directly assesses the haemodynamic significance of RAS (peak systolic pressure gradients (DP) ≥ 20 mmHg) (5). The threshold for significant RAS is defined by a ratio of distal renal pressure to aortic pressure (Pd/Pa) < 0.90 (5).

Differential diagnosis

Renovascular hypertension should be distinguished from primary aldosteronism and pheochromocytoma (resistant hypertension) (19-21).

Primary aldosteronism is the most common form of secondary hypertension. The main causes of primary aldosteronism are adrenal adenoma that produces aldosterone and idiopathic bilateral adrenal hyperplasia (19, 20). The prevalence of primary aldosteronism in a hypertensive patient population is 3.5% (19, 20). Screening for primary aldosteronism should be performed in

patients with resistant hypertension, moderate or severe hypertension and hypertension associated with hypokalaemia and in patients younger than 40 years with cerebrovascular events due to hypertension. Measurement of plasma aldosterone concentration (PAC) and the ratio of PAC/PRA are used as screening tests for the diagnosis of primary aldosteronism (19, 20). Adequate preparation of patients is required. Two weeks before the test, antihypertensive drugs that affect renin and aldosterone concentrations in plasma are terminated (such as angiotensin converting enzyme inhibitors, angiotensin receptor II blockers, beta blockers, and direct renin blockers). Spironolactone should be excluded at least two months before blood sampling for screening tests (19, 20). Alpha-1-blockers (doxazosin), hydralazine, and calcium channel blockers have the least impact on the plasma concentrations of renin and aldosterone (19, 20). The test is considered positive if the PAC/PRA ratio is > 20 and the PAC is > 15 ng/dL (> 416 pmol/L). In patients with a positive screening test, confirmatory testing, such as a captopril suppression test (CST) should be conducted (19, 20). When a CST is performed, serum aldosterone concentration is measured prior to, and two hours after, administration of a 25 mg dose captopril. The test is positive if the PAC is > 15 ng/dL (> 416 pmol/l) (19, 20). If the screening and confirmation tests are positive, then additional diagnostic procedures for adrenal visualization are indicated (such as: an adrenal ultrasound, CT, or NMR of the adrenals), as well as tests to assess lateralization (such as measuring the concentration of aldosterone in adrenal vein blood samples). In patients with adrenal adenomas (identified from a positive lateralization test), unilateral laparoscopic adrenalectomy is indicated, and in patients with idiopathic bilateral hyperplasia, medical therapy should be applied (such as the mineralocorticoid receptor antagonists spironolactone and eplerenone) (19, 20).

A pheochromocytoma is a rare, catecholamine-secreting tumour derived from chromaffin cells (21). It is also designated as an intraadrenal or intraglandulary paraganglioma, unlike extraadrenal sympathetic, parasympathetic, and extraglandular paragangliomas (21). The prevalence of a pheochromocytoma in hypertensive patients is 0.2-0.5%. Due to paroxysmal release and increased concentration of catecholamines in serum, its clinical features are characterized by the "5Ps": paroxysmal hypertension (hypertension jumps), palpitations (due to tachycardia), perspiration, paleness (vasoconstriction due to decreased blood flow) and a pulsating headache (21). Screening for pheochromocytoma should be applied in patients with: resistant hypertension, characteristic "5P" clinical features, positive family history of pheochromocytoma, and genetic syndromes that are known to be associated with pheochromocytoma (such as MEN 2, von Hippel Lindau syndrome, and neurofibromatosis). Two screening tests are available: assessment of the plasma and 24 h urine sample concentrations of metanephrine and normetanephrine. Plasma concentrations of metanephrine > 0.31 nmol/L and normetanephrine > 0.61 nmol/L suggest increased catecholamine secretion. A concentration of urine metanephrine > 0.7 µmol/24 h and normetanephrine > 1.7 µmol/24 h confirms the presence of pheochromocytoma (21). If the screening test is positive, additional diagnostic procedures for visualization of the adrenals should be conducted (abdomen and pelvis CT or NMR). If test is negative, ^{123}I-metaiodbenzylguanidine (MIBG) adrenal scintigraphy should be performed (21). Treatment of pheochromocytoma is surgical, with adequate patient preoperative preparation. Adrenoceptor antagonists (doxazosin, α_1-blocker) are used to prevent the effects of catecholamines suddenly liberated (preventing the development of hypertensive crisis and cardiac rhythm disorders) (21).

Treatment of renovascular hypertension

In patients with a stenosis of one renal artery, use of angiotensin converting enzyme inhibitors (ACEI) and angiotensin II receptor blockers (ARBs), with appropriate monitoring of serum creatinine concentration is feasible (22). These drugs are contraindicated if stenosis of both renal arteries is present, due to high risk of developing acute kidney injury (5, 23-25). In patients with atherosclerosis due to RAS, primary prevention of progression of stenosis with statins and secondary prevention of the development of renal and cardiac events with low-dose aspirin are indicated (23-26).

Revascularization of RAS (which includes angioplasty with or without stenting or surgical bypass) is indicated by the following: RAS ≥ 50% (systolic pressure gradient (DP) ≥ 20 mmHg and a Pd/Pa ratio < 0.9 indicates haemodynamically significant stenosis (RAS) > 60%), resistant hypertension, loss of renal function after administration of angiotensin converting enzyme inhibitors (ACEI) or angiotensin receptor II blockers (ARBs) (≥ 30% decrease in GFR compared to the values measured before ACEI/ARB administration, an increase in serum creatinine concentration of 0.5 - 1.0 mg/dL (44.2 - 88.4 ìmol/L) after administration of ACEI/ARB), or recurrent flash pulmonary oedema associated with bilateral renal artery stenosis (5, 23-26).

The contraindications for renal artery revascularization include longitudinal diameter of the affected kidney < 8.0 cm, resistance index measured from the blood flow curve through the segmental arteries (RI) > 0.8, chronic kidney disease (GFR < 30 ml/min/1.73 m^2) or negative captopril scintigraphy (lack of lateralization) (23-26).

CONCLUSION

Early diagnosis of renovascular hypertension and timely implementation of appropriate therapeutic procedures ensures optimum control of blood pressure, prevents ischemic nephropathy progression and prevents the development of cardiovascular morbidity and mortality in the hypertensive patient population.

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UPDATES ON THE TREATMENT OF PTERYGIUM

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NOVINE U LEČENJU PTERIGIJUMA

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ABSTRACT

Pterygium is an ocular disease characterised by the growth of fibrovascular conjunctiva on the cornea. It occurs more often in men, at an older age, and in individuals exposed to ultraviolet radiation. Surgical treatment is the primary treatment for pterygium and there are two common procedures for pterygium excision. In the first method, the head of the pterygium is separated from the corneal surface using a surgical blade. The second method is based on avulsion. Other approaches to excising the pterygium include the use of argon laser and excimer laser. Because of a high recurrence rate, adjuvant therapies, including radiotherapy, chemotherapy, and graft procedures, are used after pterygium excision. These procedures have become the standard long-term treatments for pterygium. Radiotherapy is based on beta irradiation. Chemotherapy includes the use of mitomycin C, 5-fluorouracil, bevacizumab, and loteprednol etabonate. Graft procedures include amniotic membrane grafts and conjunctival autografts. Many surgeons believe that using mitomycin C and conjunctival autografts provides the best outcomes in terms of recurrence, cosmetics and patient satisfaction.

Keywords: *pterygium, excision, adjuvant therapy, recurrence*

SAŽETAK

Pterigijum je očno oboljenje i predstavlja rast fibrovaskularne konjunktive preko rožnjače. Češće se javlja kod muškog pola, starijih ljudi, kao i kod onih koji su izloženi utraljubičastom zračenju. Hiruški tretman predstavlja osnovni tretman pterigijuma. Postoje dve vodeće procedure za eksciziju pterigijuma. U prvoj metodi glava pterigijuma razdvaja se od površine rožnjače korišćenjem hiruškog noža. Druga metoda zasnovana je na avulziji. Drugi pristupi eksciziji pterigijuma uključuju argon laser i egzajmer laser. Zbog visoke stope recidiva, nakon eksizije pterigijuma, koriste se adjuvantne terapije koje uključuju radioterapiju, hemoterapiju, procedure sa plasiranjem graftova. One su postale standardni modaliteti dugoročnog tretmana pterigijuma. Radioterapija je zasnovana na beta zračenju. Hemoterapija uključuje upotrebu Mitomicina C, 5-flurouracila, Bevacizumaba, Loteprendol etabonata. Procedure sa plasiranjem graftova su amnionski membranski graft i konjunktivalni autograft. Mnogi hirurzi smatraju da upotreba Mitomicina C i konjunktivalnog autografta daje najbolje ishode u smislu recidiva, kozmetskog izgleda i zadovoljvstva pacijenta.

Ključne reči: *pterigijum, ekscizija, adjuvantna tarapija, recidiv*

ABBREVIATIONS

anti-VEGF - anti-vascular endothelial growth factor
MMC - mitomycin C
5-FU - 5-fluorouracil

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INTRODUCTION

The term "pterygium", which is a latinized version of the Greek term "pterygion", meaning "small wing", describes a wing-shaped growth of fibrovascular conjunctiva on the cornea that most commonly appears on the nasal side (Figure 1) (1). Its presence is not just a cosmetic issue, as it also indicates a clinical condition that affects vision by blocking the visual axis, causing tear film instability, or inducing corneal astigmatism (1). The prevalence rates vary widely, but they are generally more frequent in the equatorial belt and are associated with high levels of ultraviolet radiation, which is the most important etiological factor of pterygium occurrence (2). Earlier epidemiological studies indicated a higher prevalence in rural regions, with age and in men, which often correlates with outdoor work (3, 4).

Despite an extensive presence in the literature, pterygium still remains somewhat of an enigma. The first known authors who described pterygium and its surgical management were Hippocrates, Celsus, Pallus, Sushruta and Aetius (3). Even then, they recognised that its treatment was difficult, and the recurrence or loss of vision was almost certain (1). Until the 20th century, there were few improvements in the treatment and prevention of pterygium recurrence. In fact, some treatment success was achieved only by introducing new drugs and methods (1).

PTERYGIUM EXCISION

Surgical treatment remains the primary treatment for pterygium. Simple excision combined with adjuvant therapy, such as anti-VEGF agents, or grafts that include amniotic membrane grafts or conjunctival autografts provide the best results for the long-term success of pterygium surgery (5, 6).

The main goal of surgical excision is to completely remove all parts of the pterygium head, neck and body (7).

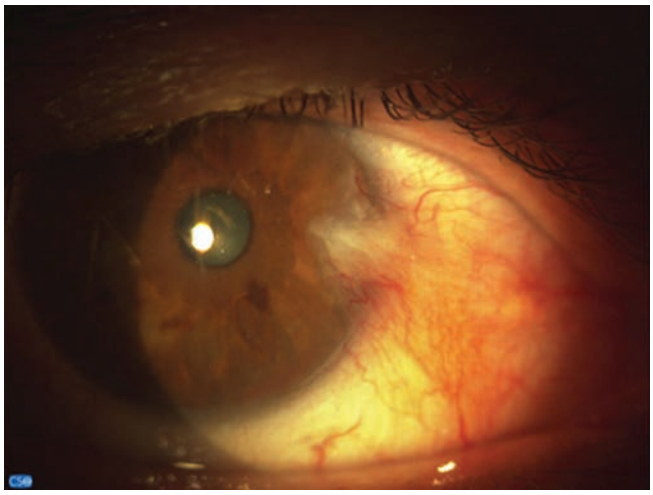

Figure 1. Pterygium- a wing-shaped growth of fibrovascular conjunctiva on to the cornea, most commonly on the nasal side.

There are two procedures that are frequently performed for pterygium excision. In the first method, after administering an appropriate local anaesthesia, the head of the pterygium is grasped with forceps and separated from the corneal surface using a surgical blade. The rest of the pterygium is dissected with scissors posteriorly up to approximately five to seven millimetres from the limbus and then cut (8). The second method is based on avulsion. Subsequent to undermining and dissecting below the body of the pterygium, an unsharpened instrument, such as a spatula, is inserted underneath the pterygium body. In the next step, the body of the pterygium is pulled with forceps, and the head is cut away from the cornea (8). The aim for both methods is a clear corneal bed. This is achieved by using a surgical blade to scrape off the remaining parts of the pterygium and by cauterisation any bleeding vessels (8).

Other approaches to excising the pterygium include argon laser and an excimer laser blade (9, 10). These methods are used after pterygium removal to achieve a fine corneal surface. In some conditions, these lasers can be used to completely excise the pterygium, but these procedures are usually associated with a higher risk of complications (10). Problems that can occur with pterygium excision are an inability to identify a good separation area during blunt dissection and pterygium tissue remnants on the cornea (9, 10). Applying ethanol before surgical excision can improve these techniques (11). Ethanol separates corneal epithelial cells by destroying their junctions, which makes it easier to remove the pterygium from the underlying cornea with a spatula. Additionally, prior ethanol application establishes a better separation area (11). This method is especially suitable for patients with recurrent pterygium in which the cornea has become thin or with a double-headed pterygium (11).

ADJUVANT THERAPIES

A high recurrence rate associated with the bare sclera technique led to the development of several procedures for managing pterygium after its excision (8). These include radiotherapy, chemotherapy and graft procedures (12). Even these procedures have some concerns regarding safety, patient comfort, surgery costs and duration, or whether the results of the surgical treatment are improved. These procedures have become standard for the long-term treatment of pterygium (12).

BETA IRRADIATION

Application of beta irradiation to the bare sclera, usually as a single dose, was shown to be an effective postoperative therapy, especially when it was applied just after pterygium surgery or within 24 hours (13). Additionally, beta irradiation can be combined with other adjuvant methods (14). Radiotherapy has become unpopular among surgeons because of previously documented complications that include conjunctival inflammation, scleral melting, cataract, and uveitis (15).

CHEMOTHERAPY

Over the years, many substances have been used in attempts to prevent recurrence after pterygium excision. Triethylene thiophosphoramide (thiothepa) was one of the first known chemotherapeutic agents used for this purpose (16). Afterwards, other drugs were found to play a role in reducing the recurrence rate, which include doxorubicin and steroids, and more recently, alcohol and anti-VEGF (17, 18). In the last few years, it was reported that the level of VEGF in pterygium tissue was increased compared with the levels in the normal conjunctiva, which justified the use of anti-VEGF to treat pterygium (19).

It is well known that recurrent pterygium has a more aggressive form, which seems to be a strong motivator for investigators to find an adequate adjuvant treatment to prevent pterygium regrowth (8). Many anti-VEGF drugs have been used for this purpose worldwide, but mitomycin C and 5-fluorouracil are the most popular (20).

MITOMYCIN C

MMC is a natural antibiotic-antineoplastic compound derived from *Streptomyces caespitosus* (21). It is an alkylating agent, rather than an antimetabolite, that selectively inhibits DNA replication, mitosis and protein synthesis. MMC inhibits proliferation of fibroblasts and suppresses vascular ingrowths (21). The first known use of MMC in pterygium management was described in 1963 (22). Two approaches have been developed for applying mitomycin C, which include postoperative use of topical eye drops and intraoperative use of sponges soaked in 0.02% mitomycin C (dose, 0.2 mg/ml) applied directly to the bare sclera for three to five minutes (23). This could be used as a primary adjuvant treatment, or an additional graft of conjunctiva or amniotic membrane could be used to cover the bare sclera (24). However, adjuvant mitomycin C treatment is not without risk. It is associated with prolonged, irreversible stem cell damage that can lead to chronic keratopathy and toxic keratoconjunctivitis (21). It can also cause aseptic scleral necrosis, infectious sclerokeratitis, and secondary glaucoma (23). An important fact to remember when using MMC is to be aware of complications as they arise, which can occur many months after MMC application (22). It is recommended to use MMC intraoperatively more than postoperatively because of better control over the dose. Overdose is not uncommon in the postoperative period when mitomycin C is prescribed to the patient (20).

5-FLUOROURACIL

5-FU, a pyrimidine analogue, inhibits DNA synthesis (25). Its effect is expressed in S phase of the cell cycle (25). It also blocks proliferating fibroblast cells that are activated in response to inflammation (25). As previously mentioned for MMC, 5-FU can be applied as a single adjuvant therapy, or it can be combined with a grafting procedure after pterygium excision (20). 5-FU use is related to a few transient complications, but there is still no common opinion among investigators as to how the long-term safety and efficacy of 5-FU application can be adequately evaluated in pterygium treatment (23).

BEVACIZUMAB

Bevacizumab is a recombinant humanised murine monoclonal immunoglobulin G1 that inhibits the VEGF-A isoform, the main stimulator of angiogenesis (26). Significant regression of limbal-conjunctival neovascularisation and a delayed recurrence were reported in patients with recurrent pterygium who were treated with bevacizumab (26). Intraoperatively, bevacizumab is often used as a subconjunctival injection, disabling neovascularisation of the cornea and conjunctiva (27). This method of bevacizumab administration can be performed alone or in combination with argon laser phototherapy to obliterate specific conjunctival feeder vessels (27). Bevacizumab applied topically can prevent corneal neovascularisation (28, 29).

LOTEPREDNOL ETABONATE

An improved understanding of the roles that inflammation plays in the pathogenesis and surgery for pterygium in recent years has led to the use of topical corticosteroids, such as loteprednol etabonate, in pterygium management protocols (30). Compared with other corticosteroids, loteprednol etabonate has a unique structure, which allows it to readily penetrate cell membranes (31). In addition, it has a strong potential for glucocorticoid receptors, whereas the detached drug is rapidly converted into inactive metabolites, preventing any unwanted side-effects of ocular corticosteroids, such as intraocular pressure elevation and cataractogenesis (32). Currently, there are no significant clinical trials that have revealed positive correlations between loteprednol etabonate use and a reduction in the recurrence of pterygium, which leaves possibilities for future investigators (33).

GRAFT PROCEDURES

Amniotic membrane graft and conjunctival autograft have become standard pterygium treatments for many surgeons (23). After pterygium excision, these grafts can be implemented alone or combined with other adjuvant therapies. The currently preferred method for graft procedures is to attach the graft with fibrin glue rather than sutures because of its superior intraoperative and postoperative characteristics (34). These advantages include reductions in the operation time, postoperative inflammation and recurrence rate (35)

AMNIOTIC MEMBRANE GRAFT

The first documented usage of the amniotic membrane graft is connected to its original description in 1947 (1). The amniotic membrane is composed of three different layers: an epithelial layer, basement membrane and an avascular stroma (36). Its useful characteristics include anti-inflammatory, anti-scarring, and anti-angiogenic properties, which make amniotic membranes compatible for pterygium treatment. The amniotic membrane can be applied when it is fresh or cryopreserved. In developing countries, fresh amniotic membrane is usually unavailable because of the need to test it in many types of infections (23). It serves as an alternative to conjunctival tissue in cases where there is a large conjunctival defect and to cover the bare sclera. The amniotic membrane has no human leucocyte antigens, so it has no risk of rejection (36). The advantages of amniotic membrane transplantation over other grafting procedures are a shorter surgical time, less eye pain, faster recovery, and usually better cosmetic outcomes (37).

CONJUNCTIVAL AUTOGRAFT

For the last thirty years, since its first introduction by Kenyon et al. (38) conjunctival autograft has become probably the most effective treatment for pterygium due to the transplantation of autologous tissue. Covering the bare sclera could be done by a primary direct closure, a sliding conjunctival flap, or by a free conjunctival autograft that is usually taken from the superior bulbar conjunctiva (39). It was shown that sliding and free grafts are more effective than direct conjunctival closure (39). The reported recurrence after this procedure is 0-39% (40). The recurrence rate can be decreased by the application of fibrin glue or alcohol while performing pterygium excision and by subsequently covering the bare sclera with conjunctival autograft (34). Minimal use of cautery, ensuring the graft is tendon free, and removal of excess fibrin are important factors for successful conjunctival graft transplantation (39). Limbal-conjunctival grafts, which include about two millimetres of limbal tissue in graft, allows for the damaged limbal cells to be filled with fresh tissue, which minimises the tendency for recurrence (41).

CONCLUSION

Direct comparisons among studies remain difficult because of the differences in the techniques of pterygium excision, durations and types of adjuvant therapies used. It is accepted that various adjuvant therapies and their combinations have significantly improved treatment outcomes in terms of recurrence, cosmetics and patient satisfaction. Many surgeons believe that using the mitomycin C and conjunctival autograft techniques provides the most satisfactory results (42). Other anti-VEGF agents, steroids and ethanol will probably further improve pterygium surgery when several clinical trials are finished.

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DIFFICULTIES IN PREVENTING REPEATED GENITAL SELF-MUTILATION

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TEŠKOĆE U PREVENIRANJU PONOVLJENIH GENITALNIH SAMOPOVREĐIVANJA

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ABSTRACT

Self-mutilation is self-inflicted and intentional damage done to one's body or one's body parts without a conscious suicidal intention. The first case of genital self-mutilation was published in 1846, and the first scientific description of genital self- mutilation was written by Stroch in 1901. Since the first case has been described, there have been a relatively small number of described cases of genital self-mutilation in both genders; there have been an even smaller number of cases of repeated genital self-mutilation and only a few descriptions of repetitive forms of male genital self-mutilation in the literature. The aim of our study is to present difficulties in preventing repeated male genital self-mutilation of a patient with an intellectual disability who was diagnosed and treated for epilepsy and psychosis in early adult life and had a previous history of self-destructive behaviour during childhood. Previous literature does not contain many repeated cases of male genital self-mutilation. After evaluating the contribution of each individual factor in the aetiology of self-mutilation, we concluded that every individual factor is significant in the aetiology of self-mutilation; however, no single factor, as well as all the factors put together, is not enough for prevention of self-mutilation. Our conclusion is that all the presented factors in our research (intellectual disability, epilepsy, psychosis, self-destructive tendencies in childhood) have their place in the aetiology of male genital self-mutilation, but none of them are determining factors. This confirms that it is necessary to conduct further research in the field of aetiology of male genital self-mutilation, which would contribute towards more adequate prevention.

Keywords: *repetitive male genital self-mutilation, psychosis, epilepsy, intellectual disability*

SAŽETAK

Samopovređivanje je namerno oštećenje tela ili delova tela, bez svesne suicidalne intencije. Prvi slučaj genitalnog samopovređivanja objavljen je 1846.godine, a Stroch je 1901 godine dao prvi naučni opis ovog fenomena. Nakon opisanog prvog slucaja u literaturi se pominje relativno mali broj slucajeva genitalnog samopovredjivanja, a jos manji broj slucajeva ponovljenog genitalnog samopovredjivanja od kojih je samo par zabelezeno kod osoba muskog pola. Cilj rada je da se ukaze na teskoce u preveniranju ponovljenog genitalnog samopovređivanja kod muškarca sa intelektualnim deficitom, koji je lečen od epilepsije i psihoze u ranom odraslom dobu, sa istorijom autodestruktivnih postupka u detinjstvu. U literaturi je malo opisa repetativnih formi genitalnog samopovređivanja kod muškaraca. Nakon alize svakog faktora ponaosob u etiologiji samopovredjivanja zakljucili smo da je svaki od njih znacajan za etiologiju samopovredjivanja; medjutim nijedan od njih pojedinacno kao ni svi zajedno nisu dovoljni da se prevenira samopovredjivanje. Naš zaključak je da svi navedeni faktori u našem istraživanju (intelektualni deficit, epilepsija, psihoza i autodestruktivne tendencije u detinjstvu) imaju svoje mesto u etiologiji genitalnog samopovredjivanja kod muskaraca, ali nijedan od njih nije determinišući. Ovim se potvrđuje potreba za daljim istraživanjima u oblasti etiologije genitalne automutilacije kod muskaraca, koja bi doprinela adekvatnoj prevenciji.

Ključne reči: *ponovljeno muško genitalno samopovređivanje, psihoza, epilepsija, intelektualni deficit*

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INTRODUCTION

Self-mutilation is self-inflicted and intentional damage done to one's body or one's body parts without a conscious suicidal intention (1).

The first case of genital self-mutilation was published in 1846 (2), and the first scientific description of genital self-mutilation was written by Stroch in 1901 (3). Stroch linked the cause of genital self-mutilation of a 27 year old man with his failures in everyday life, not with sexual failure (3).

Since the first case was described, there has been a relatively small number of described cases of genital self-mutilation in both genders; a total of 110 cases have been described in the literature so far (4-7). There has been an even smaller number of cases of repeated genital self-mutilation reported in the literature. Genital self-mutilation is most common in the male population in their twenties and thirties, using a kitchen knife (8-10).

There were three described cases of male genital self-mutilation in Serbia (11). Interestingly, two cases happened within the same family, with both patients suffering from psychosis (11). In the third case, the patient did not suffer from a mental illness. A kitchen knife was used in all three cases (12).

Out of 87% cases of genital self-mutilation that are linked with psychosis, most of the patients were diagnosed with schizophrenia (23%) (4). Religious delusions were the central psychopathological phenomenon in psychosis linked with genital self-mutilation (11).

Feelings of guilt caused by sexual conflicts accompanied by frequent masturbation in patients with psychosis can cause a strong and overwhelming need for self-punishment (11). Failure in partner relationships can be connected with genital self-mutilation, even in patients without psychosis (11).

Temporal lobe epilepsy can, in some cases, be linked with male genital self-mutilation (13). Reyazuddin described a case of genital self-mutilation in a patient with epilepsy that did not have any evident epileptic symptoms in the last six months before the genital self-mutilation. The author notes that genital self-mutilation might have been a byproduct of chronic epilepsy (7).

The connection between male genital self-mutilation and an intellectual disability is not clear. Some authors think that the connection between male genital self-mutilation and an intellectual disability is based on lack of insight and acuteness of the patient, while others see genital self-mutilation as a way of counterbalancing intrinsic aggressive tendencies (8).

The literature contains few descriptions of repetitive forms of male genital self-mutilation. There is no recent research on this subject, and only a few cases were described before 2002 (12).

The aim of our study is to present difficulties in preventing repeated male genital self-mutilation of a patient with an intellectual disability who was diagnosed and treated for epilepsy and psychosis in early adult life and had a previous history of self-destructive behaviour during childhood.

CASE REPORT

Patient, 32 years old, single, unemployed, Orthodox. The patient lives with his parents in a family house. The patient was born from a controlled pregnancy, naturally, on the due date, as the youngest of two children by married parents. He spoke his first words in time and started walking in his second year. The patient did not gain control of urinating during childhood, more precisely until he was 20.

In his preschool period, the patient occasionally hit his head on the wall without any known or understandable reason (this behaviour stopped around the time he turned 15). He started school on time but was restless and often started fights with other children. When he was 8 years old, the patient was diagnosed with grand mal epilepsy. Despite being treated with antiepileptic drugs, the disease was not under control, and because of multiple weekly seizures, he stopped attending school. The patient was never hospitalized or properly diagnosed with epilepsy during a period of over twenty years.

The patient was not recruited for the military. The patient's mother describes him as a person who is extremely obedient, peaceful and reserved. The patient did not have any emotional relationships or sexual experience.

He does not smoke, drink or use psychoactive substances.

His great-grandfather committed suicide, and his uncle suffers from epilepsy.

When the patient was 30 years old (a year before the first self-mutilation), his parents noticed the first psychological symptoms; during a visit to his relatives, without any cause, the patient stopped all verbal communication. As his regression continued, periods of him talking to himself occurred, and he was brought to a psychiatrist. After being treated with antipsychotics, he started communicating with people in his environment once again.

At the time of the actual visit to the psychiatrist, his parents noted that when he was 31 years old, the patient hurt his genitals for the first time by making a small cut at the base of his penis. His father noticed shallow cuts while helping him maintain personal hygiene. The patient negated genital self-mutilation at first. Later on, he admitted to doing the act of genital self-mutilation in the bathroom using a kitchen knife while urinating. After the first genital self-mutilation, his parents did not ask for medical help and did not mention what occurred to the psychiatrist.

Seven months later, new genital self-mutilation occurred, after which the patient was hospitalized. Both cases of genital self-mutilation occurred after his parents stopped helping the patient in maintaining personal hygiene. The first genital self-mutilation occurred after his mother stopped her involvement, and the other occurred after his father stopped his involvement in this type of help.

Approximately ten days before the incident, the patient experienced disappointment after being rejected by a woman, which resulted in increased tension and anxiety. During the same period of time, the patient's interest in pornographic magazines and watching scenes with nudity on

TV intensified. He masturbated multiple times a day when he felt increasingly nervous and anxious, which he did not mention to his parents. His patients thought that these events were the cause of the genital self-mutilation. The patient had no explanation for his behaviour. At the time of the patient's hospitalization, his parents informed us that they noticed that the patient had thoughts about cutting off his penis – he was visibly upset, and he often pulled up his pants to observe his genital area. During family meals, he showed increased anxiety while looking at the kitchen knife. He insisted that the knife stay on the table, but right after the lunch, he would remove the knife from the table first. Later on, he used the same knife to hurt himself.

On the day of the actual genital self-mutilation, the patient, while in his room, cut his penis with a kitchen knife, and then he walked into the living room with his pants lowered down and full of blood. The injury was surgically taken care of, and after consultation with a surgeon and urologist, he was taken to a psychiatrist. According to the psychiatrist's evaluation, the patient was upset and anxious and required hospital treatment, even though clear psychotic symptoms were not present. At the admission, the patient looked upset and tense; verbalization was limited and contact with him was superficial. He did not look like someone under the influence of psychotic symptoms, and he did not have suicidal thoughts or tendencies. At first, he did not want to talk about the act of genital self-mutilation. His anxiety reached psychotic level.

During the hospitalization of the patient, delusions or hallucinations were not present. Additionally, we did not notice any epileptic seizures during that period of time. After the first day in hospital, having adjusted his antipsychotic drug doses, the patient became more compliant endless tense, and his anxiety decreased. His laboratory results were normal. Examinations were performed in consultation with a neurologist, surgeon and a specialist of internal medicine, and they did not show any deviant findings important for the case of genital self-mutilation. Examination by a psychologist showed an intellectual disability (Intelligence quotient=45-50) and potential organic changes.

During the hospitalization, he was treated with an antipsychotic (Pills Haloperidol 7.5 mg), antiepileptic (Carbamazepine 800 mg, Valproate 1000 mg) and an anxiolytic (Clonazepam 3 mg).

DISCUSSION

The patient, 32 years old, male, committed genital self-mutilation on two separate occasions. He has been diagnosed with an intellectual disability, epilepsy and psychosis. In a way, his diagnoses belong to all the aetiological frames that have a connection with male genital self-mutilation.

In the case of the patient, there is a similarity to the first scientific description of male genital self-mutilation given by Strochiz in 1901 (3). In both cases, genital self-mutilation was linked with bad experiences and failure in everyday life.

During the early childhood of the patient, he showed auto-destructive tendencies (1), such as hitting his head into the wall repeatedly, which can be related to his intellectual disability and explained as a way of modulating increased anxiety.

Additionally, data from the literature (11) show that one of the dominant factors that lead to male genital self-mutilation in patients with psychosis is the feeling of guilt because of sexual conflicts and failures. Frequent masturbation that follows these conditions causes a large amount of guilt and a strong, uncontrollable urge for self-punishment. In the case of this patient, we can assume that failure in obtaining contact with a woman led to frequent masturbation followed by an increased feeling of guilt and a strong urge for genital self-mutilation, but the patient did not confirm this.

Psychosis, a disorder most commonly associated with male genital self-mutilation, was diagnosed in the patient (4). However, in the period before and after the actual genital self-mutilation, no psychotic symptoms were manifested; neither could they be foreseen by observing his behaviour. Specifically, there were no religious delusions, which are the psychotic symptoms most commonly linked with male genital self-mutilation.

The intellectual disability that was diagnosed during the patient's early childhood by itself does not explain the genital self-mutilation. Its effect is on lowering his frustration threshold and limiting capacities for adaptation of his personality, especially while feeling neglected or denied.

The patient has also been diagnosed with grand mal epilepsy since he was eight years old. In the period before the genital self-mutilation, he did not have any epileptic seizures and was receiving a stable treatment of antiepileptic drugs. Therefore, genital self-mutilation in the patient cannot be linked with epilepsy.

Genital self-mutilation of the patient occurred during the period in which his parents stopped helping him maintain personal hygiene. We think that the act of genital self-mutilation was either a type of protest for feeling rejected by his parents or a compensation for his sexual impulses and emotions towards a young woman. The latter seems more plausible.

All of the analysed factors, which were previously mentioned and described, including auto destructive tendencies during childhood, psychosis, intellectual disability and epilepsy, cannot be directly linked to the actual genital self-mutilation.

Additionally, in an earlier study case that described genital self-mutilation of two men from the same family, none of the above mentioned factors could be directly linked with the actual genital self-mutilation (11).

CONCLUSION

These types of studies can help us discover potential causes of male genital self-mutilation.

However, after taking into consideration all of the previously mentioned risk factors, we can conclude that they are relevant, but they not sufficient enough by themselves in determining the aetiology of male genital self-mutilation. Thus, attempts at creating strategies aimed at prevention of male genital self-mutilation should not be diminished.

This study also opens up a field of research for other potential causes, which could then help create adequate programmes for prevention and identification of men with a higher risk for genital self-mutilation.

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We obtained informed consent from the parents of the patient we presented.

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PNEUMOTHORAX RELATED TO MECHANICAL VENTILATION: SILENT ENEMY

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PNEUMOTORAKS UDRUŽEN SA MEHANIČKOM VENTILACIJOM PLUĆA

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ABSTRACT

Pneumothorax is well known and described complication in intensive care unit patients (ICU). Incidence of this complication is higher in patients with underlying pathology. As it can be occult, it is of the most importance to think of it in patients on mechanical ventilation. In this case report we well present ventilator-related pneumothorax in infant: clinical presentation, diagnosis and management

Keywords: *pneumothorax, mechanical ventilation, pediatric patients*

SAŽETAK

Pneumotoraks je dobro poznata komplikacija kod bolesnika u jedinicama intenzivnog lečenja. Incidenca ove komplikacije je veća kod bolesnika na mehaničkoj ventilaciji. Pneumotoraks može biti klinički teško prepoznatljiv u početku, ali veoma je važno misliti na njega kod bolesnika na mehaničkoj ventilaciji. Prikazaćemo slučaj deteta sa pneumotoraksom udruženim sa mehaničkom ventilacijom pluća, kliničku slku, dijagnozu i terapijski pristup .

Ključne reči: *pneumotoraks, mehanička ventilacija, pedijatrjski bolesnici*

INTRODUCTION

Pneumothorax is well known and described complication in intensive care unit patients (ICU). It can be classified as spontaneous, traumatic or iatrogenic. Iatrogenic pneumothorax has been reported in up to 3% of adult patients admitted in ICU.[1,2] Estimations were made that the incidence in mechanically ventilated patients is 4%-15% [3,4] Pneumothorax is potentially lethal complication and if not recognized and treated, it can progress to tension pneumothorax and increase morbidity and mortality of ICU patients.

CASE REPORT

A 42 days old male infant, TM 3,8kg, with postnatal made diagnosis of Truncus arteriosus communis, ventricular and atrial septal defect and interrupted aortic arch was admitted to pediatric cardiac intensive care unit after complete correction of congenital heart defect. Previously he was operated and had devided colostomas. He was treated for sepsis and other nosocomial infections and had one episode of rightsided pneumothorax. From the day he was born, he was on mechanical ventilation, never took a breath on his own. After the operation his sternum stayed opened as a standard procedure, to relieve pressure on the heart. Measured pressure in pulomary artery was 70% of systolic pressure. Initial ventilator settings were: PCV, FiO_2 85%, Pinsp 23-25cmH2O, PEEP 5-6cm H2O, Tinsp 0,7s, RR 26/min, achieved Vt 8ml/kg. The goal was to achieve PaO_2> 80 mmHg, normocarbia and pH 7,45-7,50. He was countinously sedated with midasolam, analgesia was provided with countinous infusion of fentanyl and muscle relaxation achieved by intermittent boluses of rocuronium. Inotropic support and afterload reduction was made by milrinione, diuresis was regulated by countinous infusion of furosemide. On the first postoperative day sternum was closed without impact on hemodynamics and lung function.

The child was stable in following days with average TA 86/33/51 mmHg, HR 130/min, CVP 11-12cm H2O , diuresis ≥ 1ml/kg/h, SpO2 98%, FiO2 80%, blood gas analysis (BGA) pH 7,51 , PaO2 90 mmHg, PaCO2 35 mmHg, HCO3 28, BE 4,8, Hgb 12 g/dl, SaO2 98%. On auscultation of lungs one could hear equal breathing sounds on both sides with lot of crackles. In the evening on the third postoperative day sudden desaturation

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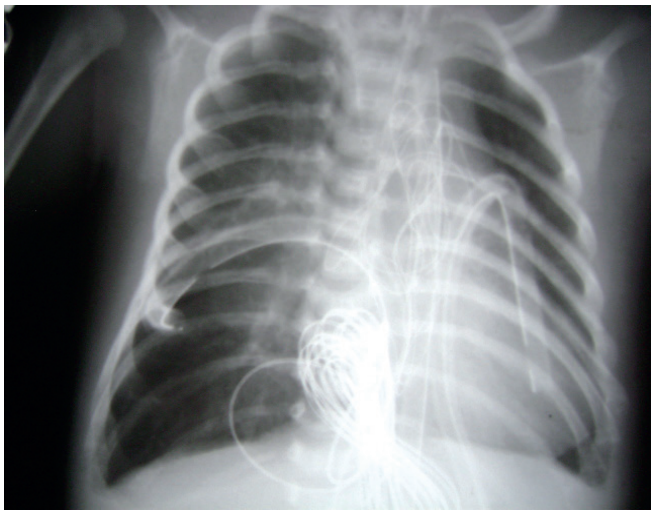
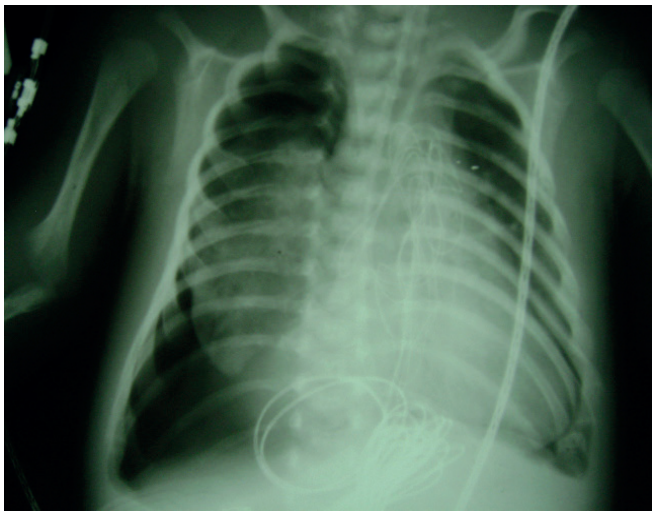
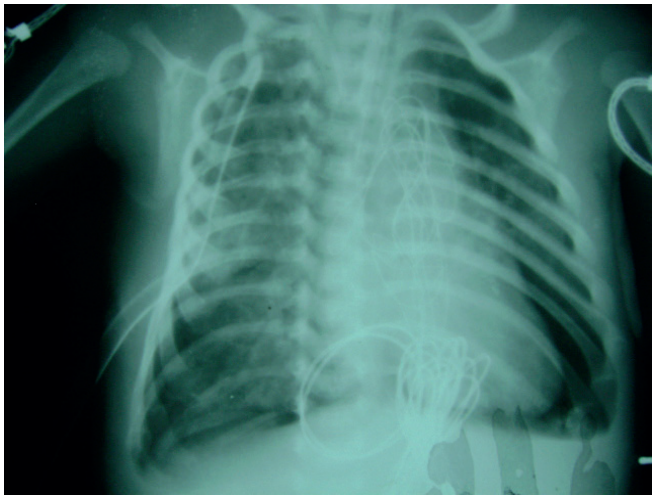


Picture 1. First postoperative day



Picture 2: Third postoprative day : pneumothorax on right side, more than 20% of cavity



Picture 3: Reexpansion of the lung after 12 hours

occurs with SpO_2 80%, FiO_2 80%. Desaturation was followed by increase in blood and central venous pressure - TA 95/36/56 mmHg, HR 156/min , CVP 14-15 cmH2O. BGA showed respiratory acidosis (pH 7,26, PaO2 51 mmHg, PaCO2 83mmHg, HCO3 26,3, BE 5,3, SaO2 77%, Hgb 11,8mg/dl), auscultation of lungs confirmed slightly decreased, symmetrical breathing sounds. On manual ventilation with 100% oxygen lungs were stiff, bolus of fentanyl was given, and after aspiration mucus plug was evacuated. The episode resolved in 30 minutes, and child was stable again but maximal measured saturation on FiO2 100% oxygen was 93%. This made attending ICU doctor unsatisfied and alert to reassaess after 30 minutes again. This time on auscultation breathing sound was diminished on right side. Radiography was made and diagnosis of pneumothorax was obvious. Complete right lung was collapsed leaving more than 20% of cavity filled with air. Immediate chest tube was inserted

Staisfactory reexpansion was achieved after 12 hours, with significant improvement in oxygenation, BGA : SpO2 98%, FiO2 60%, pH 7,53, PaO2 110 mmHg, PaCO2 36mmHg, HCO3 30, BE 6,8, SaO2 99%, Hgb 11,2 mg/dl.

DISCUSSION

In critical illness, pneumothoraces may be difficult to diagnose if they have atypical presentation and are complicated by underlying disease in unconscious patients.[5,6] Unrecognized pneumothorax in patients on mechanical ventilation could rapidly progress to tension pneumothorax which is more common in this patient population, occurring in 30%-97% of all pneumothoraces.[7-9]. If barotaruma is complication of mechanical ventilation, the mortailty rates are high, ranging from 46%-77%.[4,7-9]. Having this data in mind, pneumothorax should not be an issue in patients on mechanical ventilation. In another words, awareness of this lethal complication should be present at all times especially in those who have underlying disease, since barotrauma has been more related to changes in lung parenchyma and lung compliance than ventilator settings alone.[10]

The diagnosis of pneumothorax includes patient`s history, examination, and radiological investigations. In our patient, data of prolonged mechanical ventilation, sepsis, nosocomial infections, recent cardiopulmonal bypass with its` effect on lungs and history of previous pneumothorax alerted us to be more carefull. Therefore, we applied, as described above, lung protective strategy during mechanical ventilation and performed repeated auscultation of the chest. Often reassessment of this child and high level of awareness of pneumothorax possibility made diagnosis prompt. Once suspected, pneumothorax conformation with chest X ray should be made, since it's reliable, cheap and easy to perform diagnostic tool. According to British Thoracic Society : "Standard practice is to place a chest tube for any pneumothorax occurring during mechanical ventilation, due to the risk of positive pressure expanding the pneumothorax into a tension pneumothorax" implying that size of pneumothorax is not determinant of management itself. [11]

CONCLUSION

As in all other diseases and conditions we deal with in everyday practice, prevention of pneumothorax comes in the first place. Applying lung protective strategies decrease incidence of pneumothorax but doesn't exclude one, and should be always applied according to recommendations. Besides this, recognition of risk factors (e.g. underlying pathology) is important also in reducing possibility of this serious complication. In pediatric population, risk factors related to pneumothorax are: extremely low birth babies (ELBW), premature babies, neonates delivered by cesarian section, neonates with RDS, aspiration of meconium syndrome, chlidren with underlynig lung pathology,cardiothoracic procedures and prolonged mechanical ventilation and previous episodes of pneumothorax. There is no definite consensus how to treat pneumothorax in conscious patients, especially one that is < 15% of thorax cavity, but there has definite recommendation has been made to decompress pneumothoraces in patients on mechanical ventilation.

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RETRACTION

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Marija Popovic, Mladen Tasic and Milena Grubisa.
EFFICACY AND SAFETY OF IVUS-GUIDED PERCUTANEOUS CORONARY INTERVENTIONS.
Ser J Exp Clin Res 2015; 16 (2): 115-119.
DOI: 10.1515/SJECR-2015-0015

This is a notice of retraction of the article: EFFICACY AND SAFETY OF IVUS-GUIDED PERCUTANEOUS CORONARY INTERVENTIONS, published in the SJECR in 2015, Ser J Exp Clin Res 2015; 16 (2): 115-119. The Editor-in-Chief has been informed that this paper plagiarizes an earlier paper: Mladen Tasić, Nevena Tasić, Nikola Jagić. Efficacy and safety of intravascular ultrasound-guided percutaneous coronary intervention. PONS Med C 2013 / PONS Med J 2013. This claim is correct and the entire paper, including the abstract, is a verbatim copy of the earlier one. After confirmation of this fact, the Editor-in-Chief of the Serbian Journal of Experimental and Clinical Research has decided to retract the paper immediately.

We apologize to the readers of the journal that it took almost a year to notice this error and to retract the paper. We request readers of the journal to directly get in touch with the editorial office and the editors of the journal for similar cases in the future, so that they can be handled promptly.

INSTRUCTION TO AUTHORS FOR MANUSCRIPT PREPARATION

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The introduction is concise, and states the reason and specific purpose of the study.

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The selection of patients or experimental animals, including controls, should be described. Patients' names and hospital numbers are not used.

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ACKNOWLEDGMENTS

This section gives possibility to list all persons who contributed to the work or prepared the manuscript, but did not meet the criteria for authorship. Financial and material support, if existed, could be also emphasized in this section.

REFERENCES

References should be identified in the text by Arabic numerals in parentheses. They should be numbered consecutively, as they appeared in the text using the Vancouver style. Personal communications and unpublished observations should not be cited in the reference list, but may be mentioned in the text in parentheses. Abbreviations of journals should con form to those in Index Serbian Journal of Experimental and Clinical Research. The style and punctuation should con form to the Serbian Journal of Experimental and Clinical Research style requirements. The following are examples:

Vancouver style Article in a journal: You CH, Lee KY, Chey WY, Menguy R. Electrogastrographic study of patients with unexplained nausea, bloating and vomiting. Gastroenterology 1980;79:311-4; DOI:10.2478/ s11533-007-0023-3.

Book: Eisen HN. Immunology: an introduction to molecular and cellular principles of the immune response. 5th ed. New York: Harper and Row; 1974.

1. Introduction

This document describes standards for preparing the references in the APA style. The following sections give detailed instructions on citing books, journal articles, newspaper articles, conference papers, theses, webpages and others.

Please provide all the required elements in the references to your paper. Please pay particular attention to spelling, capitalization and punctuation. Accuracy and completeness of references are the responsibilities of the author. Before submitting your article, please ensure you have checked your paper for any relevant references you may have missed.

A complete reference should give the reader enough information to find the relevant article. And most importantly, complete and correct references may allow automatic creation of active links by the MetaPress technology that we use for making the electronic version of our journal. Active reference linking is regarded as the greatest benefit of electronic publishing and it adds a lot of value to your publication.

2.Book

a. Book (one author)

Format:

Author. (Year of publication). *Book title*. Place of publication: Publisher.

Example:

Baxter, R. (1982). *Exactly Solvable Models in Statistical Mechanics.* New York: Academic Press.

b. Book (two or more authors)

Format:

Author1, Author2 & Author3. (Year of publication). *Book title*. Place of publication: Publisher.

Example:

Kleiner, F.S., Mamiya C.J. & Tansey R.G. (2001). *Gardner's art through the ages* (11th ed.). Fort Worth, USA: Harcourt College Publishers.

c. Book chapter or article in an edited book

Format:

Author(s) of chapter. (Year of publication). Chapter title. In Editors of the book (Eds.), *Book title* (Chapter page range). Place of publication: Publisher.

Example:

Roll, W.P. (1976). ESP and memory. In J.M.O. Wheatley & H.L. Edge (Eds.), *Philosophical dimensions of parapsychology* (pp. 154-184). Springfield, IL: American Psychiatric Press.

d. Proceedings from a conference

Format:

Author(s). (Year of publication). Title. In Conference name, Date (Page range). Place of publication: Publisher.

Example:

Field, G. (2001). Rethinking reference rethought. In Revelling in Reference: Reference and Information Services Section Symposium, 12-14 October 2001 (pp. 59-64). Melbourne, Victoria, Australia: Australian Library and Information Association.

e. ebook

Format:

Author(s). (Year of publication). *Title*. Publisher. Retrieving date, http address. DOI.

Example:

Johnson, A. (2000). *Abstract Computing Machines*. Springer Berlin Heidelberg. Retrieved March 30, 2006, from SpringerLink http://springerlink.com/content/w25154. DOI: 10.1007/b138965.

f. Thesis

Format:

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.

TABLES

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.

If magnification is significant (photomicrographs) it should be indicated by a calibration bar on the print, not by a magnification factor in the figure legend. The length of the bar should be indicated on the figure or in the figure legend.

Two complete sets of high quality unmounted glossy prints should be submitted in two separate envelopes, and shielded by an appropriate cardboard. The backs of single or grouped illustrations (plates) should bear the first authors last name, figure number, and an arrow indicating the top. This information should be penciled in lightly or placed on a typed self-adhesive label in order to prevent marking the front surface of the illustration.

Photographs of identifiable patients must be accompanied by written permission from the patient.

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

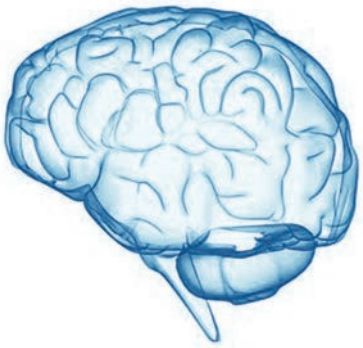
Color prints are available by request at the authors expense.

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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