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GALECTIN-3 IN OBESITY AND TYPE 2 DIABETES

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GALEKTIN-3 U GOJAZNOSTI I TIPU 2 DIJABETESA

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ABSTRACT

Galectin-3 is an important regulator of inflammation and acts as a receptor for advanced-glycation (AGE) and lipoxidation end-products (ALE). Evidence indicates a significant upregulation in circulating levels and visceral adipose tissue production of Galectin-3 in obesity and type 2 diabetes. Recent studies demonstrate development of obesity and dysregulation of glucose metabolism in Galectin-3 "knockout" (KO) mice, which also develop accelerated and more severe pathology in models of atherosclerosis and metabolically-induced kidney damage. Thus, evidence that Galectin-3 is an important player in metabolic disease is accumulating. This review discusses current evidence on the connection between Galectin-3 and metabolic disease, focusing on mechanisms by which this galectin modulates adiposity, glucose metabolism and obesity-associated inflammatory responses.

Keywords: *glucose metabolism, inflammation, obesity, diabetes*

SAŽETAK

Galektin-3 ima važnu ulogu u regulaciji inflamacije i predstavlja receptor za završne produkte glikacije (AGE) (engl. advanced-glycation end-products) i lipoksidacije (ALE) (engl. advanced-lipoxidation end-products). Dosadašnja istraživanja ukazuju da su cirkulišući nivoi i produkcija Galektina-3 u visceralnom masnom tkivu povišeni u gojaznosti i tipu 2 dijabetesa. Noviji eksperimentalni rezultati pokazuju ubrzan razvoj gojaznosti i poremećen metabolizam glukoze u Galektin-3 deficijentnih miševa, koji razvijaju teže oblike ateroskleroze i oštećenja bubrega u sklopu metaboličkih poremećaja, što govori u prilog značajne uloge Galektina-3 u metaboličkim bolestima. U ovom revijskom radu će biti prikazana sadašnja znanja o povezanosti Galektina-3 i metaboličkih poremećaja sa fokusom na mehanizme kojima ovaj galektin modulira visceralnu gojaznost, metabolizam glukoze i inflamaciju povezanu sa gojaznošću.

Ključne reči: *metabolizam glukoze, inflamacija, gojaznost, dijabetes*

INTRODUCTION

Metabolism and immunity preserve internal homeostasis in response to diverse environmental challenges such as excess nutrients or microbial agents by employing a similar hierarchy of processes thus exhibiting a close connection. Immune-mediated metabolic control is exerted through a complex network of immune cells located in metabolic tissues, including adipose tissue and the liver, that maintain homeostatic control under conditions of chronic overnutrition. Regulatory components that are shared between metabolism and immunity are yet incompletely understood (1). Galectins, evolutionarily conserved lectins that are produced by various cell types including immune cells and adipocytes, participate in immunometabolism (Fig. 1). This review addresses the role of Galectin-3 (Gal-3), a member of the galectin family of β-galactoside-binding proteins, in metabolic disease.

Galectin-3

A broad spectrum of roles has been attributed to galectins, including regulation of embryogenesis, angiogenesis, neurogenesis, and immunity. Their expression and secretion is altered during tumorigenesis, neurodegeneration and inflammation. The "galectin signalosome" has a role in

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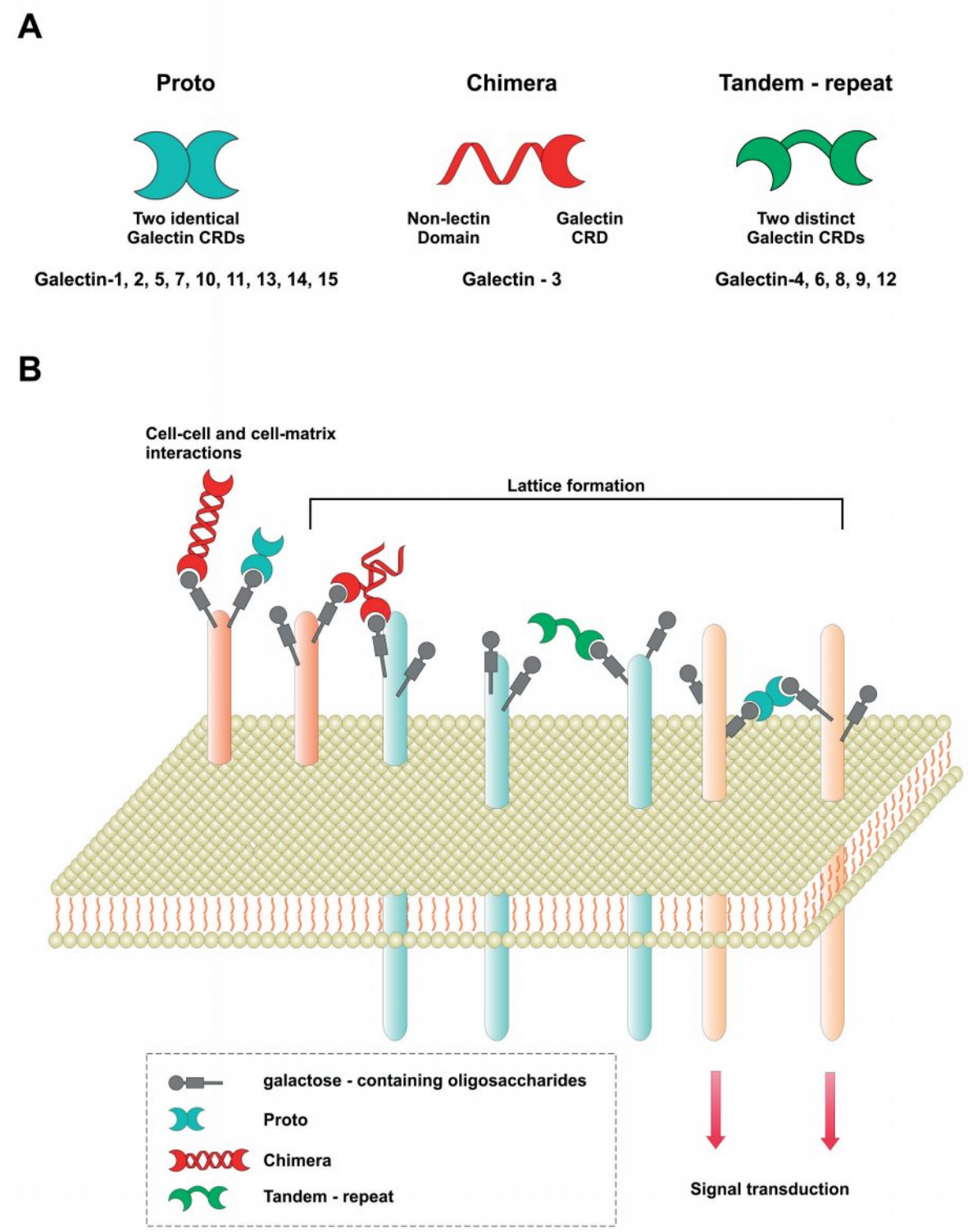

Figure 1. Galectins

Panel A: Galectins are a family of glycan binding lectins which recognize carbohydrates by conserved carbohydrate-recognition domains (CRDs). The 15 galectins which have been identified in mammals are widely distributed and have multiple roles in innate and adaptive immune responses and have been implicated in the pathogenesis of inflammatory, autoimmune and malignant disorders. Galectins are classified on the basis of their structure into three groups: prototypical galectins that contain one CRD (Galectin-1, 2, 5, 7, 10, 11, 13, 14 and 15); Galectin-3, a chimeric galectin which consists of one CRD covalently linked to tandem repeats of proline- and glycine-rich short domains; and tandem repeat galectins that contain two covalently linked CRDs connected by a small peptide domain of up to 70 aa (Galectin-4, 6, 8, 9 and 12).

Panel B: Prototypical galectins exist as dimers. Galectin-3 can both dimerize and oligomerize when it binds to multivalent carbohydrate chains, while tandem repeat galectins have two carbohydrate-binding sites. Galectins interact with transmembrane glycoconjugates and trigger intracellular signaling events; they can also bridge two cells or cells to extracellular matrix proteins and can be secreted in the extracellular space.

Table 1. Response of Gal-3 KO mice in models of metabolic disease

Model	Strains and treatments	Outcome of Gal-3 KO mice compared to WT mice	Reference
Obesity and insulin resistance	WT and Gal-3 KO mice on chow or HFD	Increased adiposity and inflammation, impaired glucose metabolism	[38,39]
Atherosclerosis	WT and Gal-3 KO mice on atherogenic diet	Increased severity	[20]
	Apo-E KO and Apo-E/Gal-3 KO on chow or high cholesterol diet	Decreased severity	[19,56]
Renal disease	WT and Gal-3 KO mice with age- , chemical- or diet-induced glomerular lesions	Increased severity	[13-15,17]
	WT and Gal-3 KO mice with ischemia-reperfusion renal injury	Decreased severity	[57]
Liver disease	WT and Gal-3 KO mice with age- or diet-related NAFLD/NASH	Increased severity	[21,22,58]
	WT and Gal-3 KO mice with age-related NAFLD/NASH	Decreased severity	[18]

many physiological and pathological conditions and better understanding of its functions could lead to development of novel therapeutic approaches that will enable control of systemic and tissue specific expression of galectins.

Galectin-3 has a unique structure in the galectin family, having both lectin-like and carbohydrate-recognition domains (CRD) (Fig. 1). Gal-3 can be present on the cell surface and intracellularly, both in the cytoplasm and the nucleus, and can also be secreted in extracellular spaces, including the systemic circulation. Gal-3 recognizes endogenous lectins, specifically cell surface β-galactosides and N-acetyllactosamine (LacNAc), and modulates intracellular signaling pathways upon cell activation, proliferation and apoptosis. In addition, Gal-3 exerts important cell-cell and cell-extracellular matrix pro-adhesive roles, while also acting as a scavenger molecule for glucose and lipid adducts and binding microbial products, including endotoxin (2).

Production of Gal-3 is altered in a variety of pathophysiological conditions in humans, including cancer, autoimmunity, endurance exercise and others (3,4). Importantly, Gal-3 is considered an excellent prognostic marker in patients with heart failure (5). Here we will focus on the regulation and role of Gal-3 in obesity and Type 2 diabetes (T2D).

Galectin-3 and inflammation

Production of Gal-3 is highly increased during inflammation in both humans and experimental animals, but the role of this galectin in modulating inflammation depends on the cell type, localization and pathophysiological condition. Thus, Gal-3 exerts pro-inflammatory effects in a variety of *in vivo* experimental models, including autoimmune disorders, acute liver injury, bacterial infections and malignancies, as demonstrated by reduced disease severity in Gal-3 KO mice (4,6-10). This evidence has led to the suggestion that Gal-3 may function as an alarmin, a family of endogenous immunomodulatory molecules that belong to the larger family of danger-associated molecular patterns (DAMPs) (11). However, evidence that Gal-3 KO mice have exacerbated sensitivity to endotoxin (12) and heightened inflammation in response to some metabolic stimuli questions this classification (Table 1). In fact, Gal-3 KO mice develop accelerated glomerular injury induced by diabetes (13,14), advanced glycation end-products (AGE) (15,16) or ageing (17). However, in the context of atherosclerosis and hepatic steatosis, both reduced (18,19) and more pronounced (20-22) disease has been reported in Gal-3 KO mice. In situations where increased pathology is observed in Gal-3 KO mice this has been attributed to elevated oxidative stress and inflammatory responses as a result of decreased scavenging of AGE and lipoxidation end-products (ALE), increased expression of receptor for AGE (RAGE) and the consequent RAGE-dependent inflammation. Other potential protective effects of Gal-3 include its ability to enhance production of the anti-inflammatory cytokine IL-10 while suppressing the pro-inflammatory IL-17 pathway in response to microbial stimulation (23-25). Since Gal-3 directly interacts with the microflora and a variety of pathogenic bacteria (26,27), the contradictory results obtained when examining the role of Gal-3 in inflammation using Gal-3 KO mice may at least in part be the consequence of different microbial populations in colonies reared apart and/or the involvement of specific commensals in the disease pathogenesis under different experimental conditions. Given the important involvement of the microflora in a variety of pathologies, including those of metabolic origin, a better understanding of the cross-talk between Gal-3 and commensal bacteria is necessary to clarify these issues.

Galectin-3 and obesity

White adipose tissue is the main site for energy storage, where insulin controls uptake and storage of glucose

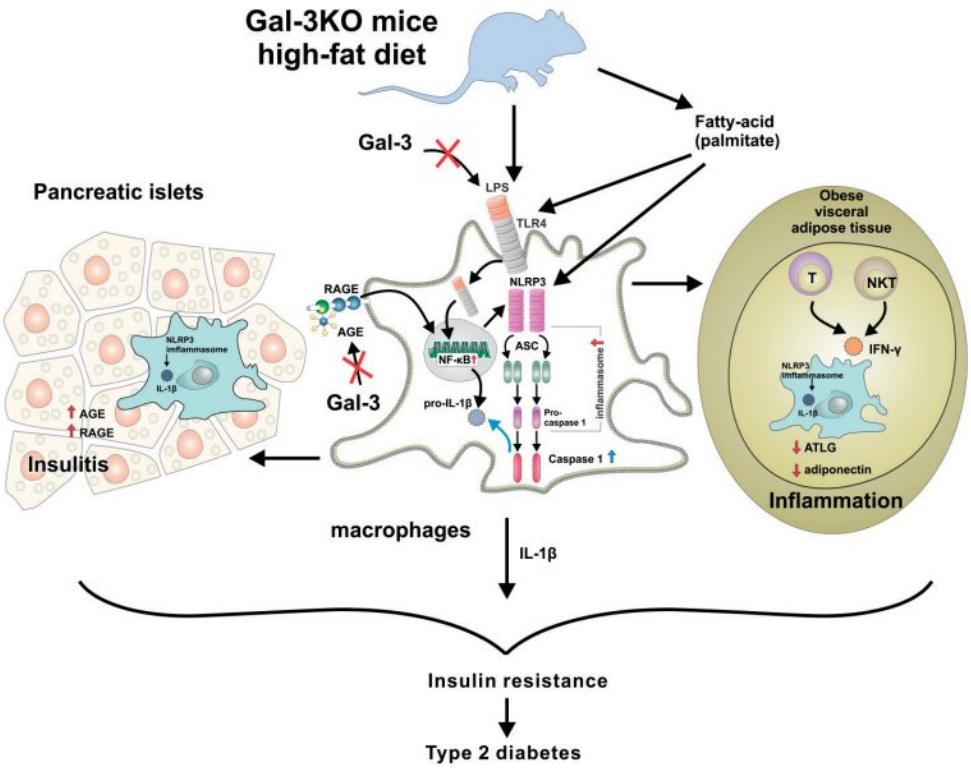

Figure 2. Involvement of Galectin-3 in obesity and type 2 diabetes
Galectin-3 deficient mice develop accelerated obesity and increased adipose mass, dysregulated glucose metabolism and enhanced inflammation in adipose tissue and pancreatic islets when placed on high-fat diet. Galectin-3 acts as a scavenger molecule for advanced glycation end-products (AGE) and binds lipopolysaccharide (LPS). In the absence of Gal-3, AGE bind to receptor for advanced glycation end-products (RAGE) and gut microbiota derived LPS binds to TLR4 to promote activation of the NFκB pathway in macrophages, leading to secretion of proinflammatory cytokines. LPS also upregulates the NLRP3 inflammasome which, when activated by exogenous danger signals such as saturated fatty acids (palmitate) from a high-fat diet, triggers release of IL-1β, that in turn deregulates insulin signaling and potentially leads to insulin resistance. Enhanced expression of the NLRP3 inflammasome in adipose-tissue macrophages during an obese state in Gal-3 KO mice is associated with activation of T and NKT cells, including production of IFN-γ in adipose tissue which promotes further macrophage activation and systemic inflammation. Obese adipose tissue in Gal-3 KO mice is characterized by decreased expression of adipose tissue triglyceride lipase (ATGL) and adiponectin. Pancreatic islets of obese Gal-3 KO mice are infiltrated with macrophages that express active IL-1β and exhibit increased deposition of AGE and upregulated RAGE. The increase of systemic IL-1β levels and a decrease of anti-inflammatory IL-10 and IL-13 levels contribute to metaflammation in obese Gal-3 KO mice, pointing to Gal-3 as an important regulator of diet-induced obesity and type 2 diabetes.

and fatty acids and inhibits lipolysis (28). Through secretion of adipokines, cytokines and hormones, mature adipocytes contribute to maintaining overall energy balance. During obesity adipocytes become dysfunctional and adipose tissue is infiltrated by pro-inflammatory CD11c$^+$ macrophages and other leukocytes that produce pro-inflammatory cytokines which, among other things, interfere with insulin signaling, while expression of the protective adipokine adiponectin is reduced (29). Enhanced release of fatty acids and inflammatory cytokines leads to development of low-grade systemic inflammation, termed metaflammation, in overweight/obese subjects (30,31).

Increased serum and monocyte-associated levels of Gal-3 are present in obese and T2D subjects and experimental animals (32-35). In obese subjects, circulating levels of Gal-3 positively correlate with serum leptin, resistin, IL-6, and age (35), while in the general population they positively correlate with blood pressure, serum lipids, renal function and age (32).

Both human and murine white adipose tissue expresses Gal-3, with higher levels present in the stromovascular fraction (SVF) compared to mature adipocytes (33,36). Obesity leads to progressive increase of Gal-3 expression particularly in visceral (VAT), but also in subcutaneous (SAT), adipose tissue in mice (33,36). Higher expression of Gal-3 in VAT compared to SAT is also observed in humans (35), although how adipose tissue levels of Gal-3 are regulated during obesity in humans remains to be elucidated. Regulation of Gal-3 during obesity in mice is leptin-independent, with proinflammatory CD11c$^+$ macrophages being the main producers of this galectin (33). The anti-diabetic, PPARγ activators thiazolidinediones (TZD) stimulate adipocyte differentiation, induce adiponectin, decrease adipocyte size and increase insulin sensitivity (37). Administration of the TZD rosiglitazone decreases Gal-3 expression in VAT of HFD-fed mice, with no change in circulating levels, thus pointing to differential regulation and/or release of Gal3 by different tissues and cell types (33). The inhibiting effect of TZD on adipose Gal-3 ex-

pression are likely secondary to their adiponectin-inducing properties (37), since adiponectin directly suppresses Gal-3 expression in monocytes and adipocytes (34,35).

Gal-3 KO mice develop accelerated obesity and significantly increased adipose mass, with higher leptin levels, when placed on high-fat diet (HFD) (38,39). The increase in adiposity is not related to increased food intake and the difference in body weight between the two genotypes is already observed after three weeks of HFD. Despite markedly increased adiposity, Gal-3 KO mice have only a non-significant trend towards larger adipocytes, possibly due to reduced factors involved in adipogenesis, such as PPARγ (38). Incubation of preadipocytes with recombinant Gal-3 stimulates proliferation of preadipocytes *in vitro* through the CRD (36). However, whether Gal-3 directly affects adipogenesis in vivo remains to be addressed. Reduced lipolysis may also contribute to development of obesity in Gal-3 KO mice, as significantly reduced expression of adipose tissue triglyceride lipase, the rate-limiting enzyme in lipolytic pathways, is present in Gal-3 KO mice compared with diet-matched groups (38). A possible direct role for Gal-3 in modulating adipocyte lipolysis has not yet been elucidated.

A two-way cross-talk likely exists between Gal-3 and adiponectin (Fig. 2). In fact, as mentioned above, adiponectin suppresses Gal-3 production (34,35), while both lean and obese Gal-3 KO mice have significantly reduced mRNA expression of adiponectin in VAT, and their adipose tissue cultures *ex vivo* produce lower amounts of adiponectin (38). Although neither circulating nor monocyte-derived Gal-3 correlates with circulating levels of adiponectin in humans (34,35), whether Gal-3 and adiponectin reciprocally regulate each other in adipose tissue remains to be investigated.

In summary, Gal-3 KO mice fed HFD develop accelerated obesity associated with changes of biomarkers of adipose tissue metabolism, pointing to a role of Gal-3 in adipose tissue remodeling and metabolism during obesity.

Galectin-3 regulates inflammatory responses in adipose tissue

Under healthy nutrient intake, the immune microenvironment in adipose tissue adopts a regulatory phenotype that actively maintains high adipocyte insulin sensitivity. This is characterized by the presence of adipose tissue-associated T regulatory cells (Tregs), Type 2 T helper (Th) cells, alternatively activated macrophages and high levels of the anti-inflammatory cytokine IL-10 (40). However, chronic overnutrition results in persistent cellular stress that leads to an increase in the number of proinflammatory adipose tissue leukocytes that establish inflammation and contribute to development of insulin resistance (41,42).

Adipose tissue in obese Gal-3 KO mice has increased percentages of T lymphocytes and NKT cells expressing interferon (IFN) γ, with significantly reduced adipose tissue and splenic Tregs compared to WT mice (39). Feeding a HFD also significantly increases $CD4^+PD1^+$ cells in VAT and spleen of Gal-3 KO mice, suggesting that activation of T cells is increased in the absence of Gal-3, possibly due to enhanced TCR-mediated signaling (43). This is accompanied by the increase of adipose tissue $F4/80^+CD11c^+CD206^+$ macrophages and $F4/80^+CD11b^+CD11c^+$ bone marrow-derived cells and markedly reduced alternatively activated M2 macrophages in obese Gal-3 KO mice (39), in agreement with previous studies demonstrating that Gal-3 is an important factor in modulation of M1/M2 polarization (44).

The NLRP3 inflammasome, a multimolecular complex that catalytically activates caspase-1 causing the release of IL-1β, IL-18 and, through a distinct mechanism, IL-1α, participates in sensing metabolic danger molecules (45). Visceral adipose tissue of obese Gal-3 KO mice has increased percentages of macrophages expressing NLRP3 inflammasome and IL-1β (39). Increased protein expression of NLRP3 inflammasome and active caspase-1, together with increased NF-κB activation, are observed in VAT from obese Gal-3 KO mice (39) (Fig. 2). Moreover, Gal-3-deficient peritoneal macrophages exhibit higher caspase-1 activity and secrete higher amounts of caspase-1-dependent IL-1β in response to stimulation with endotoxin and/or saturated fatty acids compared with cells obtained from WT mice. Silencing the NLRP3 inflammasome attenuates IL-1β production by Gal-3 KO macrophages, suggesting that Gal-3-deficient cells have enhanced NLRP3 inflammasome activation (39). Galectin-3-deficient peritoneal macrophages also produce increased amounts of IL-1β and IL-6 upon stimulation with endotoxin *in vitro*, possibly as a result of the inhibitory effect of Gal-3 when it binds to endotoxin (12). Furthermore, expression of IL-6 and TNFα in VAT of lean and obese Gal-3 KO mice is significantly increased than in respective WT controls (38). Gal-3 KO mice also develop age-related systemic inflammation, irrespective of diet, evidenced by a significant elevation in circulating levels and hepatic mRNA expression of acute-phase proteins, elevation of serum IL-1β and IL-6, with significantly reduced IL-10 and IL-13, as well as hematological alterations, compared to diet-matched WT controls (38,39). Thus, Gal-3 contributes to control excessive activation of NFκB, the NLRP3 inflammasome and downstream inflammation by endotoxin and free fatty acids, most likely through the TLR4 signaling pathway that has been linked to development of insulin resistance and regulation of NLRP3 inflammasome expression (46-48). Collectively, the amplified obesity-induced inflammation of Gal-3 KO mice suggests a regulatory and protective role for Gal-3 in the development of metaflammation.

Galectin-3 and glucose metabolism

Subjects with T2D have elevated circulating levels of Gal-3 (35,49). However, whereas a negative correlation between Gal-3 and glycated hemoglobin (HbA1c) is observed in overweight/obese Caucasian diabetics (35), a positive correlation between Gal-3, HbA1c and vascu-

lar complications is observed in a population of normal-weight Asian T2D subjects (49). Therefore, whether obesity and/or ethnicity modulate production of Gal3 in T2D remains to be clarified. Monocyte-associated Gal-3 levels are also elevated in overweight/obese subjects with T2D, with a blunted effect of AMPK-activating compounds on Gal-3 production (34).

Gal-3 deficiency in mice leads to dysregulated glucose metabolism, as reflected by the presence of hyperglycemia and impaired glucose tolerance, already noticeable in young Gal-3 KO mice on chow diet and more pronounced with older mice on HFD developing metaflammation (38). The chronic elevation of glucose levels is confirmed by increased levels of HbA1c, hyperinsulinemia and insulin resistance (39). However, expression of gluconeogenic enzymes in the liver and of Glut1 in VAT are not altered in Gal-3 KO mice, suggesting the gluconeogenic response in the liver is not enhanced and insulin-independent disposal of glucose to adipose tissue is not affected in the absence of Gal3 (38). Treatment with antibiotics leads to normalization of hyperglycemia in Gal-3 KO mice, a result that points to the potential role of the microbiota in modulating glucose metabolism, possibly through pattern recognition receptors (38).

Galectin-3 and the pancreas

Overnutrition-related metabolic triggers such as lipotoxicity and glucotoxicity, oxidative stress, endoplasmic reticulum stress, and perturbed autophagy induce inflammatory responses in pancreatic islets, characterized by accumulation of macrophages, enhanced or reduced insulin secretion and β cells apoptosis during T2D progression (50,51).

Obese Gal-3 KO mice exhibit severe insulitis, also present in a proportion of islets in lean Gal-3 KO mice, compared to their respective WT controls (39). In WT mice HFD upregulates Gal-3 protein within pancreatic islets (39). Enhanced expression of Gal-3 protects rat pancreatic β-cells from the cytotoxic effect of IL-1β (52), and Gal-3 is highly expressed in islet endothelial cells in obesity-induced diabetes in mice (53), but its exact role in diabetes progression is poorly understood. Type 2 diabetes may be classified as an autoinflammatory disease with a central role for NLRP3-ASC inflammasome–mediated IL-1β production (54). Increased expression of ASC, mature caspase-1 and active NFκB in pancreatic tissue of obese Gal-3 KO mice compared to WT controls suggests that Gal-3 may be an important player in the pathogenesis of T2D (39). Moreover, islets of obese Gal-3 KO mice have elevated deposition of AGE and increased RAGE expression, which could be an additional mechanism for NFκB activation and IL-1β secretion within islets. Under an inflammatory milieu, ablation or pharmacological inhibition of Gal-3 prevents apoptosis of β cells exposed to pro-inflammatory cytokines (55), suggesting that endogenous Gal-3 may act to protect β cells from inflammatory stimuli.

Concluding remarks and future perspectives

In conclusion, Gal-3 plays an important role in regulation of adiposity and glucose metabolism in mice. Gal-3 KO mice develop accelerated obesity, metaflammation and systemic inflammation as animals age, which is more pronounced in the presence of metabolic stress induced by HFD.

Future studies will help to elucidate the role of Gal-3 in other metabolic tissues in the course of diet-induced obesity or aging. In particular, clarifying the mechanisms for the protective role of Gal-3 in pancreatic islets in the course of obesity would be of great importance. Obesity, diabetes, heart failure and other diseases associated with inflammation in humans are associated with elevated levels of Gal-3 and further studies are needed to better understand the role of Gal-3 in these conditions, especially in the light of current development of pharmacological inhibitors of Gal-3 for treatment of cancer and fibrosis.

Acknowledgments

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IMMUNOHISTOMORPHOMETRIC CHARACTERISTICS OF PITUITARY GH CELLS IN INFANT AND PERIPUBERTAL FEMALE RATS AFTER TREATMENT WITH ESTRADIOL OR HUMAN CHORIONIC GONADOTROPIN

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IMUNOHISTOMORFOMETRIJSKE KARAKTERISTIKE GH ĆELIJA HIPOFIZE KOD VEOMA MLADIH I PERIPUBRTALNIH ŽENKI PACPVA TRETIRANIH ESTRADIOLOM ILI HUMANIM HORIONSKIM GONADOTROPINOM

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ABSTRACT

The effects of estradiol-dipropionate (EDP) or human chorionic gonadotropin (hCG) on immunohistomorphometric characteristics of pituitary GH cells in infant and peripubertal female rats were investigated. The first group of females received five injections of EDP (0.25 mg/kg b.w.) during the neonatal period of life, and was further divided into two subgroups which were sacrificed at the infantile period (17th day) or at the peripubertal period (38th day). The second group received two doses of hCG (50 IU/kg b.w.) on the 15th and 16th day of life in the first subgroup, and on the 36th and 37th days of life in the second subgroup, while they were sacrificed 24 h after the last treatment, respectively. The control females were injected with an equivalent volume of the vehicle and sacrificed according to the appropriate schedules as the hormone treated rats. EDP treatment decreased GH cell volume density in infant and peripubertal females, by 38% and 76% ($p<0.05$) respectively, in comparison with the controls. The number of GH cells per mm^2 in infantile and peripubertal period was decreased in EDP treated animals by 26% and 53% ($p<0.05$) respectively, compared to the controls. Also, upon EDP treatment in both periods, GH cells were diminished in size and less intensely immunolabelled than in the control groups. The morphometric parameters in animals treated with hCG were insignificantly changed in both analyzed periods, in comparison with the controls. Unlike hCG, EDP manifested clear inhibitory effects on the immunohistomorphometric characteristics of GH cells in examined female rats.

Key words: *female rat, infant, peripubertal, GH cells, estradiol, human chorionic gonadotropin*

SAŽETAK

U studiji su ispitivani efekti estradiol dipropionata (EDP) i humanog horionskog gonadotropina na imunohistomorfometrijske karakteristike hipofiznih GH ćelija veoma mladih i peripubertalnih ženki pacova. Prva grupa ženki je tokom neonatalnog perioda života primila pet injekcija EDP-a (0.25 mg/kg b.w.), a naknadno je podeljena na dve podgrupe koje su žrtvovane kao veoma mlade (17. dan) ili u peripubertalnom periodu (38. dan). Druga grupa je primila dve doze hCG-a (50 IU/kg b.w.) 15. i 16. dana života (prva podgrupa), odnosno 36. i 37. dana života (druga podgrupa), a žrtvovane su 24h nakon poslednjeg tratmana, ponaosob. Kontrolne ženke pacova su primile ekvivalentan volumen rastvarača i žrtvovane su po obrascu koji je važio za hormonima tretirane grupe. Tretman EDP-om je prouzrokovao smanjenje volumenske gustine GH ćelija kod veoma mladih i peripubertalnih ženki pacova za 38% odnosno 76% ($p<0.05$) u poređenju sa kontrolama. Broj GH ćelija po mm^{2}kod veoma mladih i peripubertanih životinja je smanjen nakon EDP tretmana za 26% odnosno 53% ($p<0.05$) poredeći sa kontrolnim vrednostima. Takođe, tretman EDP-om u oba perioda je izazvao smanjenje dimenzija i intenziteta imunobojenja GH ćelija u odnosu na kontrole. Morfometrijski parametri kod životinja tretiranih hCG-om u oba perioda nisu značajno promenjeni u poređenju sa kontrolnim vrednostima. Za razliku od hCG-a, EDP je ispoljio jasne inhibitorne efekte na imunohistomoprfometrijske karakteristike GH ćelija kod ispitivanih ženki pacova.

Ključne reči: *ženke pacova, veoma mlade, peripubertalne, GH ćelije, estradiol, humani horionski gonadotropin*

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INTRODUCTION

In the present study we aimed to investigate the immunohistomorphometric changes of pituitary growth hormone (GH) producing cells upon application of synthetic hormones, estradiol-dipropionate (EDP) or human chorionic gonadotropin (hCG), to infant and peripubertal female rats. GH cells represent the GH/insulin-like growth factor (IGF1) axis specific operative modul, being controlled by hypothalamic, intrapituitary and peripheral signals in the function of somatic development (1). The period of growth and development of individuals from birth to sexual maturation is particularly sensitive when it comes to GH/IGF1 axis functioning and considers the prolonged phases of GH cells intensive activity as well as their "silencing" (2). Namely, besides the daily-based pulsatile GH secretion in young individuals of both genders (3), maturing females express the luteinizing hormone (LH) increase coinciding decline in circulating GH/IGF1 levels (4, 5), while high circulating estradiol is followed with GH elevation (6, 7, 8). Generally, it is well founded that estradiol has stimulatory effect on the pituitary weight of female rats when applied in critical neonatal period of life (9, 10, 11, 12) as well as that increases the number of chromophobes, prolactin (PRL), luteinizing hormone- (LH) and follicle stimulating hormone- (FSH) producing cells (9, 13, 14, 15, 16). On the other hand, immunohistomorphometric features of pituitary adrenocorticotropic (ACTH) cells of infant and peripubertal female rats appear decreased upon application of estradiol (11, 12). It was reported that, unlike PRL, FSH and LH cells, less than 5% of ACTH cells express estrogen receptor α (ERα) in the human pituitary (17). Also, some novel studies suggest that ERs are substantially involved in GH gene expression (18, 19).

It should be also pointed out that hCG is a heterodimeric glycoprotein, composed of 244 amino acids, with two subunits: α (alpha), identical to that of FSH, LH and thyroid stimulating hormone (TSH); and β (beta), responsible to hormone-specific functions (20). hCG interacts with the luteinizing hormone/choriogonadotropin (LHCG) receptor (21), while at the level of young female rat pituitary significantly increases the number of FSH and LH, and volume of TSH cells (9, 10). ACTH cells upon hCG treatment of juvenile female rats express decreased volume density, while the treatment of peripubertal female rats with the same hormone causes the increase of ACTH cell morphometric parameters (11, 12).

As a continuation of our previous studies in the field of EDP and hCG effects on various pituitary hormone-producing cell histological features in the sensitive periods of female rat life cycle (9, 10, 11, 12), this study is focused on the same, less studied features of developmentally relevant GH cells and relies on up-to-date immunohistomorphometric approach, as a gold standard in modern quantitative histology.

MATERIAL AND METHODS

Animals

In the experiment were used infant (sacrificed at the 17th day of life) and peripubertal (sacrificed at the 38th day of life) Wistar female rats, bred in the Institute for Biological Research "Siniša Stanković", Belgrade, Serbia. All animals were individually in cages, under controlled conditions (12:12 light/dark, room temperature - 22°C), with free access to food (a product of the Veterinary Institute Subotica, Subotica, Serbia) and water.

All animal procedures complied with the European Communities Council Directive (86/609/EEC) and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković", University of Belgrade (No 2–12/13).

The first group of female rats received five intraperitoneal (*i.p.*) injections of estradiol dipropionate (EDP; 0.25 mg/kg b.w., ICN-Galenika Pharmaceuticals, Belgrade, Serbia) every second day from the 4th to the 14th day after birth. After the treatment with EDP, the animals were further divided into two subgroups which were sacrificed at the infantile period (17thday) and at the peripubertal period (38thday). The control females were injected with an equivalent volume of sterile olive oil and sacrificed according to the same schedule as the EDP treated rats.

The second group of females *i.p.* received two doses of pregnyl-gonadotrophinumchorionicum (hCG; 50 IU/kg b.w.; N.V. Oregon, Netherlands) on the 15th and 16th day of life in the first subgroup, and on the 36th and 37th days of life in the second subgroup. Treated females from both subgroups were sacrificed 24 h after the last treatment *i.e.* on the days 17th (infantile period) and 38th (peripubertal period). The control females were injected with an equivalent volume of the vehicle and sacrificed according to the same schedule as the hCG treated rats.

Light microscopy and immunocytochemistry

After decapitation the pituitary glands were excised, weighted, fixed in Bouin's solution for 48h, dehydrated in a series of increasing concentrations of ethanol and enlightened in xylol. After embedding in paraplast, each tissue block was serially sectioned at 5 μm thicknesses on a rotary microtome (RM2125 RT Leica Microsystems, Wetzlar, Germany). Sections were deparaffinized in xylol, rehydrated in a series of decreasing concentration of ethanol and immunolabelled using the peroxidase-antiperoxidase (PAP) method of Sternberger et al. (22). The immunohistochemicall procedure is described in detail in our previous work (23). Digital images of the pituitary gland immunolabeled sections were taken using a LEITZ DM RB light microscope (Leica Mikroskopie & Systems GmbH, Wetzlar, Germany), a Leica DFC320 charged coupled device (CCD) Camera (Leica Microsystems Ltd., Heerbrugg, Switzerland) and the Leica DFC Twain Software (Leica, Germany).

Morphometry

Morphometric assessment was performed using a light microscope (Olympus, BX-51, Olympus, Japan) equipped with a microcator (Heidenhain MT1201, Heidenhain, USA) to control movements in the z-direction (0.2 µm accuracy), amotorized stage (Prior, Prior Scientifi Inc., USA) for stepwise displacement in the x–y direction (1 µm accuracy), and a CCD video camera (PixeLink, PixeLINK, Canada) connected to a 19" computer monitor. Stage movement was controlled by the newCAST stereological software package (VIS – Visiopharm Integrator System, ver.2.12.1.0; Visiopharm; Denmark) running on a personal computer.

Volume density estimation was used to determine the percentage of immunopositive GH cells in the anterior pituitary gland of experimental and control females. Two pituitary sections from the superior, three from the middle and two from the inferiorpart (seven horizontal sections) of the rat pituitary glands were analyzed (the same sections were used in the subsequent estimation of number of GH cells *per* unit area-mm^2). The counting area was defined using a mask tool. An interactive test grid with uniformly spaced test points for histomorphometric assessment was provided by the newCAST softare.

Test points hitting the immunopositive GH cells and to the uncolored phase of anterior pituitary were determined. Volume densities (V_V) of GH cells were calculated as the ratio of the number of points hitting immunopositive GH cells with nuclei divided by the number of points hitting the uncolored phase of the anterior pituitary:

V_V (%) = Pp / Pt x 100.

Pp - points hitting the immunopositive GH cells with nuclei,

Pt-points of the test system hitting the uncolored phase of anterior pituitary.

Volume density of GH cells was calculated for each analyzed section. Then, the average value for seven analyzed sections was calculated and represents the volume density of GH cells in pituitary gland (23).

The number of GH cells *per* mm^2 was also calculated. In the first step, the areas of analyzed sections were determinated by Measure Properties option (Polygon area) and then, by simple point counting, the number of immunopositive GH cells was estimated. Additionally, the number of GH cells was expressed *per* unit area (mm^2).

RESULTS

Immunopositive GH cells in infant and peripubertal rats were located in *pars distalis* of pituitary gland and appeared ovoid or pyramidal in shape, with pronounced round nuclei (1 A, D). In animals treated with EDP, in both infantile and peripubertal subgroup, GH cells were diminished in size and less intensely immunolabelled than GH cells in the control groups (Fig. 1B, E). In peripubertal EDP treated subgroup of female rats the individual GH cells with cytoplasmatic processes can be observed (Fig. 1E). GH cells in hCG treated animals in both periods were similar in size and shape as in the controls (Fig. 1C, F), but the immunolabelling for GH cells was less intense in peripubertal subgroup than in the controls (Fig.1F, D).

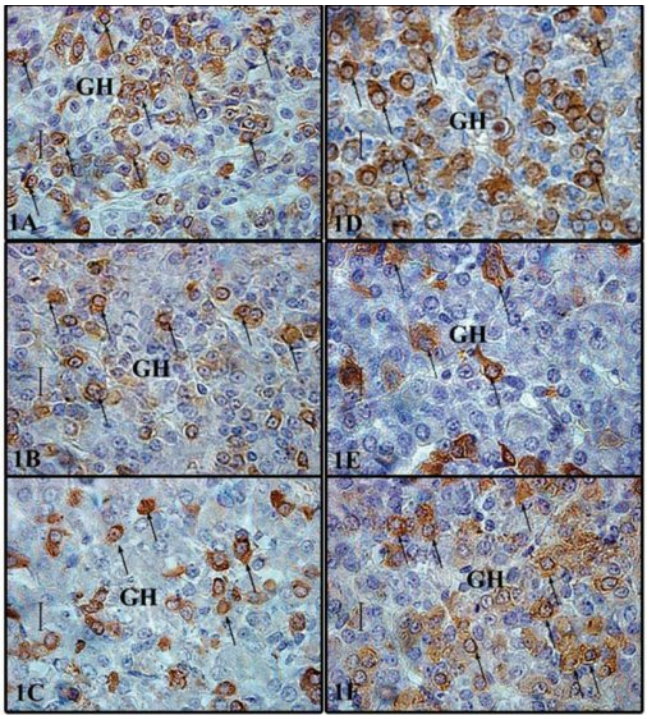

Figure 1. GH-immunopositive cells in the *pars distalis* of pituitary gland from **A**- control infant females, **B** - infant females treated with EDP, **C** – infant females treated with hCG; **D** - peripubertal control females, **E** - peripubertal females treated with EDP, **F** – peripubertal females treated with hCG.

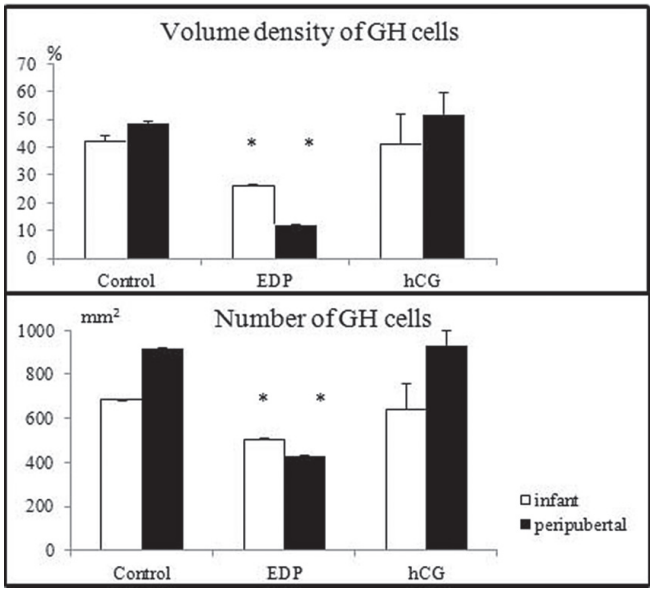

Figure 2. Volume density (A) and number of GH cells per mm^2 (B) in control, estradiol dipropionate (EDP) and human chorionic gonadotropin (hCG) treated infant and peripubertal female rats. All values are means ± standard deviation, n = 5 animals *per* group, *p<0.05 *vs.* control.

Morphometric analysis has shown that EDP treatment significantly decreased volume density in infant (by 38%; $p<0.05$) and peripubertal females (by 76%; $p<0.05$), in comparison with the controls (Fig. 2A). This parameter in animals treated with hCG was insignificantly ($p>0.05$) changed in both analyzed periods, in comparison with the controls (Fig. 2A). The number of GH cells *per* mm^2 in infantile and peripubertal period was significantly decreased in EDP treated animals by 26% and 53% ($p<0.05$) respectively, compared to the controls (Fig. 2B). This parameter in hCG treated subgroups was not significantly ($p>0.05$) changed, in comparison with the adequate control animals (Fig. 2B).

DISCUSSION

The aim of this study was to examine the effects of the treatments with synthetic estradiol or hCG on the immunohistomorphometric features of pituitary GH cells during the infantile and peripubertal period of female rat development. Using a gold-standard approach in modern quantitative histology, we have shown that the EDP treatment caused significant reduction in volume density and number *per* mm^2 of GH cells in infant and peripubertal females. Treatment with hCG didn´t affect on examined immunohistomorphometric parameters in the same stages of development.

The influence of sex steroids on GH/IGF1 axis during puberty is well documented in animal and human studies (24). During that period of life estrogen induces the stimulation of the GH/IGF1 axis and a pubertal growth spurt (25). Although the pubertal rise in sex steroids and activation of GH/IGF1 axis is fundamental for attainment of a normal pubertal growth spurt in both sexes, exposure to sex steroids during the neonatal period is also required (6). Namely, the number and organization of GHRH and SS neurons, GH cells and GH secretory pattern are affected by the neonatal sex steroid environment (24). The number of GHRH mRNA containing neurons in the hypothalamus and GH cells in anterior pituitary of adult male rats is significantly greater compared to that found in females (26). Indeed, the number of GHRH neurons in the early neonatal period does not differ between male and female rats, but in females a progressive loss of neurons occurs until approximately 20 days of age (24). Reduction of GHRH neurons and GH cells is gender-specific and may be caused by the action of estrogen in neonatal period. It is possible that the exogenous estrogen in our experimental conditions exhibits pronounced endogenous estrogen-like effect on the GHRH neurons and GH cells, which caused a reduction in volume density and the number of GH-cells in the anterior pituitary of infant and peripubertal female rats. It was demonstrated that estrogen stimulates the hypothalamic production of somatostatin (27), which also may contribute to the reduction of morphometric parameters of GH cells in our study. These results indicate that the exposure to exogenous estrogen during neonatal period has some permanent effects on the immunostaining properties, volume density and number of GH cells, which partially determines the ability of this gland to produce and secrete growth hormone throughout life.

Human chorionic gonadotropin is a heterodimeric glycoprotein with a numerous functions, mainly related to the maintenance of pregnancy. In our experimental conditions hCG did not affect the morphometric features of GH cells in infant and peripubertal female rats. The available literature data didn't indicate the connection between hCG and GH cells, so we can conclude that GH cells supposedly don't figure as the target cells for applied hCG in the analyzed periods.

Based on these results, it can be concluded that EDP has an inhibitory effect on GH cell immunohistomorphometric characteristics, while the treatment with hCG didn't affect this histological aspect of GH cells in infant and peripubertal female rats.

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APPROPRIATE BIOMARKERS FOR OXIDATIVE STRESS IN PATIENTS WITH END STAGE RENAL DISEASE

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ODGOVARAJUĆI BIOMARKERI OKSIDACIONOG STRESA KOD PACIJENATA U TERMINALNOJ FAZI BUBREŽNE INSUFICIJENCIJE

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ABSTRACT

The appropriate biomarkers for oxidative stress (OS) in patients with end stage renal disease (ESRD) are important in renal pathology. Patients (56) with ESRD were investigated (35 men and 21 women). Patients, with mean age of 45±17 years, defined education, specific HD duration and calculated body mass index (BMI), were exposed to a polysulphone type HD membrane for approximately 4 hours per HD session, 3 times per week. The control group was composed of 31 healthy volunteers. The total antioxidative capacity (TAC) and the antioxidative (AO) enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx), were assessed. Analyses included Randox Crumlin GB; lipid peroxidation (LP) using its end product, malonyldialdehyde (MDA) (fluorimetric); and a LDL-ox immunoassay (Biomedica gruppe, Vienna, Austria). The TAS was higher in ESRD patients before HD (1.63±0.1 mmol/L) compared to the control group (1.23±0.03 mmol/L) (p

SAŽETAK

Za renalnu patologiju i pacijente u terminalnoj fazi bubrežne insuficijencije (ESRD) od velikog je značaja pravilan pristup korišćenja biomarkera oksidacionog stresa (OS). U studiji je učestvovalo 56 pacijenata sa ESRD (35 muškaraca i 21 žena), prosečnih godina starosti 45±17 sa definisanim: obrazovanjem, trajanjem hemodijaleze (HD) i indeksom telesne mase (BMI). Svi pacijenti bili su izloženi hemodijalizi pomoću polisulfonske membrane u trajanju od 4 sata, 3 puta nedeljno. Zdravi volonteri, njih 31, činilo je kontrolnu grupu. Praćeni su sledeći parametri: totalni antioksidacioni kapacitet (TAC), superoksid dismutaza (SOD), glutation peroksidaza (GPx), lipidna peroksidacija (LP) merena preko njenog produkta malonildialdehida (MDA). Vrednosti parametara određivani su fluorimetrijskom metodom i oksidovanim LDL imunoesejom. TAC je bio statistički značajno viši kod pacijenata sa ESRD pre HD (1.63±0.1 mmol/L) u poređenju sa kontrolnom grupom (1.23±0.03 mmol/L).

INTRODUCTION

Oxidative stress (OS) has been reported in end stage renal disease (ESRD) patients undergoing haemodialysis (HD). It may be a principal risk factor for cardiovascular morbidity and mortality of these patients, and it demands appropriate treatment (1, 2). Inflammation and fibrosis in ESRD patients are related to OS. ESRD is also linked to tubulointerstitial inflammation and fibrosis, tubular atrophy, glomerulosclerosis, renal vasculopathy, and fibrous tissue. The pathogenesis suggests a possible unifying mechanism with cardiovascular disease and its progression (3, 4).

OS may be caused by the activation of polymorphonuclear cells, which may be activated by the HD membrane and/or the type of anticoagulant. Anticoagulants function via complement system activation (5). Direct contact between neutrophils and the HD membrane initiates degranulation, which stimulates their oxidative metabolism. HD and uremic factors may influence monocyte activation. Interleukin-1 (IL-1) and tumour-necrotizing factor α (TNF-α) increase free radicals (FR) and induce OS. An imbalance exists between FR production and antioxidant defence. Raddeke HH et al, 1990 established that human mesangial cells can generate FR, as a response to IL-1 and TNF-α, in amounts comparable to monocyte production (6). Uremic toxin retention may trigger a lymphocyte response. This response results in the production of IL-1β and IL-8, which provokes innate immunity through CD8+ cells. Uric toxins may also produce a superoxide ion FR via xanthine oxidoreductase activity, and can no longer bind NADH. Reduced L-arginine production leads to decreased NO activity, which is vital to endothelial function. This cascade is further affected by the atherosclerosis risk factor ox-LDL. The largest source of FR may be the mitochondria within the respiratory chain. Oth-

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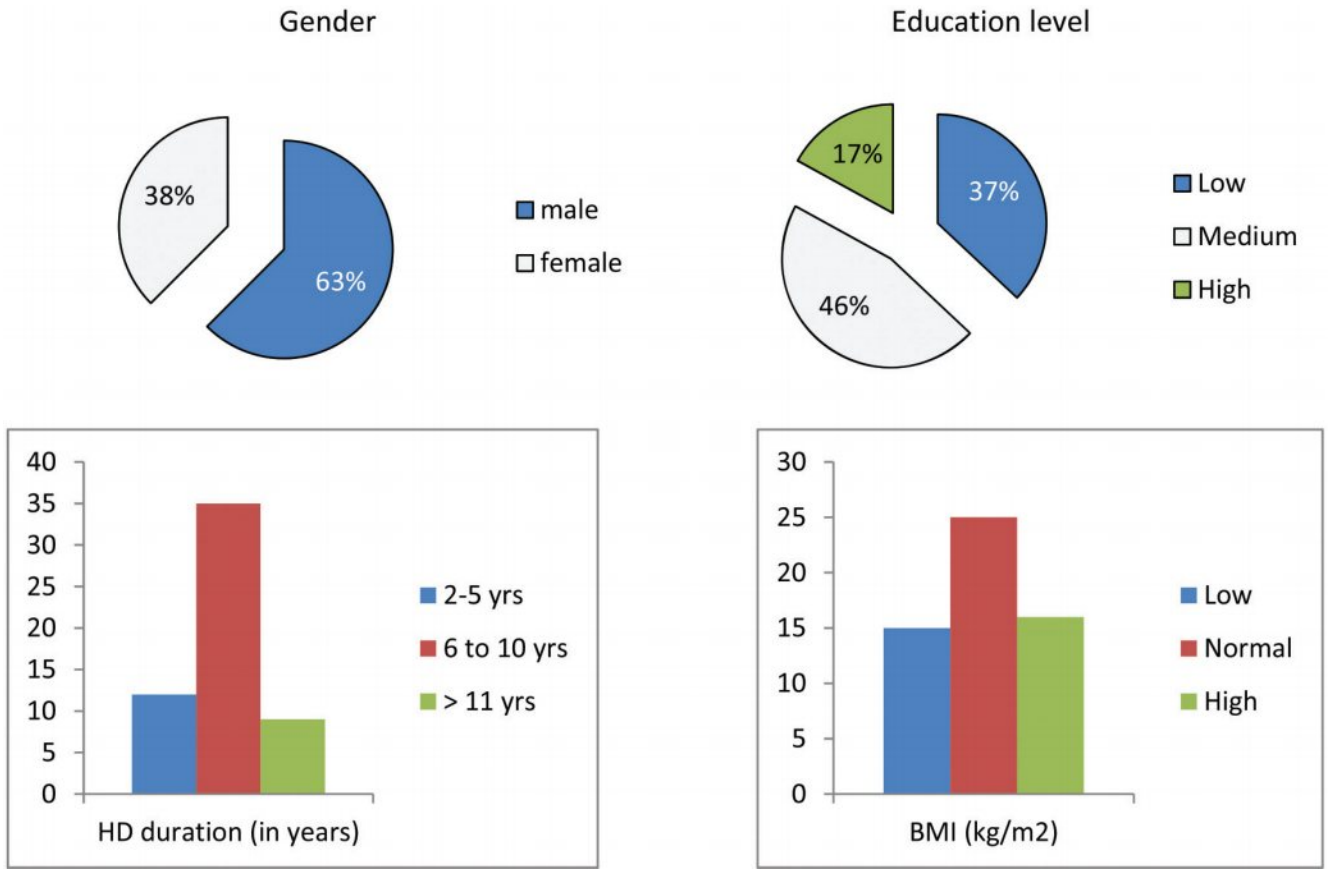

Fig 1. Description of material used, related to: gender (%), educational level (%), HD duration (in years), body max index - BMI (kg/m^2)

er factors, such as obesity, metabolic disease, genetic factors and lifestyle, may cause OS (Fig. 1).

The antioxidant system (enzymatic and non-enzymatic) scavenges FR and prevents OS. The first defence of the antioxidant system is superoxide dismutase (SOD), which accelerates the dismutation of the superoxide anion to hydrogen peroxide. Glutathione peroxidase (GPx) reduces organic lipid peroxide, which requires glutathione as a hydrogen donor. The total antioxidant system contains vitamins, uric acid, bilirubin, transport proteins, etc. The level of OS is determined by lipid peroxidation through its end products malonyldialdehyde and LDL-ox (3).

Previous research has attempted to identify the appropriate biomarkers for OS because of the short half-life of FR. Quantification of the redox-sensitive proteins of signalling microdomains is not straightforward. Our approach measured the stable end products in circulation during OS (7). We investigated appropriate markers of OS in ESRD patients.

MATERIAL AND METHODS

Patients (56) with ESRD, and undergoing HD, were investigated. The study contained 35 men and 21 women with an average age of 45±17 years. Patients had a low (n=37), intermediate (n=46) or high (n=17) level of education. Patients underwent HD (polysulphone type membrane) for a period of 2-5 years (n=12), 6-10 years (n=35) or 11+ years (n=9). Patients exhibited a body mass index (BMI) that was low (n=15), normal (n=25) or high (n=16) (Fig. 2). HD was performed for approximately 4 hours per session, 3 times per week. For inclusion in the study, patients were required to be more than 25 years old and to have been receiving stable HD for more than 2 years. A polysulphone type membrane was used for HD.

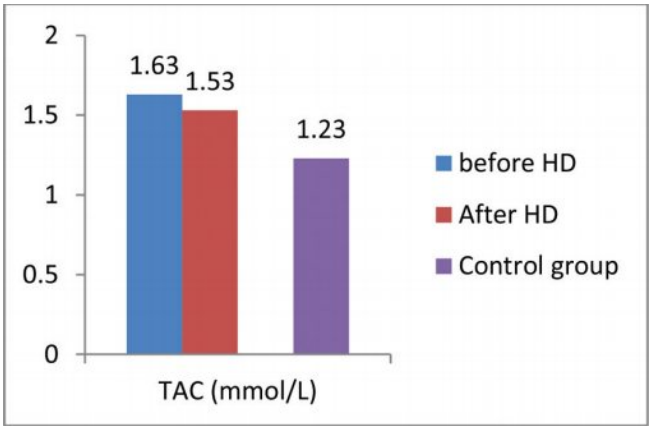

Fig. 2. TAC in ESRD patients (before and after HD) and in control group

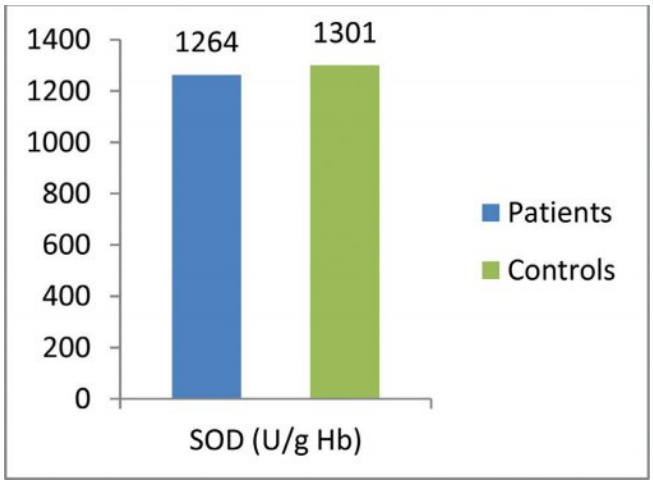

Fig. 3. Superoxide dismustase (SOD) in HD patients and in control group

Patients with other chronic diseases, such as diabetes mellitus or heart failure, and those using supplement therapy, such as antioxidants, vitamins, iron and erythropoietin, were excluded from the study. The control group was age- and sex-matched with 31 healthy volunteers. The enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx), and the combination kits by Randox, Crumlin, Great Britain were used to determine the total antioxidative capacity (TAC). The lipid peroxidation (LP) was determined by a fluorimetric method (Yagi et al, 1967) using the end product malonyldialdehyde (MDA) as a substance that reacts with thiobarbituric acid. The immunoassay by Biomedica gruppe, Vienna, Austria was used to examine the LDD-ox and anti–LDL-ox antibodies (anti LDL-ox Ab). Student t-test was used ($p<0.05$) for statistical analysis.

RESULTS

ESRD patients demonstrated a higher value of TAS (1.63±0.1 mmol/L (before HD)) compared to the control group (1.23±0.03 mmol/L) ($p<0.001$). After HD, the TAO value decreased to 1.53±0.1 mmol/L ($p<0.05$) (Fig. 3).

The antioxidative enzymes SOD (1264 ± 124 U/g) and GPx (54.8± 12 U/g Hb) did not demonstrate statistical significance compared to the control group (Fig. 4, Fig. 5).

Lipid peroxidation resulted in a higher value of TAS (4.52±0.22 μmol/L) compared to the control group (3.81±0.18 μmol/L) ($p<0.01$) (Fig. 6). The TAS value increased (220±125 mU/ml) compared to the control (201±83 mU/ml) for Anti LDL-ox Ab ($p<0.05$) (Fig. 7).

DISCUSSION

ESRD patients exhibit an imbalance of pro-oxidant and anti-oxidant systems, which leads to OS and its consequences. Nguyen et al, 1985 first identified increased FR in the circulation of patients during HD. The activation of

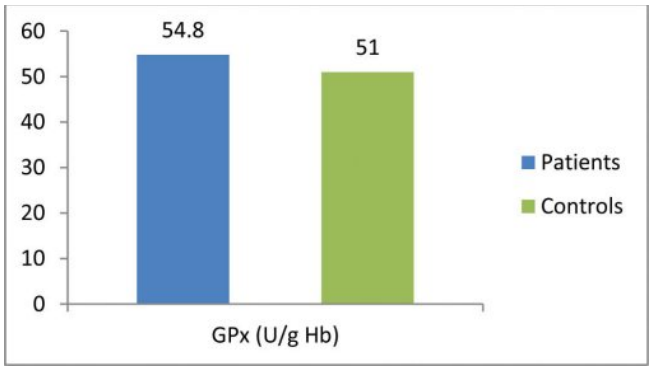

Fig. 4. Glutathion peroxidase (GPx) in patients on HD and in control group

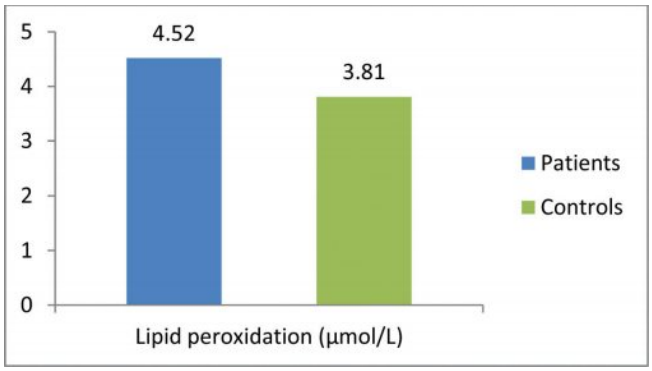

Fig. 5. Lipid peroxidation (MDA) in HD patients and in control group

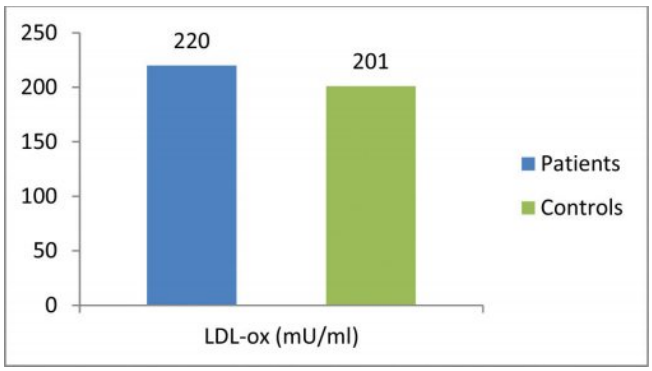

Fig. 6. LDL-ox in HD patients and in control group

phagocyte oxidative metabolism is induced by the haemodialysis membrane (8). Thus, inflammatory response is an important source of increased OS and endothelial dysfunction in ESRD patients. This means that inflammation, OS and atherosclerosis are closely related and have much in common. The TAC is based on small plasma molecules, such as vitamins (A, C, E), uric acid, bilirubin, transferrin, ceruloplasmin, etc. The TAC level increased despite OS in ESRD patients. This finding is consistent with the results published by Montazerifar F et al, 2010 (9), in which the TAC level and vitamin A were significantly higher than the controls. In spite of increased TAC in these patients, the depletion of key chain breaking antioxidants is apparent, which may induce accelerated atherogenesis in these patients (10, 11). In this study, the TAC level was higher for the ESRD patients than

the controls. The level decreased after HD, but it remained higher than the controls. The higher TAC value may be related to concomitant fluctuations of plasma urates. This finding requires further study and interpretation. There is a strong relationship between the TAC and the MDA, haemoglobin, haematocrit and the serum albumin level (12, 13, 14). No changes were observed for the antioxidative enzymes SOD and Gpx in HD patients. Knap B, et al, (15) reported a decrease in SOD activity and no difference in Gpx activity compared to controls; however, MDA and LDL-ox were increased in HD patients. This result indicated the presence of OS. Despite no change in antioxidative enzymes and increased TAC, HD patients demonstrated an impaired balance of FR production and scavenge, which means that the antioxidative defence was not appropriate. Antioxidant loss, through membranes during the HD process, may be related to higher LP level. This increased level in HD patients affects atherosclerosis development (16). An increased level of LDL-ox in HD patients confirmed that FR impaired the LDL molecule through oxidation. This impairment might have occurred because of the released myeloperoxidase from activated neutrophils (from the HD process) (17). Both LP and LDL-ox demonstrated elevated values in HD patients compared to the controls. OS is progressive and may induce complications such as the dysfunction of lipids and other molecules, accelerated tissue or cell apoptosis and endothelial impairment with possible cardiovascular morbidity. These last parameters are important biomarkers for the severity of OS, and the morbidity and mortality of these patients. Future studies are needed to clarify the appropriate biomarkers that define a HD patient's condition, and to demonstrate the benefits of antioxidative therapy.

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ANALYSIS OF CLINICAL, HAEMATOLOGICAL AND BIOCHEMICAL PARAMETERS IN PATIENTS WITH INFECTIOUS MONONUCLEOSIS

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ANALIZA KLINIČKIH, HEMATOLOŠKIH I BIOHEMIJSKIH PARAMETARA KOD BOLESNIKA SA INFEKTIVNOM MONONUKLEOZOM

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ABSTRACT

Primary infection with Epstein-Barr virus (EBV) usually occurs in early childhood and often does not present clinical symptoms. More than 90% of adults are infected with this virus. A primary infection that occurs in adolescence or adulthood is usually clinically presented as infectious mononucleosis with a triad of symptoms: fever, lymphadenopathy and pharyngitis. Our retrospective study included 51 patients with a median age of 17 (9-23) years and serologically confirmed infectious mononucleosis. All patients with infectious mononucleosis were treated at the Clinic for Infectious Diseases at the Clinical Center in Kragujevac during 2013. We analysed the clinical, haematological and laboratory parameters of patients. The aspartate-aminotransferase levels were increased in 40 patients, with a mean value of 116.24 (±93.22); the alanine-aminotransferase levels were increased in 44 patients, with a mean value of 189.24 (±196.69). Lymphadenopathy was the most common clinical feature upon admission in 49 patients (96%); 38 patients (74.5%) had splenomegaly, and 20 (39%) had hepatomegaly. Twenty-six patients (51%) had leukocytosis with lymphocytosis, while 15 (75%) of the 20 who had a normal leukocyte count also had lymphocytosis. In the present study, we updated the clinical, haematological and laboratory parameters, which may lead to the establishment of an accurate diagnosis and promote further treatment of the patients.

Keywords: *Epstein-Barr virus, Infectious mononucleosis, Aspartate-aminotransferase, Alanine-aminotransferase, Reticuloendothelial system, Young adults*

SAŽETAK

Primarna infekcija Epstein-Barr virusom (EBV) uglavnom nastaje u ranom detinjstvu i najčešće protiče klinički inaparentno. Više od 90% odraslih osoba je inficirano ovim virusom. Ukoliko se primarna infekcija odigra u adolescentnom ili odraslom dobu, najčešće se klinički prezentuje kao infektivna mononukleoza, u vidu povišene telesne temperature, limfadenopatije i angine. Naša retrospektivna studija je obuhvatila 51 pacijenta prosečne starosti 17 (9-23) godina sa serološki potvrđenom infektivnom mononukleozom. Svi pacijenti su lečeni na Klinici za infektivne bolesti Kliničkog centra u Kragujevcu, tokom 2013. godine. Analizirani su klinički, hematološki i laboratorijski parametri. Enzimska aktivnost aspartat-aminotrasferaze povećana je kod 40 pacijenata, srednja vrednost iznosila je 116.24 (±93.22); aktivnost alanin-aminotrasferaze povećana je kod 44 pacijenta, srednja vrednost iznosila je 189.24 (±196.69). Limfadenopatija je najčešći klinički znak koji je na prijemu bio prisutan kod 49 pacijenata (96%), 38 pacijenata (74,5%) imalo je splenomegaliju i 20 pacijenata (39%) imalo je hepatomegaliju. Dvadeset šest pacijenata (51%) imalo je leukocitozu sa limfocitzom dok je 15 pacijenata (75 %) od 20 koji su imali normalni broj leukocita takođe imalo limfocitozu. U ovoj studiji prikupili smo kliničke, hematološke i laboratorijske parametare koji mogu da pomognu u uspostavljanju tačne dijagnoze i daljeg adekvatnog tretmana pacijenata.

Ključne reči: *Epstein-Barr virus, Infektivna mononukleoza, Aspartat - aminotrasferaza, Alanin - aminotrasferaza, Reticuloendotelni sistem, Mladi*

ABBREVIATIONS

ALT – alanine aminotransferase
AST – aspartate aminotransferase
EA – early antigen
EBV – Epstein-Barr virus
ELISA – enzyme-linked immunosorbent assay
IM – Infectious mononucleosis
LYDMA – lymphocyte-determined membrane antigens
NA – nuclear antigen
VCA – viral-capsid antigen

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INTRODUCTION

Primary infection with Epstein-Barr virus (EBV) usually occurs in early childhood and often does not present clinical symptoms. More than 90% of adults are infected with this virus, which is demonstrated by serological reactions (1). Primary infections that occur in adolescence or adulthood are usually clinically presented as infectious mononucleosis (IM) (1, 2) with a triad of symptoms: fever, lymphadenopathy and pharyngitis (3, 4). However, splenomegaly, hepatomegaly and palatal petechiae are each present in more than 10 percent of patients. Less common complications include haemolytic anaemia, thrombocytopenia, aplastic anaemia, hepatitis, splenic rupture and rash (3). As a member of the Herpesviridae (5, 6) family, EBV possesses the ability to establish a latent infection with the possibility of later reactivation, which may be clinically manifested as recurrent parotitis, uveitis or interstitial pneumonia (5). EBV also leads to an aetiological relationship with some carcinomas such as nasopharyngeal carcinoma, Burkitt's tumour, Hodgkin's disease and B-cell lymphoma in HIV-infected patients (7).

EBV has a specific affinity for B-lymphocytes and epithelial cells in the oropharynx that bind to the CD21 receptor. Infection is most often transmitted by the saliva. The antigenic structure of EBV is quite complex. It possesses capsid antigen (EBV-VCA), nuclear antigen (EBV-NA), early antigen (EBV-EA) and lymphocyte-determined membrane antigens (LYDMA) (7).

Haematological analysis is a characteristic test for diagnosing IM with leukocytosis and lymphocytosis (more than 10% atypical lymphocytes). Biochemical analyses in approximately 90% of cases show increased aminotransferase and alkaline phosphatase activity in the serum as a result of liver damage (8, 9). The Paul-Bunnell-Davidson test is used in the diagnosis of IM to identify the heterophilic antibodies in the patient's serum but the test is not specific. In the first week of the disease, the test is positive in 40% of patients, and it is positive in the third week in 80-90% of patients (7). The most important analyses for acute IM diagnosis assess the IgM class of antibodies that bind to VCA, which is present in the first two months of the illness. The IgG class of antibodies that binds to VCA is mainly used for diagnosing IM that persists for life (8).

IM is a self-limiting disease with a good prognosis. Fatal cases are extremely rare and are usually caused by complications in the central nervous system, spleen rupture or obstruction of the upper respiratory tract.

The aim of this study was to highlight the most important clinical, haematological and biochemical abnormalities in patients with IM and to determine the frequency of these abnormalities in young adults.

PATIENTS AND METHODS

Participants

Our retrospective study included 51 patients with serologically confirmed IM. The study was conducted at the Clinic for Infectious Diseases at the Clinical Center in Kragujevac and included all patients with IM treated in the period from 1 January 2013 to 31 December 2013. The subjects had a median age of 17 (9-23) years and included 24 (47%) males and 27 (53%) females. In all cases, the diagnosis was established by the detection of EBV-VCA IgM antibodies using an enzyme-linked immunosorbent assay (ELISA).

We analysed the following clinical parameters: activity of the liver enzymes in the blood (aspartate aminotransferase - AST, alanine aminotransferase - ALT), the number of leukocytes and lymphocytes, body temperature, the reticuloendothelial system (liver, spleen, lymph nodes), pharyngitis, edema of the eyelid, and rash.

Statistical analysis

The experimental data were analysed using basic descriptive statistics: the mean value (X) ± standard deviation (SD). The chi-squared test was performed to compare categorical variables between groups. A database analysis of the results was performed using the SPSS version 18 software package (SPSS Inc., Chicago, IL, USA).

RESULTS

Clinical characteristics

The study was performed in the Clinic for Infectious Diseases at the Clinical Center in Kragujevac. The records of 51 patients hospitalized with a documented EBV infection over a 1-year period were retrospectively reviewed.

Sore throat was noted in 37 patients (72.5%). Twenty-six patients (51%) suffered from fever, 5 (10%) from edema of the eyelid, and 3 (6%) from rash.

Lymphadenopathy was the most common clinical feature upon admission and was detected in 49 patients (96%). Thirty-eight patients (74.5%) had splenomegaly and 20 (39%) had hepatomegaly. The median enlargement of the spleen, identified by ultrasonography, was 13.3 cm (10.2–20), while the median enlargement of the liver was 14.1 cm (9.6-16.5). The triad of symptoms (fever, lymphadenopathy, and sore throat) was noted in 21 patients (41%) (Table 1).

Laboratory findings

From the total number of patients, 31 (61%) had leukocytosis (mean value: 11.3; range: 4.1-24.8 x 10^9/L), while 41 (80.5%) had lymphocytosis (mean value: 54.4;

range: 24.2-81%). Twenty six patients (51%) had leukocytosis with lymphocytosis. while 15 (75%) of the 20 who had a normal leukocyte count also had lymphocytosis (Table 2).

The AST levels were increased in 40 patients (78.4%), and the ALT levels were increased in 44 patients (86.3%) (Table 3).

Treatment and outcome

All patients received supportive care. Empirical antimicrobial regimens were given to 38 (74.5%) patients before the establishment of the diagnosis. No cases of fulminant hepatitis or liver failure were observed. None developed chronic liver disease. Finally, no cases of EBV-induced hepatitis-related mortality were noted among the study patients. All patients fully recovered, as confirmed by follow up visits in the outpatient clinic.

Table 1. Frequency of the associated signs and symptoms in patients[a] with EBV infection.

Signs and symptoms	No of patients (%)
Sore throat	37 (72.5)
Fever	26 (51)
37-38°C	14 (54)
38-39°C	11 (42)
39-40°C	1 (4)
>40°C	0
Edema of the eyelid	5 (10)
Rash	3 (6)
Lymphadenopathy	49 (96)
Splenomegaly	38 (74.5)
Hepatomegaly	20 (39)
Triad of symptoms[b]	21 (41)

[a] Total number of patients: 51.
[b] The triad of symptoms includes patients who had a fever, lymphadenopathy and a sore throat.

Table 2. Laboratory values on admission of the 51 patients with EBV infection.

Laboratory value	Mean (±SD)
Leukocytes, cells x10^9/l	11.29 (4.47)
Lymphocytes, in %	54.36 (12.32)
[a]AST (U/I)	116.24 (93.22)
[b]ALT (U/I)	189.24 (196.69)

[a]AST: aspartate-aminotransferase.
[b]ALT: alanine-aminotransferase.

Table 3. Number of patients (%) with different levels of enzyme elevation (AST, ALT) during EBV infection.

	AST[a] (%)	ALT [b](%)
Normal	11 (21.6)	7 (13.7)
< 2x	11 (21.6)	(17.6)
2-3x	8 (15.7)	9 (17.6)
3-5x	15 (29.4)	9 (17.6)
5-10x	4 (7.8)	12 (23.5)
>10x	2 (3.9)	5 (9.8)

[a] AST: aspartate-aminotransferase.
[b] ALT: alanine-aminotransferase.

DISCUSSION

As previously mentioned, IM manifests with characteristic clinical, haematological and serological parameters after an incubation period, which may last up to 50 days (8). The manifestation usually begins with fever, pharyngitis and enlargement of the liver, spleen and lymph nodes (10). IM is generally a self-limiting disease with a good prognosis. Complications are rare, but they can be very serious. In less frequent cases, the complications may be the only clinical manifestation of the disease (11).

Due to the various clinical, haematological and biochemical manifestations, in this study, we wanted to determine the frequency and level of these abnormalities.

Regarding the hepatic complications, it is known that 80 - 90% of patients with IM have moderately increased aminotransferase levels, which indicates liver lesions. In cases when there are no clinical signs of liver damage, there is always a characteristic histopathological change (6, 8, 12). Consistent with those studies, our results are similar in that the liver enzymes were increased. The AST levels were increased in 40 (78.4%) patients, while the ALT levels were increased in 44 (863%) patients. During EBV infection, transaminases are typically elevated less than five-fold compared with the normal levels (13). Our data showed that the AST and ALT levels were elevated approximately 5-fold above the normal limits, a finding that is consistent with the existing literature (6, 14, 15). Elevations greater than 10-fold over the normal levels are less likely (16), but our results have shown that the AST levels in 3.9% of patients and ALT levels in 9.8% of patients were greater than 10-fold the normal levels, which correlates with the results of Yang et al (12). The incidence of serum ALT levels greater than 1000 IU/L was similar to the result of a previous study (17). It is believed that EBV does not have a direct cytocidal effect on hepatocytes, but that damage is caused by free radicals (8). It is believed that another possible cause of liver damage during EBV is type II (cytotoxic) hypersensitivity reactions (18).

Leukocytosis was noted in 61% of patients, while Yang et al noted leukocytosis in 86.1% of children who had IM (12). Furthermore, leukocytosis with lymphocytosis occurred in 51% of patients, which is similar to the results of Kofteridis et al, who investigated the same aged population (6).

The reticuloendothelial system is usually affected, as evidenced by generalized splenomegaly, hepatomegaly and lymphadenopathy (19).

When we analysed the size of spleen, we found similar results as the study by Kofteridis et al, who found that approximately two-thirds of the patients developed splenomegaly (6). Regarding the clinical and laboratory manifestations, our results differed somewhat from earlier studies (12, 20). Hepatomegaly in young adults has been found in 36.7% of patients with diagnosed IM (20), which is similar to the results in our study, where we had 39% of patients with an enlarged liver.

Lymphadenopathy is most common in the anterior and posterior cervical lymph nodes, the submandibular lymph nodes, and occasionally the axillary and inguinal lymph nodes. Traditionally, epitrochlear lymphadenopathy is suggestive of IM because this finding is uncommon in generalized lymphadenopathy (19). In our study, this was the most common symptom. A difference was noted in rate of lymphadenopathy (96% in our cases vs. 78-89% in earlier reports) (6, 20, 21), while our result was similar with the percentage of patients with lymphadenopathy in a study of preschool children (21).

The onset of illness was defined as the first day of sore throat and fever. Fever was the most common feature in previous studies (6, 12, 21), while in our study, 51% of patients had a body temperature higher than 37°C. The physical examination typically reveals pharyngitis that is often is accompanied by moderate-to-marked tonsillar enlargement, occasionally with exudates that cannot be distinguished from streptococcal pharyngitis (19). Our results have shown that fewer patients had pharyngitis compared to previous studies (20, 21).

Rash occurs in 3% to 15% of patients and is usually maculopapular (19). In our study, the percentage of patients who had this symptom was less than has been observed in previous research (6, 12, 20, 21).

Tanner has logically divided the ocular manifestations of IM into two groups: 1) those due to direct EBV infection of the eye and ocular adnexa and 2) those affecting vision and the neuroophthalmologic apparatus from a remote focus, particularly the central nervous system. Symptoms of pain upon rotation of the eyes, deep orbital pain, photophobia, and an epiphora of short duration have all been noted (22). Edema of the eyelids and periorbital tissue, which may be pronounced, is observed in 25 to 40% of patients (23). Only 6% of patients had this symptom in our study, which was similar to the adult patients in another study and six times higher than that observed in preschool children with IM (21).

CONCLUSIONS

In conclusion, the present study, despite its limitations, namely the small number of patients and its retrospective nature, provides updated clinical, haematological and laboratory parameters, which may lead to the establishment of an accurate diagnosis and promote further treatment of IM patients.

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THE EFFECTS OF L-ARGININE AND L-NAME ON CORONARY FLOW AND OXIDATIVE STRESS IN ISOLATED RAT HEARTS

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EFEKTI L-ARGININA I L-NAME NA KORONARNI PROTOK I OKSIDACIONI STRES IZOLOVANOG SRCA PACOVA

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ABSTRACT

The aim of this experimental study was to assess the effects of the acute administration of L-arginine alone and in combination with L-NAME (a non-selective NO synthase inhibitor) on the coronary flow and oxidative stress markers in isolated rat hearts. The experimental study was performed on hearts isolated from Wistar albino rats (n=12, male, 8 weeks old, body mass of 180-200 g). Retrograde perfusion of the isolated preparations was performed using a modified method according to the Langendorff technique with a gradual increase in the perfusion pressure (40–120 cmH_2O). The following values were measured in the collected coronary effluents: coronary flow, released nitrites (NO production marker), superoxide anion radical and the index of lipid peroxidation (measured as thiobarbiturate reactive substances). The experimental protocol was performed under controlled conditions, followed by the administration of L-arginine alone (1 mmol) and L-arginine (1 mmol) + L-NAME (30 μmol). The results indicated that L-arginine did not significantly increase the coronary flow or the release of NO, TBARS and the superoxide anion radical. These effects were partially blocked by the joint administration of L-arginine + L-NAME, which indicated their competitive effect. Hence, the results of our study do not demonstrate significant effects of L-arginine administration on the coronary flow and oxidative stress markers in isolated rat hearts.

Key words: *L-arginine, L-NAME, redox status, isolated heart, rats*

SAŽETAK

Cilj ovog istraživanja je bio procena efekata akutne administracije L-arginina na koronarni protok i markere oksidacionog stresa, samostalno i/ili u prisustvu L-NAME (neselektivni inhibitor NO sintaze), na izolovanim srcima pacova. Ovo je eksperimentalna studija, koja je sprovedena na izolovanom srcu Vistar albino soja pacova (n = 12, muški, 8 nedelja, telesna masa 180-200g). Retrogradna perfuzija izolovanih organa se sprovodila modifikovanom tehnikom prema Langendorfu, sa postepenim povećanjem perfuzionog pritiska (40–120 cmH_2O). Nakon izmerenog koronarnog protoka, u prikupljenim uzorcima koronarnog efluenta mereni su sledeći parametri: nivoi azot monoksida (u formi nitrita), superoksid anjon radikala i indeksa lipidne peroksidacije (meren kao TBARS). Eksperimentalni protokol je sproveden pod strogo kontrolisanim uslovima, i podrazumeva administraciju samo L-arginina (1 mmol), i administraciju L-arginina (1 mmol) u kombinaciji sa L-NAME (30 μmol). Rezultati ovog istraživanja ukazuju na to da L-arginin neznatno povećava koronarni protok, neznatno povećava nivoe azot monoksida, TBARS-a i superoksid anjon radikala. Ovakav efekat je delimično blokiran u slučaju zajedničke administracije L-arginin+L NAME što ukazuje na njihovu kompetitivnost. Dakle, rezultati našeg istraživanja ne pokazuju statistički značajne efekte primene L-arginina na koronarni protok i markere oksidacionog stresa izolovanog srca pacova.

Ključne reči: *L-arginin, L-NAME, redoks status, izolovano srce, pacovi*

INTRODUCTION

L-arginine (2-amino-5-guanidinovaleric acid) is a basic, conditionally essential amino acid that enters an organism via the diet or is obtained by the degradation of body proteins or endogenous *de novo* synthesis (1). This semi-essential amino acid takes part in numerous key biochemical and physiological activities.

During the last decades of the 20th century, L-arginine was identified as a precursor of nitric oxide synthesis (NO) (2). Specifically, it represents the key source of NO synthase in many cells of an organism (3). NO is produced during the transformation of L-arginine to L-citrulline in a reaction catalysed by NO synthase (NOS) (4–7).

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The L-arginine/NO system is one of the crucial players in the maintenance of microvascular homeostasis. Additionally, NO causes vasodilatation, improves microcirculation by stimulating endothelial proliferation and angiogenesis, and inhibits endothelial apoptosis, the release of endothelin-1, the proliferation of smooth muscular cells and thrombocyte aggregation and adhesion (8).

Endothelial dysfunction is one of the earliest markers of vascular abnormality. It is present in cardiovascular diseases linked to the increased production of reactive oxygen species (ROS) or the state of oxidative stress (9, 10). Cell damage caused by ROS (the most significant among which are the superoxide anion radical and hydroxyl radical) is a significant causal factor of heart diseases, particularly those that present with myocardial ischemia-reperfusion damage (11). Many authors have demonstrated the production and release of free radicals in the ischemic heart, including their intensive release during the reperfusion period (12, 13, 14). The rapid recovery of blood flow increases tissue oxygenation with a consequential secondary production of ROS, leading to reperfusion injury (15). One of the possible mechanisms underlying ROS-mediated cardiovascular diseases is the reduced production of endothelium-dependent vasodilatory substances (16), of which NO is the most significant (17). Moreover, the L-arginine-dependent enzyme arginase is up-regulated in response to the reduction in NO bioavailability during oxidative stress (9).

Because NO is an endothelial-dependent relaxing factor that plays an essential role in the regulation of the vascular tonus and haemodynamics, there has been interest for decades in the application of L-arginine for the prevention and treatment of cardiovascular diseases (18). L-arginine appears to provide "hope" for the treatment of cardiovascular diseases. Based on results obtained to date, oral or parenteral administration of this amino acid seems to recuperate endothelial function and improve coronary microcirculation. L-arginine affects atherosclerotic risk factors (hypercholesterolemia, hypertension, and smoking) by improving endothelial functions in these patients (8).

However, the exact role of the L-arginine/NO system within the coronary circulation is still unknown due to reports of controversial data.

The aim of the present study was to examine the effects of L-arginine alone or in combination with a non-selective NOS inhibitor (N^G-nitro-L-arginine monomethyl ester, L-NAME) on the coronary flow, oxidative stress markers and nitrites in hearts isolated from rats.

MATERIAL AND METHODS

Preparation of isolated rat hearts

Isolated hearts (total number n=12, 6 preparations for each experimental group; rejected hearts did not contribute to the total number) were obtained from Wistar albino rats (male, 8 weeks old, body mass of 180 - 200 g; obtained from the VMA - Military Medical Academy, Belgrade, Serbia) and perfused with a modified apparatus according to the method of Langendorff (Hugo-Sachs Electronik-Harvard Apparatus GmbH, March- Hugstetten, Germany). The animals were euthanised by cervical dislocation following administration of a short ether anaesthetic with the anticoagulant heparin (Schedule 1 of the Animals/Scientific Procedures, Act 1986, United Kingdom). Following the emergency thoracotomy and the induction of heart failure via the superfusion of cold physiological dissolvent, the heart was quickly prepared and isolated by the removal of all redundant parts (with the exception of ascending aorta, which was cannulated to provide retrograde perfusion under gradually increasing coronary perfusion pressure (CPP)). Krebs-Henseleit buffer was used for retrograde perfusion (in mmol/l: NaCl 118, KCl 4.7, $CaCl_2$ x $2H_2O$ 2.5, $MgSO_4$ x $7H_20$ 1.7, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11, and pyruvate 2). The buffer was balanced with 95% O_2 and 5% CO_2, with a pH value of 7.4 and temperature of 37°C. In all preparations, an electrostimulator (Hugo-Sachs Electronik-Harvard Apparatus GmbH) ensured the heart rate and its regularity (5 V, 320 bpm) via electrodes set in the atrial region.

Physiological examination and experimental protocol

Following the establishment of heart perfusion, the preparations were stabilised within 30 minutes with a basal coronary perfusion pressure of 60 cmH_2O. During the stabilisation of the preparations, the reactivity of the coronary blood vessels was examined by short occlusion of coronary flow (5-30 s) and bolus injection of 5 mmol/l adenosine (60 µl at a flow rate of 10 ml/min to obtain the maximum flow). The preparations were rejected (approximately 25%) unless an increase in the flow of 100% was achieved compared to the control values for both tests. Following the stabilisation period, the perfusion pressure was reduced to 50 and 40 cmH_2O and then gradually increased to 70, 80, 90, 100, 110 and 120 cmH_2O to establish coronary autoregulation. At each given value of coronary perfusion pressure a value of flow was noted for at least 5 minutes. When the flow was determined to be stable, samples of coronary effluent were collected for each value of perfusion pressure. The correctly performed control experiment (control values in each experimental group) included the double examination of coronary perfusion pressure/coronary flow in the absence of any medication. The main goal was to confirm that the preparation was stable and that the response between the first and second series of changes in perfusion pressure were not significantly different. Following the control experimental protocol, the preparations were perfused with L-arginine (1 mmol) and L-arginine (1 mmol) plus an NO synthesis inhibitor (30 µl L-NAME). Testing started immediately after the control experiment to avoid unwanted time-de-

pendent consequences. The administration of medicines lasted until the achievement of a stable flow but not under 5 minutes for each value of perfusion pressure. The results obtained during the experimental protocol (coronary flow, superoxide anion radical concentration, released nitrites and index of lipid peroxidation) were compared to the results obtained after the administration of L-arginine and L-arginine + L-NAME.

Biochemical analysis

Samples of coronary venous effluent were collected after the stabilisation of the coronary flow for each value of the gradually increased perfusion pressure. We performed the spectrophotometric determination of nitrites, superoxide anion radicals and the index of lipid peroxidation indirectly via reactive thiobarbituric substances (TBARS) for all samples.

Determination of nitrites

Nitric oxide quickly decomposes into stable metabolite nitrites/nitrates. Nitrites are used as an index of NO production via a spectrophotometric method using the Griess reagent. Briefly, 0.5 ml of the perfusate is precipitated with 200 µl of 30% sulfosalicylic acid, mixed for 30 minutes and centrifuged at 3000 x g. Equal volumes of the supernatant and Griess reagent are mixed and stabilised for 10 minutes in the dark, and then the sample is measured spectrophotometrically at a wavelength of 543 nm. The nitrite concentrations are determined using sodium nitrite as the standard (19).

Determination of superoxide anion radicals

Superoxide anion radical concentrations are measured using the NTB (Nitro Blue Tetrazolium) reagent in TRIS buffer (assay mixture) with coronary venous effluent. The measurement was performed at a wavelength of 530 nm. The Krebs-Henseleit solvent was used as the blank control (20).

Determination of the index of lipid peroxidation (TBARS)

The index of lipid peroxidation was determined indirectly by measuring the products of the reaction of lipid peroxidation with thiobarbituric acid (TBARS or Thiobarbituric Acid Reactive Substances). Briefly, 1% thiobarbituric acid (TBA) in 0.05 M NaOH is incubated with coronary venous effluent at 100°C for 15 minutes and then spectrophotometrically measured at a wavelength of 530 nm. The Krebs-Henseleit solvent was used as the blank control (21).

Reagents

The L-arginine and L-NAME solvents were obtained as a gift from the Biomedical Sciences Department of the Academy of Sciences of Slovakia (Bratislava, Republic of Slovakia). A set of reagents for the spectrophotometric determination of nitrites (naphthyl ethylenediamine dihydrochloride and sulfosalicylic acid) were purchased from Sigma-Aldrich Chemie GmbH. Sulfanilamide, phosphorous acid, NTB, TRIS-puffer and TBA were purchased from Merck KGaA Company (Dermstadt, Germany).

Statistical analysis

Values were expressed as the arithmetic mean + S.E.M. A multifactorial analysis of variance with repeated measures was performed. In this model, different values of CPP were given as within-subject factors, whereas the application of a treatment was provided as a measurement of the difference between subjects. A p value less than 0.05 was considered statistically significant.

RESULTS

Coronary flow

The coronary flow exhibited a significant increase that was proportional to the coronary perfusion pressure over the whole range of perfusion pressure values studied in both the control and study groups. Under the control conditions, the coronary flow varied in the range from 3.00 ± 0.86 ml/min/g of tissue mass (wt) at 40 cmH_2O to 8.57 ± 1.77 ml/min/g wt at 120 cmH_2O. L-arginine did not induce a significant change in the coronary flow (range from 3.65 ± 1.02 at 40 cmH_2O to 10.93 ± 2.80 ml/min/g wt at 120 cmH_2O) (Fig. 1).

L-arginine + L-NAME did not induce a significant reduction in the coronary flow compared to the control group. Under the control conditions, the coronary flow varied in the range from 3.15 ± 0.66 ml/min/g wt at 40

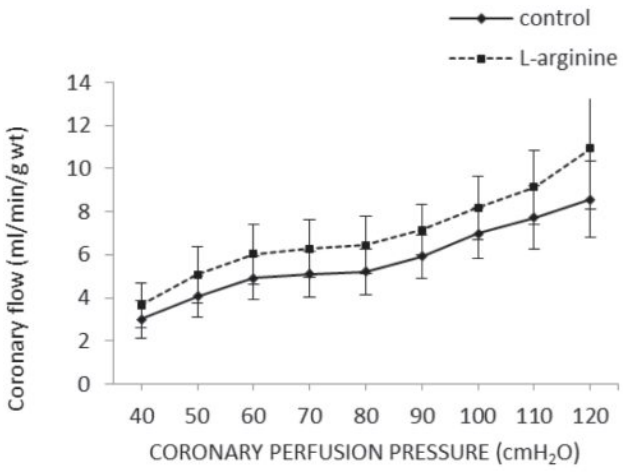

Figure 1. Effects of L-arginine (1 mmol) on the coronary flow at different coronary perfusion pressures (CPP). Each value represents the mean ± SE and is expressed relative to the control.
A p value < 0.05 was considered to be significant. *p < 0.05.

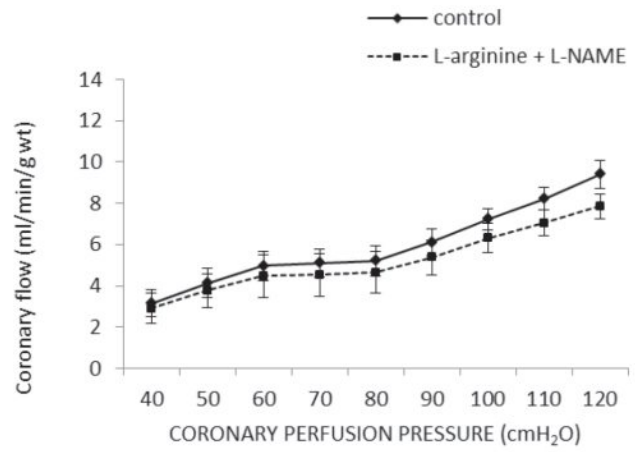

Figure 2. Effects of L-arginine + L-NAME (1 mmol + 30 µmol) on the coronary flow at different coronary perfusion pressures (CPP). Each value represents the mean ± SE and is expressed relative to the control. A *p* value < 0.05 was considered to be significant. *p < 0.05.

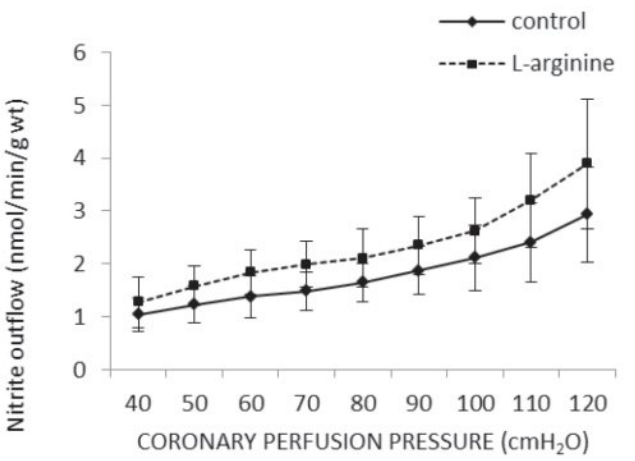

Figure 3. Effects of L-arginine (1 mmol) on the nitrite outflow at different coronary perfusion pressures (CPP). Each value represents the mean ± SE and is expressed relative to the control. A *p* value < 0.05 was considered to be significant. *p < 0.05.

cmH$_2$O to 9.40 ± 0.67 ml/min/g wt at 120 cmH$_2$O. In the treated group, the flow ranged from 2.90 ± 0.72 ml/min/g wt at 40 cmH$_2$O to 7.85 ± 0.60 ml/min/g wt at 120 cmH2$_O$ (Fig. 2).

Nitrite outflow

Under the control conditions, the nitrite outflow varied from 1.04 ± 0.32 nmol/min/g wt at 40 cmH2$_O$ to 2.93 ± 0.90 nmol/min/g wt at 120 cmH2$_O$. L-arginine did not induce a significant increase in the nitrite outflow (range from 1.28 ± 0.48 nmol/min/g wt at 40 cmH2$_O$ to 3.89 ± 1.23 nmol/min/g wt at 120 cmH2$_O$) (Fig. 3). Additionally, there was no significant difference between the groups in the dynamics of the increase in the nitrite outflow with increasing CPP.

L-arginine + L-NAME induced a significant decrease in the nitrite outflow compared to the control group. Under the control conditions, the values changed in the range from 1.00 ± 0.13 nmol/min/g wt at 40 cmH2$_O$ to 3.40 ± 1.50 nmol/min/g wt at 120 cmH2$_O$; in the treated group, the values changed from 0.86 ± 0.21 nmol/min/g wt at 40 cmH2$_O$ to 2.46 ± 0.58 nmol/min/g wt at 120 cmH2$_O$ (Fig. 4). The nitrite concentrations increased as the CPP increased in both groups.

Superoxide anion production

L-arginine did not induce significant changes in the superoxide anion radical (O_2^-) levels. However, a significant increase in O_2^- levels was noted in both groups as the CPP increased. Under the control conditions, O_2^- production

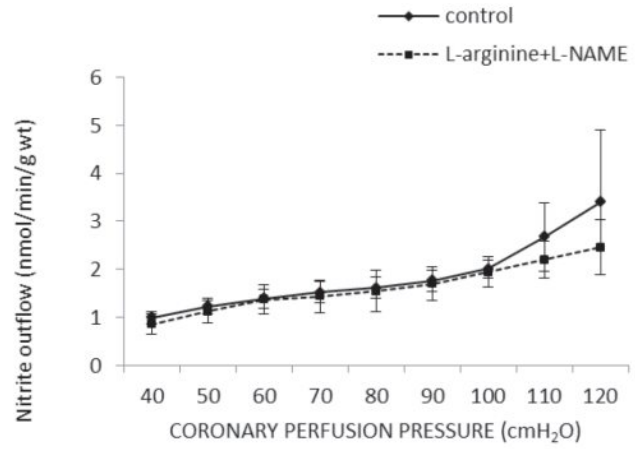

Figure 4. Effects of L-arginine + L-NAME (1 mmol + 30 µmol) on the nitrite outflow at different coronary perfusion pressures (CPP). Each value represents the mean ± SE and is expressed relative to the control. A *p* value < 0.05 was considered to be significant. *p < 0.05.

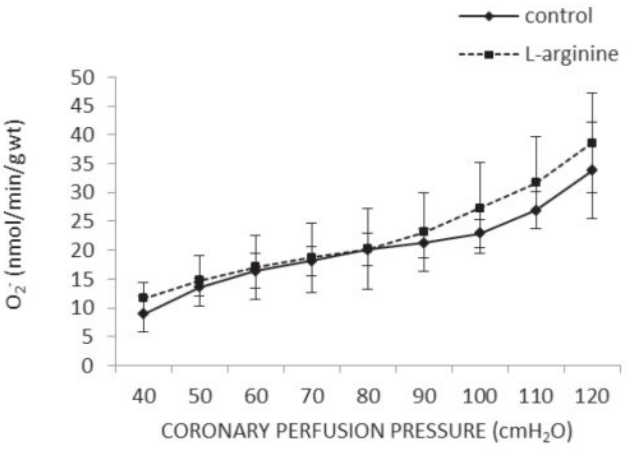

Figure 5. Effects of L-arginine (1 mmol) on superoxide anion production at different coronary perfusion pressures (CPP). Each value represents the mean ± SE and is expressed relative to the control. A *p* value < 0.05 was considered to be significant. *p < 0.05.

varied from 8.90 ± 3.09 nmol/min/g wt at 40 cmH2$_O$ to 33.79 ± 8.40 nmol/min/g wt at 120 cmH2$_O$; in the treated group, O_2^- production varied from 11.63 ± 2.67 nmol/min/g wt at 40 cmH2$_O$ to 38.61 ± 8.67 nmol/min/g wt at 120 cmH2$_O$ (Fig. 5).

L-arginine + L-NAME also did not significantly affect superoxide anion production compared with the control values (Fig. 6).

Index of lipid peroxidation (TBARS production)

Under the control conditions, the TBARS production varied from 0.51 ± 0.40 µmol/min/g wt at 40 cmH2$_O$ to 1.38 ± 0.69 µmol/min/g wt at 120 cmH2$_O$. L-arginine did not significantly affect the TBARS production at any CPP value (range from 0.57 ± 0.16 µmol/min/g wt at 40 cmH2$_O$ to 1.80 ± 0.88 µmol/min/g wt at 120 cmH2$_O$) (Fig. 7).

Conversely, L-arginine + L-NAME significantly decreased the TBARS production compared with the control values (decreased from 32.7% at 40 cmH2$_O$ to 50.2% at 120 cmH2$_O$) (Fig. 8). Indeed, the difference in TBARS production between the groups increased concomitantly with the CPP values.

DISCUSSION

The present study was performed to assess the intracoronary effects of the acute administration of L-arginine and L-arginine in combination with L-NAME (a non-specific NO synthase inhibitor) on isolated rat hearts under different coronary perfusion pressure conditions (40–120 cmH2$_O$). The results obtained under the control conditions were compared with those obtained after the administration of L-arginine and L-arginine + L-NAME. Variations in the tested parameters in different groups of animals under the control conditions were presented for the purpose of biological diversity.

Our results showed that the acute administration of L-arginine (compared to the control group for all values of applied CPP) did not significantly increase the coronary flow or any of the estimated oxidative stress parameters (NO, O_2^-, and TBARS) (Figs. 3-8).

The administration of L-arginine + L-NAME did not significantly reduce the coronary flow compared to the control group, although the differences were more evident at the higher CPP values (CPP 90–120 cmH2$_O$, which were out of the autoregulatory range). Moreover, the NO and O_2^- concentrations were not significantly reduced compared to the control group. However, L-arginine + L-NAME significantly reduced the TBARS value, especially at higher CPP values (CPP 90–120 cmH_2O).

The L-arginine/NO system plays an important role in the control of the basal tonus of coronary blood vessels and is involved in the coronary autoregulation of the

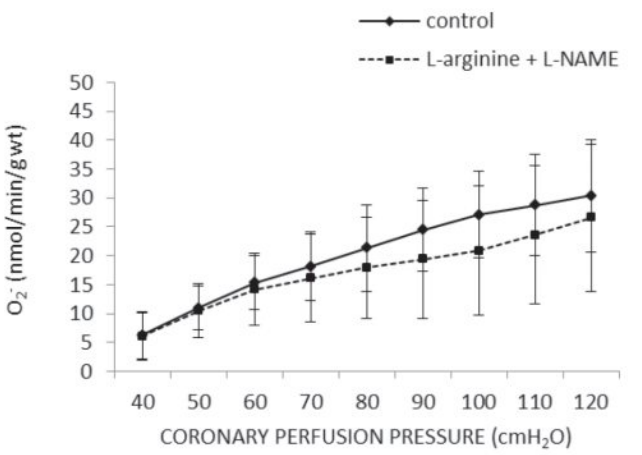

Figure 6. Effects of L-arginine + L-NAME (1 mmol + 30 µmol) on superoxide anion production at different coronary perfusion pressures (CPP). Each value represents the mean ± SE and is expressed relative to the control. A *p* value < 0.05 was considered to be significant. *p < 0.05.

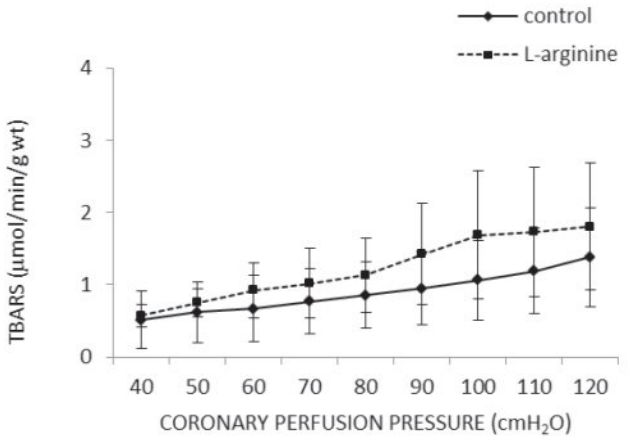

Figure 7. Effects of L-arginine (1 mmol) on TBARS production at different coronary perfusion pressures (CPP). Each value represents the mean ± SE and is expressed relative to the control. A *p* value < 0.05 was considered to be significant. *p < 0.05.

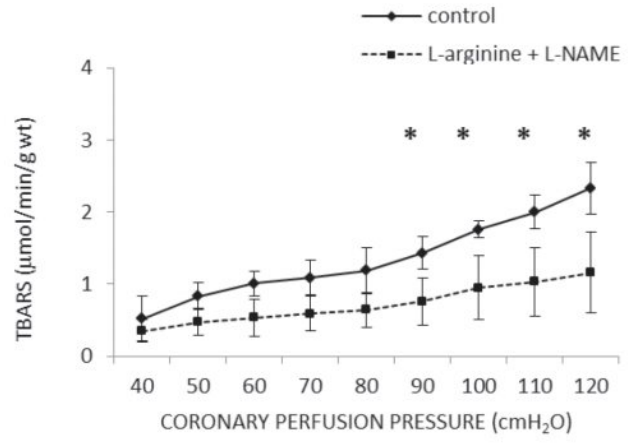

Figure 8. Effects of L-arginine + L-NAME (1 mmol + 30 µmol) on TBARS production at different coronary perfusion pressures (CPP). Each value represents the mean ± SE and is expressed relative to the control. A *p* value < 0.05 was considered to be significant. *p < 0.05.

isolated rat heart. The isolated rat hearts exhibited autoregulation of the coronary flow between 50 and 80 cm$H2_O$ of coronary perfusion pressure. Below the autoregulatory range the coronary flow slowly went down and above range the value more than doubled (22), which was in line with our results.

Our experimental data showed that the acute exogenous entry of arginine might increase the production of NO and NO-mediated vasodilatation despite the fact that the intracellular concentration of arginine was far beyond the *Km (Michaelis-Menten constant)* for eNOS. The Km for L-arginine is 2.9 μmol/l (8, 23–27). The intracellular concentration of arginine in endothelial cells is 0.8-2 mmol/l (8, 27), which suggests that the available intracellular L-arginine is more than sufficient for NO production. Based on this observation, intracellular arginine can provide full saturation of eNOS; thus, the endothelial production of NO should not depend on the extracellular concentration of L-arginine. However, the NO production increases in a dose-dependent manner when the concentration of L-arginine increases in endothelial cell cultures (28). Moreover, the increase in the plasmatic value of L-arginine is connected with the increase in vascular NO production. This biochemical phenomenon (or discrepancy) is designated the "arginine paradox". Theories explaining the arginine paradox include the low basal values of L-arginine in diseased states such as hypertension and hypercholesterolemia, intracellular variations in the concentrations of L-arginine or the potential presence of enzyme inhibitors. Identified competitive inhibitors of NOS include N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine (L-NNA), N^G-nitro-L-arginine monomethyl ester (L-NAME) and asymmetric dimethylarginine (ADMA).

Extracellular L-arginine appears to play a significant role in NO synthesis through membrane-linked eNOS. The constitutive transport system that facilitates the entry of arginine into endothelial cells is a cation amino acid transporter (CAT-1). CAT-1 and eNOS are physically connected in the caveolae in endothelial cells (29), which suggests the existence of a direct supply of extracellular arginine to eNOS.

Whether extracellular L-arginine changes the rate of arginine transport to the cell and contributes to the improvement in NO synthesis or whether the intracellular concentration in the microdomains of a cell plays a more important role in the modulation of NO synthesis are unknown (30).

The vascular endothelium plays an important role in vascular physiology. Attention has especially been focused on the endothelial production of NO (endogenous messenger molecules), including the different endothelial-mediated physiological effects in the vascular system. Because endothelial dysfunction is the basis of numerous diseases (atherosclerosis, hypertension, and diabetes mellitus) and is linked with the reduced production of endothelial NO, supplementation with L-arginine (donor NO) could be considered as therapeutic approach to these diseases. Therefore, many researchers are interested in the therapeutic possibilities of L-arginine, including whether supplementation with L-arginine can increase NO production and thereby improve vascular health. The effects of the oral or parenteral administration of L-arginine on vascular health and diseases have been examined in both human and animal models.

Böger et al (31) studied the clinical pharmacology of L-arginine and concluded that the response of the organism to the administration of L-arginine depended on the specific characteristics of the cardiovascular disease, vascular segments and morphology of the arteries of the examinee. Undesired effects of L-arginine administration are rare and are mainly mild and dose-dependent. The results obtained from a number of animal studies (animal models with damaged endothelial-dependent NO biological functions, including hypercholesterolemic rabbits, hypertensive rats, and hyperlipidemic monkeys) suggested that the administration of L-arginine *in vivo* improved vascular health by increasing NO production. Both acute and chronic administration of L-arginine improved endothelial-dependent vasodilatation, whereas chronic administration also modulated other NO-dependent vascular functions, such as the reduction of leukocyte adhesion, inhibition of thrombocyte aggregation and proliferation of smooth muscular cells.

In their review paper, Preli et al (27) summed up the results of studies (animal and human) involving the oral supplementation of L-arginine on the formation of atherosclerotic lesions. The results from hypercholesterolemic animals generally showed beneficial effects. L-arginine appeared to inhibit the progression of atherosclerotic plaques and protect endothelial functions. Moreover, L-arginine affected other mediators of atherosclerosis, including circulating inflammatory cells and thrombocytes. In contrast to the positive results obtained in the animal studies, differences were observed in the human studies.

Some previous experimental and clinical studies indicated that L-arginine could improve the antioxidant status (32–35). L-arginine was reported to act as a free radical scavenger, inhibit the activity of pro-oxidant enzymes and thus act as an antioxidant; these roles of L-arginine were mediated by NO. Tripathi et al (32) indicated that oral supplementation with L-arginine (3 g/day for 7 days) in ischemic patients increased the superoxide dismutase (SOD) level, total thiols (T-SH) and the plasma ascorbate levels, but these increases were not significant. This study demonstrates that L-arginine administration may be beneficial for patients with myocardial ischemic disorders, such as acute myocardial infarction and acute angina. Huang et al (36) suggested that L-arginine supplementation reduced the oxidative damage and inflammatory response of skeletal muscles, liver and kidneys caused by exhaustive exercise in young rats. The rats were fed with 2% L-

arginine diet for 30 days, and this supplementation increased the antioxidant enzyme level although the increase was not significant. In the study by Lucotti et al (33), oral supplementation with L-arginine (8.3 g/day) concurrent with a weight loss diet for 21 days increased the SOD levels in obese, insulin-resistant type 2 diabetic patients. The use of different doses and weight loss diets combined with L-arginine supplementation may explain the different results. Similarly, Jabeca et al (37) reported that the oral administration of L-arginine (2 g/day for 28 days) significantly increased the TAS (total antioxidant status) level in the plasma from patients with mild hypertension. This study confirms the hypothesis that augmented concentrations of L-arginine stimulate NO biosynthesis, which leads to a reduction in oxidative stress.

The results of our study clearly show a non-significant effect of L-arginine on the coronary flow and oxidative stress markers in isolated rat hearts. However, research interested in the application of L-arginine for the treatment of cardiovascular diseases should be continued. Long-term random clinical studies are necessary (27, 31) to obtain "broad and clear" scientific knowledge in the field.

Acknowledgments

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Conflicts of interest

The authors declare no conflict of interest.

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EFFECTS OF DIFFERENT PUFA SUPPLEMENTATION ON INFLAMMATORY RESPONSE MARKERS IN YOUNG SOCCER PLAYERS

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EVALUACIJA EFEKATA RAZLICITIH REZIMA ISHRANE NA INFLAMATORNI ODGOVOR KOD MLADIH FUDBALERA

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ABSTRACT

Considering the limited knowledge regarding the effects of n-3 and n-6 PUFAs on the inflammatory response during physical activity, we aimed to evaluate the level of pro- and anti-inflammatory cytokines in young soccer players before and after a maximal physical load test at the beginning and end of a two-month training process. The study included 75 young footballers from Football School "Kragujevac," who were followed during the two-month training programme. The subjects were divided into the following groups: 1) control group (consumed a standard diet); 2) group that consumed fish oil (2500 mg of n-3 PUFAs per day); 3) group that consumed nutritional sunflower oil (2500 mg of n-6 PUFAs daily). The maximal progressive exercise test was performed using a treadmill belt. Venous blood samples were drawn 4 times for the determination of cytokine levels (IL-6 and TNF-α): before and after the exercise load test before the two-month training programme (initial measurement) and immediately before and after the exercise load test after the two-month training programme (control measurement). Supplementation with fish oil (n-3) has been associated with reduced levels of IL-6 compared with the initial values. After an acute bout of exercise, n-3 PUFAs did not show a significant effect on inflammatory marker dynamics, whereas n-6 PUFAs slightly stimulated the production of TNF-α.

Key words: *polyunsaturated fatty acids, cytokines, inflammation, young soccer players.*

SAŽETAK

S` obzirom na ograničeno znanje o efektima polinezasićenih masnih kiselina (n-3 i n-6 PUFA-Polyunsaturated Fatty Acids), cilj ovog istraživanja bio je da se procene vrednosti pro- i anti- inflamatornih medijatora u miru i nakon maksimalnog testa opterećenja pre i nakon dvomesečnog trenažnog programa. U studiji je učestvovalo 75 mladih fudbalera omladinske fudbalske škole "Kragujevac", starosti od 18 - 19 godina, koji su bili praćeni tokom dva meseca. Oni su bili podeljeni u tri grupe: 1. kontrolna grupa (standardna ishrana), 2. grupa koja je konzumirala riblje ulje (2500mg n-3 PUFA dnevno), 3. grupa koja je konzumirala suncokretovo ulje (2500mg n-6 PUFA dnevno). Venski uzorci krvi za određivanje nivoa citokina (IL-6, TNF- α) uzimani su 4 puta: u miru i nakon testa opterećenja, pre i posle dvomesečnog trenažnog programa. Svakodnevna suplementacija ribljim uljem povezana je sa smanjenim vrednostima IL-6 u odnosu na njegove početne vrednosti. Upotreba različitih vrsta polinezasiđenih masnih kiselina nije bitnije uticala na proizvodnju citokina, neposredno nakon akutnog nastupa sportske aktivnosti (testa opterećenja).

Ključne reči: *polinezasićene masne kiseline, citokini, upala, mladi fudbaleri*

ABBREVIATIONS

ALA - α-linolenic acid
EPA - eicosapentaenoic acid
DHA - docosahexaenoic acid
IL-6 - interleukin-6
LA - linoleic acid
LTE4 - leukotriene E4
PGE2 - prostaglandin E2
PUFA - polyunsaturated fatty acid
TNF-α - tumour necrosis factor alpha
TXB2- thromboxane B2

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INTRODUCTION

Although physical activity has numerous beneficial effects on health and especially cardiovascular diseases (1, 2), in recent years, there has been growing concern about the molecular basis of its deleterious impact. Namely, intense and prolonged exercise has been associated with the onset of a condition known as "calm" inflammation, primarily of muscle tissue (3, 4). This finding is based on strenuous exercise provoking the inflammation of muscle fibres, leading to damage, fatigue and a reduction in muscle performance (3-5).

Most of the inflammatory reactions are mediated by inflammatory cytokines. Therefore, to assess muscle inflammation, the greatest attention has been paid to particularly two cytokines: interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) (6). These cytokines produce mainly inflammatory cells, such as T lymphocytes and monocytes (6). The physiological roles of these cytokines are numerous. It has been shown that IL-6 is one of the most potent stimulators of prostaglandin E2 (PGE2) production, which is directly involved in the progress of inflammatory reactions (6). However, according to the latest findings, IL-6, depending on the situation, can act as both a pro- or as an anti-inflammatory cytokine (7). By contrast, TNF-α, possesses clear pro-inflammatory potential, promoting an inflammatory response that includes muscle damage (7).

Given that polyunsaturated fatty acids (PUFAs) are essential for the overall health of athletes. In the past decade, studies have investigated the effect of PUFAs on the degree of inflammation and muscle damage. There are two classes of polyunsaturated fatty acids (PUFAs), n-6 (omega 6 fatty acids) and n-3 (omega 3 fatty acids) (8). The main representative of the group of omega-6 PUFAs is linoleic acid (LA), and that of the group of omega-3 PUFAs is α-linolenic acid (ALA) (9). Although it is known that these two types of fatty acids have opposite effects in the body (10), data regarding the specific role of n-3 or n-6 inflammatory processes are poor and controversial.

There is evidence that fish oil, which contains a high concentration of EPA eicosapentaenoic acid (EPA, n-3) and docosahexaenoic acid (DHA, n-3), can be useful in preventing or mitigating the symptoms of muscle inflammation that often occur with intense physical activity (11). The literature data show that supplementation with n-3 fatty acids indicate arterial vasodilatation, whereas n-6 fatty acids do not lead to these effects (12). Investigations on animal models have shown that fish oil may reduce the initial production of cytokines after physical activities (13). Research in the human population have confirmed this hypothesis, pointing out that supplementation with n-3 fats leads to a reduced production of IL-6 and TNF-α in healthy volunteers (14).

Considering the limited knowledge regarding the effects of n-3 and n-6 PUFAs on the inflammatory response during physical activity, the aim of this study was to evaluate the level of pro- and anti-inflammatory cytokines in young athletes before and after a maximal physical load test at the beginning and end of a two-month training process.

MATERIALS AND METHODS

Subjects

The study included 75 young soccer players from Football School "Kragujevac". All of the players were male, aged 18-19 years, well trained with a minimum sports experience of 5 years and 12 hours of training a week.

Study design

All of the participants were followed during the two-month training programme. Before the start of the study, all the players were subjected to a standard sports medical examination. The study involved only subjects who were absolutely healthy without a history of disease, any special eating habits or the use of any medications and supplements. Participants were not included in the study if routine laboratory analyses showed that they have indications of acute inflammatory processes (increased white cell counts, sedimentation, and C-reactive protein levels). The study was approved by the ethics committee of the Faculty of Medical Sciences, University of Kragujevac.

The subjects were divided into the following groups:

1. a group that, during the two-month training program, consumed a standard diet—the control group (n = 25),
2. a group that, during a two-month training program, consumed fish oil (2500 mg of n-3 PUFAs per day)—the n-3 PUFA group (n = 25),
3. a group that, during the two-month training program, consumed nutrient-rich, cold-pressed sunflower oil (2500 mg of n-6 PUFA daily)—the n-6 PUFA group (n = 25).

All of the subjects were informed about the nature, purpose, duration, expected effects and risks of research, and they and their parents provided written consent for participation in the study.

Protocol

Testing was carried out during the regular sports and medical health examination. Each participant was subjected to the measurement of body composition and the progressive, continuous maximum physical load test. The measurement of body composition was performed using an apparatus for bioelectrical impedance analysis, the Tanita BC-418 system (Tokyo, Japan), whose validity was previously confirmed (15). The maximal progressive exercise test was performed on a treadmill belt (treadmill ECG9230K; POWERJOG, Japan). The athletes were familiarized to the testing procedure. The test was performed according to the protocol by Ellestad (16) and lasted until

voluntary exhaustion. The athletes stated their subjective feeling of exhaustion using a Borg's CR10 exhaustion scale of at least 8 (17). During the test, athletes breathed through a two-way mouthpiece (Hans Rudolph, Kansas City, USA). Maximal oxygen consumption (VO_2max) and the heart rate were monitored by an automated cardiopulmonary exercise system (FitMate Pro, COSMED, Italy) whose validity, reliability, and accuracy were previously reported (18). We considered that VO_2max was reached when the oxygen consumption reached its plateau (when an increase in the workload cannot induce an increase in oxygen consumption) (18).

At the same time, venous blood samples were drawn at rest and immediately after the load to analyse the parameters of inflammation such as tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6). At the end of the two-month training programme and diet supplementation, all of the participants were subjected to an identical sports medical examination as the initial examination. The study was conducted during the competition mesocycle (Table 1).

Biochemical assays

Venous blood samples, for the analysis of biochemical parameters, were drawn before and immediately after the test load. The venous blood samples (9 ml) were drawn 4 times: before and after the exercise load test before the two-month training programme (initial measurement) and immediately before and after the exercise load test after the two-month training programme (control measurement). Blood samples were drawn from antecubital veins into Vacutainer test tubes containing sodium citrate anticoagulant. The blood samples were processed and stored immediately. Blood was centrifuged to separate plasma and red blood cells (RBCs).

Measurement of the serum levels of cytokines (TNF-α and IL-6)

The cytokine concentrations in the serum were determined using ELISA assays specific for human cytokines (Human IL-6 DUOSET ELISA Development kit, R & D Systems, USA; Human TNF-α/TNFSF1A DUOSET ELISA Development kit, R & D Systems, USA) (19, 20).

Table 1: Example of a microcycle when the match is played on Saturdays

Day	Training	Duration
Monday	REST DAY	
Tuesday	High-Intensity Training	90 min
Wednesday	High-Intensity Training	90 min
Thursday	Medium-Intensity Training	75 min
Friday	Medium-Intensity Training	60 min
Saturday	MATCH	
Sunday	Players who played: Regeneration Training	45 min
	Players who did not play: High-Intensity Training	75-90 min

The standards were dissolved before use in phosphate-buffered saline (PBS) (pH 7.2) until an initial concentration of 2,000 pg/ml. Stock solutions were serially diluted 7 times in a solvent twice (Eng. Reagent Diluent-in (1% BSA in PBS) to obtain a standard curve of 7 points. Next, 100 µl of capture antibody was added to the wells of microtitre plates (MTPs). The plates were taped with ELISA plate sealers and left overnight at room temperature, after which the wells were washed with wash buffer in an automatic washing machine for MTF. In all of the wells, block buffer was added to a final volume of 300 µl, and the MTPs were incubated for a minimum of 1 hour at room temperature. Then, the MTPs were washed with wash buffer. All of the samples were previously diluted 10 times in deionized water. The diluted samples and standards were added to the wells of the MTPs, which were covered with plate sealers and allowed to incubate for 2 hours at room temperature. After the incubation and washing of the MTPs, 100 µl of detection antibody was added to each well, and the plates were again covered with plate sealers and incubated for 2 hours at room temperature. MTPs were washed again, and 100 µl of streptavidin horseradish peroxidase was added to all of the wells. Incubation at room temperature was stopped after 20 min by washing the MTPs. In the next step, 100 µl of substrate solution (Colour reagent A + Colour reagent B, 1:1) was added. After 20 min, 50 ml of stop solution (2 N H_2SO_4) was added, and the optical density was directly measured in each well using a Microplate Reader (ZENYTH, Anthos, UK) set at 450 nm (19, 20).

All of the measured values were reduced for the absorbance values of the blanks (deionized water). A standard curve was made based on the measured values of the standards, and it was calculated as values for each individual sample. All of the samples were measured in triplicate.

Biochemical analyses of cytokine concentrations were carried out at the Laboratory of Immunology, Faculty of Medical Sciences in Kragujevac. The measurements were performed on the apparatus ZENYTH, Anthos, United Kingdom.

Statistical Analysis

Statistical analysis was performed using SPSS 20.0 for Windows. The results are expressed as the means ± standard deviation of the mean (SD). Data distribution was checked using the Kolmogorov-Smirnov or Shapiro-Wilk test and, depending on the results, the appropriate parametric or nonparametric test was used. The differences between the values of the means from two related samples were assessed by paired t-test or Wilcoxon's test. The alpha level for significance was set at $P<0.05$.

RESULTS

The levels of IL-6 in the blood of the subjects from different experimental groups at the initial (before two-

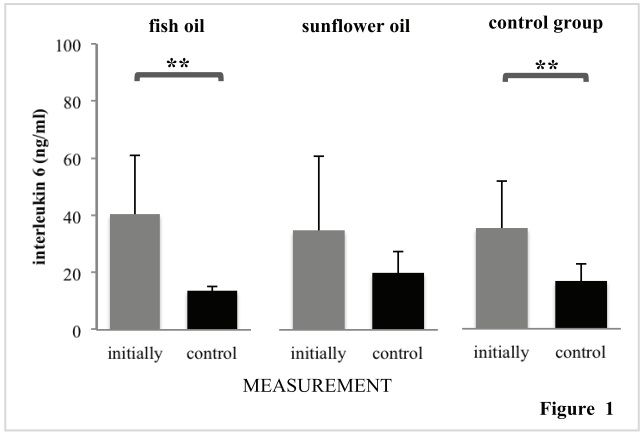

Figure 1. Level of IL-6 in the blood of subjects from different experimental groups at the initial and control measurements. The values are expressed as X ± SE; *p<0.05, **p<0.01.

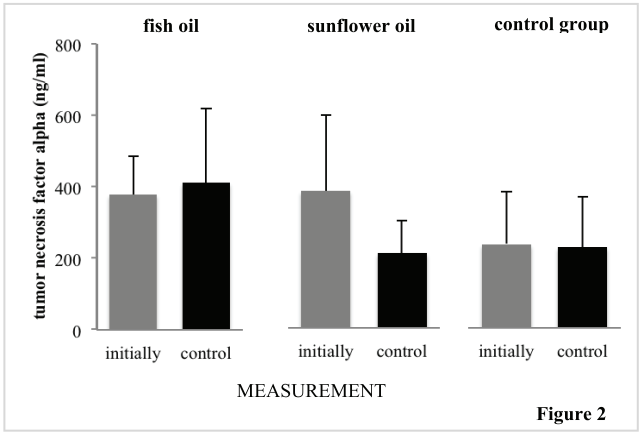

Figure 2. Level of TNF-α in the blood of subjects from different experimental groups at the initial and control measurements. The values are expressed as X ± SE; *p<0.05, **p<0.01.

month follow up period) and control measurements (after the two-month follow-up period) are shown in Figure 1. There was a significant decrease in the levels of IL-6 in the group that consumed fish oil (p<0.01) and in the control group (p<0.01). However, this reduction was more prominent in the group with fish oil supplementation (Fig 1).

The levels of TNF-α in the blood of the subjects from different experimental groups at the initial and control measurements are shown in Figure 2. None of the groups differed in the levels of this cytokine after the two-month follow-up period.

Changes in the levels of IL-6 in the blood of subjects from different experimental groups induced by physical load test (PLT) are presented in Figure 3. The levels of this inflammatory factor were not statistically changed in any group, after the physical load test, at the initial or control measurement (Fig 3).

Changes in the levels of TNF-α in the blood of subjects from different experimental groups as measured by the physical load test (PLT) are presented in Figure 4. The values of TNF-α were significantly increased after the physical load test in the control measurement in subjects who consumed sunflower oil (p<0.05) (Fig 4).

DISCUSSION

The concept of "silent inflammation" in sports has been established to note the potential dangers of an incorrectly dosed training process and overtraining that could lead to muscle damage and reduction in sports performance (21, 22). A key role in intercellular communication during the development and progression of inflammation is attributed to cytokines (23). Although some studies have suggested that TNF-α and IL-6 have pro-inflammatory effects (24, 25), others have emphasized their opposite character (26). There are several possible explanations for the different actions of these cytokines during physical activity (27). First, the type of physical activity, as well as the intensity and duration of exercise, can extremely affect the cytokine profile. Northoff and Berg were the first who noticed that IL-6 might be involved in the acute phase of inflammation after exercise (28-30).

On the other hand, the number of studies that have examined the impact of physical activity on the production of cytokines (inflammation) in young athletes-adolescents is negligible. Most children with a sports experience duration of more than five years may represent a potential critical group for the development of inflammation and its consequences for health.

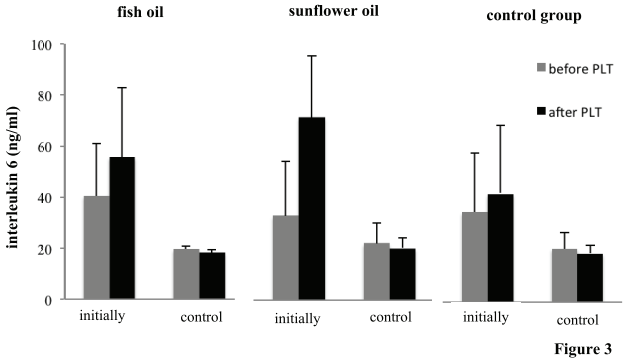

Figure 3. Levels of IL-6 in the blood of subjects from different experimental groups according to the physical load test (PLT). The values are expressed as X ± SE; *p<0.05, **p<0.01.

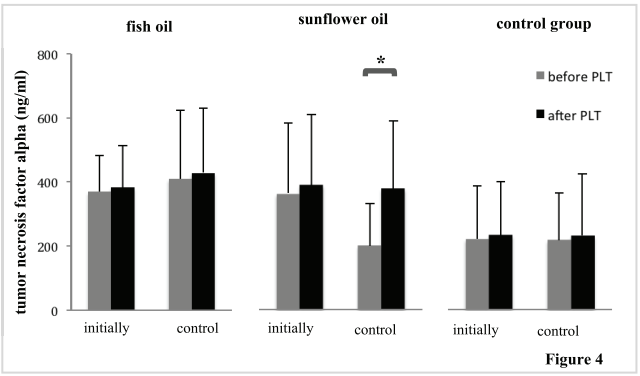

Figure 4. Levels of TNF-α in the blood of subjects from different experimental groups according to the physical load test (PLT). The values are expressed as X ± SE; *p<0.05, **p<0.01.

Additionally, supplementation of omega-3 and omega-6 fatty acids has become a trend only in recent years, and there are almost no studies on the effect of fish or sunflower oil on the production of inflammatory molecules. Because of this, the leading hypothesis of the present study was related to the potential mutual positive effect of a properly dosed training process and PUFA supplementation on the markers of inflammation in young athletes.

Indeed, after a two-month experimental period, the group that consumed fish oil had a significantly reduced level of IL-6 (Fig 1) compared with the initial measurement. Although, in the control group, we also found a decrease in the values of these cytokines, the decrease was greater in the athletes on fish oil supplementation (Fig 1). By contrast, the levels of TNF-α were not significantly changed between the groups (Fig 2). These results are in correlation with previously studies (28-30) and also show that IL-6 is probably the most sensitive marker of silent inflammation in athletes. These results also suggest that fish oil presumably has positive effects on inflammation by decreasing the levels of IL-6.

Only a few studies have examined the effects of PUFAs (mainly fish oil) on the production of cytokines. Using an animal model in rats, Robinson and Field noted that n-3 PUFAs have no significant effect on the activity of inflammatory cells in rats subjected to physical exercise (31). Tartibian and coworkers recorded that fish oil supplementation in recreational athletes prevented the occurrence of muscle fatigue and muscle damage probably by reducing the production of inflammatory cytokines (32). Mickleborough and colleagues have shown that n-3 PUFAs may reduce bronchoconstriction, which often occurs as a result of hard training in elite athletes (marathoners, triathletes), because of the increased generation of cytokines (33).

Supplementation with n-3 PUFAs (EPA and DHA), which are found in fish oils, leads to an increase in the concentration of these fatty acids in lipid membranes of inflammatory cells, thereby replacing part of the AA (34). Less substrate for the synthesis of eicosanoids from AA can result from the impaired production of its pro-inflammatory molecules: prostaglandins (PGE2) (35), thromboxane B2 (TXB2) (36), and leukotrienes (LTE4) (37). Fish oil reduces the production of cytokines from monocytes or endothelial cells also. (38). Because PGE2 and LTE4 are involved in the control of cytokine synthesis (IL-6 and TNF-α), it is assumed that one possible mechanism by which EPA and DHA reduce the production of IL-6 is related to the potential interactions of n-3 PUFAs with PGE2 and leukotrienes (39).

In our study, only the group of subjects that consumed sunflower oil experienced a significant increase in the level of TNF-α after the physical load test (Figure 4). The other experimental groups did not differ in the levels of cytokines before or after exercise load, even at the initial or control measurement (Figs 3, 4). Based on these findings, it seems that the effects of PUFAs on cytokine production after an acute bout of exercise depend on the PUFA type. Although n-3 PUFAs did not significantly affect the dynamics of the estimated inflammatory markers, n-6 PUFAs slightly stimulated it (Fig 4). On the other hand, as noted earlier, the length and type of physical activity can strongly affect the level of cytokines. Therefore, studies with protocols in which the length of the exercise test lasted longer than in our study have recorded an increase in the value of this cytokine (40, 41).

Previous studies have shown that an acute bout of strenuous exercise may lead to increased production of IL-6, which, in turn, intensifies the damage of muscle fibres (the release of creatine kinase (CK)) (40). Tofta et al and associates examined the impact of supplementation with fish oil on the cytokine synthesis in athletes for 6 weeks (42). The study was performed on marathon runners, and blood samples from the antecubital vein were collected the week before and immediately after the end of the marathon race. The results showed that the use of fish oil during this period did not induce significant changes in the production of IL-6 and TNF-α in response to the acute stress test.

Most of the research regarding the effects of n-6 fatty acids on inflammation markers were conducted in animals. For twelve weeks, Bhattacharya and colleagues treated mice with corn oil and safflower oil, which (similar to sunflower oil) contains a high proportion of LA. During the same experimental period, the animals were subjected to the treadmill test and determination of TNF-α. The results showed that the group treated with safflower oil had increased levels of TNF-α in response to the stress test (43).

One of the possible explanations for this result is that n-6 fatty acid chains, in response to the rapid onset of exercise load, can enter into potential interactions with the lipid membranes of macrophages, which normally generate TNF-α, thus potentiating its increased production (44). Nevertheless, to confirm these assumptions, more complex studies are needed that could combine the functional, histological and immunohistochemical analyses of muscles and other tissues.

CONCLUSION

Supplementation with fish oil (n-3) has been associated with reduced levels of IL-6 compared with the initial values. This oil can have a beneficial effect in preventing the acute phase of inflammation that occurs in young athletes. In this study design, the effects of PUFAs on cytokine production after an acute bout of exercise depend on the PUFA type. Although n-3 PUFAs did not have a significant effect on inflammatory marker dynamics, n-6 PUFAs slightly stimulated the production of TNF-α. This result could be of interest in determining an algorithm of PUFA supplementation in athletes in the training process or during the competitive season.

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Conflict of Interest

All of the authors declare no conflict of interest.

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EXPERIENCE OF OCULAR SYMPTOMS AMONG ALLERGIC RHINITIS PATIENTS DEPENDING ON THE TYPE OF AEROALLERGENS

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ISPOLJAVANJE OKULARNIH SIMPTOMA U ZAVISNOSTI OD TIPOVA INHALATORNIH ALERGENA

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REMARK

Part of this study was presented as an e-poster at the 2nd Meeting of the European Academy of ORL-HNS and CE ORL-HNS held in Nice, France, from 27-30 April 2013, titled "Allergic rhinitis patients and existence of ocular symptoms at ENT department of Health Centre Kragujevac".

ABSTRACT

The aim of this study was to determine the frequency of ocular symptoms and compare the demographic and clinical characteristics in AR patients depending on sensitisation to various types of aeroallergens.

Allergic rhinitis is defined as an IgE-mediated inflammation of the lining of the nose that is characterized by nasal symptoms, including nasal congestion, sneezing, itching of nose and runny nose. Patients suffering from allergic rhinitis frequently experience ocular symptoms such as ocular redness, eye itching and tears. The frequency of ocular symptoms in our study population was 27,6%. No statistical significance was found in the mean ages of the patients who did or did not experience ocular symptoms p>0,05 (p=0,243). Our results indicated that there were no statistical differences (p>0,05) among the groups of allergic rhinitis patients based on experiencing nasal symptoms according to the types of aeroallergens. Our results indicated that there were significant experiences of ocular symptoms in patients who were sensitised to outdoor aeroallergens (p<0,001) and significant sensitisation to both outdoor and indoor aeroallergens (p<0,05). Experiencing the examined ocular symptoms, including ocular redness, eye itching and tears, demonstrated highly statistical significance (p<0,001) among the groups of allergic rhinitis patients who were sensitised to indoor aeroallergens and outdoor aeroallergens, and there was statistical significance (p<0,05) among the groups of allergic rhinitis patients who were sensitised to indoor aeroallergens and both types of aeroallergens (indoor and outdoor).

Ocular symptoms are more common in patients who are sensitised to outdoor aeroallergens.

Keywords: *Allergy, rhinitis, ocular symptoms, aeroallergens.*

SAŽETAK

Cilj našeg istraživanja bio je da se utvrdi zastupljenost okularnih simptoma i korelacija u ispoljavanju okularnih simptoma kod obolelih od alergijskog rinitisa u zavisnosti od senzibilizacije na alergene spoljašnje, unutrašnje sredine ili obe grupe inhalatornih alergena.

Alergijski rinitis je IgE posredovana inflamacija sluzokože nosa koga karakterišu nazalni simptomili: kongestija, kijanje, svrab i curenje nosa. Oboleli od alergijskog rinitisa često ispoljavaju i okularne simptome: crvenilo očiju, svrab i suzenje. U ispitivanoj populaciji prisustvo okularnih simptoma uočeno je kod 26,27% obolelih od alergijskog rinitisa. Ispoljavanje okularnih simptoma nije povezano sa starošću ispitanika p>0,05 (p=0,243). Naši rezultati ukazuju da ne postoji statistički značajna razlika izmedju ispitanika koji su senzibilisani na inhalatorne alergene spoljašnje, unutrašnje sredine i senzibilisanih na oba tipa inhalatornih alergena u ispoljavanju svih nazalnih simptoma (p>0,05). Ispoljavanje okularnih simptoma je statistički visoko značajno u grupi ispitanika senzibilisanih na inhalatorne alergene spoljašnje sredine (p<0,001) i statistički značajno kod senzibilisanih na oba tipa inhalatornih alergena (p<0,05). Kod naših ispitanika u pogledu ispiljavanja crvenila, svraba očiju i suzenja postoji visoko statistički značajna razlika medju senzibilisanima na inhalatorne alergene unutrašnje i spoljašnje sredine (p<0,001) i statistički značajna razlika medju senzibilisanma na inhalatorne alergene unutrašnje sredine i na obe vrste inhalatornih alergena (p<0,05).

Ispoljavanje okularni simptomi kod obolelih od alergijskog rinitisa je u neposrednoj povezanosti sa senzibilizacijom na inhalatorne alergene.

Ključne reči: *Alergija, rinitis, okularni simptomi, inhalatorni alergeni.*

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ABBREVIATIONS

AR- allergic rhinitis
OS- ocular symptoms
SIA- sensitised to indoor aeroallergens
SMA- sensitised to both outdoor and indoor aeroallergens
SOA- sensitised to outdoor aeroallergens

INTRODUCTION

Allergic rhinitis (AR) is defined as inflammation of the lining of the nose and is characterized by nasal symptoms that may frequently be followed by ocular symptoms (OS), particularly in patients who are allergic to outdoor allergens. Allergic rhinitis is a common chronic disease that affects 10-40% of the population worldwide (1-5) and is characterised by the following nasal symptoms: rhinorrhoea, nasal congestion, sneezing and nasal itching. It is often accompanied by OS such as tearing, ocular redness and itching (3).

Allergic rhinitis is a major chronic respiratory disease due to its prevalence, impact on quality of life, impact on work/school performance and association with asthma. Allergic rhinitis is also associated with co-morbidities such as allergic conjunctivitis (3,6). Additionally, AR is one of the top ten reasons for patients visiting their general practitioner (7).

Allergic rhinitis is a multifactorial disease that is induced by gene–environment interactions (8). It is well established that aeroallergens cause AR. Aeroallergens have traditionally been subdivided into indoor and outdoor allergens. Major outdoor allergens include various types of pollens and outdoor moulds. Major indoor allergens include mites, animal dander and indoor moulds. Patients with AR can be sensitised to indoor, outdoor or both types of aeroallergens (mixed sensitisation). Aeroallergens as risk factors for AR may occur at all ages of life (3).

Based on the types of aeroallergens, AR is divided into seasonal AR and perennial AR. Seasonal AR is associated with outdoor aeroallergens, whereas perennial AR is most frequently caused by indoor or mixed aeroallergens (8,9). The Allergic Rhinitis and its Impact on Asthma workshop, in collaboration with the World Health Organization, introduced a new classification for AR based on the duration of symptoms, i.e., as either intermittent or persistent, and on the severity of symptoms, i.e., ranging from mild to moderate to severe (3).

The ocular symptoms experienced in AR, which is usually referred to as conjunctivitis, are multifactorial and can be caused by allergic agents, with various mechanisms, symptoms and signs and degree of severity (10,11). One mechanism is an acute hypersensitivity reaction with hyperaemia and chemosis accompanied by intense tearing, itching and burning of the eye following exposure to aeroallergens; alternatively, conjunctivitis may be due to the parasympathetic naso-ocular reflex (12,13).

The aim of this study was to determine the prevalence of ocular symptoms and compare the demographic and clinical characteristics in AR patients depending on sensitisation to various types of aeroallergens.

PATIENTS AND METHODS

We analysed 312 male and female consecutive patients older than 12 years who had a documented clinical diagnosis of AR at the Department of Otorhinolaryngology, Health Centre Kragujevac, Serbia, from March 2012 to 2014. All patients with OS were referred for an ophthalmological examination.

The diagnosis was based on anamnesis according to an AR questionnaire (14), clinical otorhinolaryngologic and ophthalmologic examination that included slit-lamp examination. All enrolled subjects had a positive skin prick test (weal 3 mm larger than the diluted control- histamine 1 mg/1 ml) to at least one of following aeroallergens: cat fur, moulds (indoor and outdoor), tree pollen, house dust, dog fur, weed pollen, grass pollen, Dermatophagoides pteronyssinus, plumage and cockroaches and/or serum-specific IgE. Patients who had undergone nasal surgery in the previous 6 months and patients with nasal polyps, significant deviation of the nasal septum or acute upper respiratory infection were excluded.

This population was divided into three managed groups based on the following criteria: sensitised to outdoor aeroallergens - SOA (sensitised at least to one of these aeroallergens: tree pollen, weed pollen, grass pollen), sensitised to indoor aeroallergens - SIA (sensitised to at least one of these aeroallergens: cat fur, moulds, house dust, dog fur, Dermatophagoides pteronyssinus, plumage and cockroaches) and sensitised to both outdoor and indoor aeroallergens or mixed group - SMA (sensitised to at least one of the indoor and one of the outdoor aeroallergens).

Statistics were generated using the standard statistical package SPSS (Statistical Package for the Social Sciences, version 19.0). P values less than 0,05 were considered significant, and those less than 0,001 were considered to be highly significant for all of the above tests. To describe the parameters of interest, we used the methods of descriptive analysis, chi-square test, Mann Whitney U test and ANOVA model.

We used the Mann Whitney U test to compare the experience of OS and mean ages in patients with and without OS.

The study was conducted in compliance with the Declaration of Helsinki and was approved by the local ethics committee. All participants or parents or legal guardians gave their written consent to participate in the study.

RESULTS

During the study period, a total of 312 patients had a clinical diagnosis of AR, and most of them were females

Table 1: Frequency of aeroallergens

Aeroallergens	Males N=104 n (%)	Females N=208 n (%)	Total N=312 n (%)
Weeds pollen	57(54.81)	112(53.85)	169 (54.2)
Dermatophagoides pteronyssinus	48(46.15)	97(46.63)	145 (46.5)
Plumage	44(42.31)	91(43.75)	135 (43.3)
Grass pollen	32(30.77)	67(32.21)	99 (31.7)
House dust	23(22.11)	48(23.08)	71 (22.8)
Cat fur	11(10.58)	24(11.54)	35 (11.2)
Trees pollen	11(10.58)	23(11.06)	34 (10.9)
Dog fur	10(9.61)	23(11.06)	33 (10.6)
Moulds	11(10.58)	22(10.58)	33 (10.6)
Cockroaches	6(5.77)	15(7.21)	21 (6.7)

(66,7%). The average age was 27,9 ±12,7; the ages ranged from 12 to 59 years.

The majority age group was 12–25 years, accounting for 158 (50,6%) of all cases. Based on the sensitisation to aeroallergens, the groups were SOA (30,4%), SIA (20,2%) and SMA (43,3%). No significant difference was found in the mean ages of the patients with and without experience of OS p>0,05 (p=0,243).

The frequencies of aeroallergens are shown in Table 1. Most patients were sensitised to weed pollen (54,2%), followed by Dermatophagoides (46,5%) and plumage (43,3%). The presence of sensitisation to one aeroallergen was 15,38% (48 patients), that to 2-4 aeroallergens was 52,88% (165 patients) and polysensitized (5 or more) occurred in 31,74% (99 patients).

The most common nasal symptoms were nasal congestion in 97,1% of all patients. Overall, 27.6% of all patients had OS, of which eye itching was the most common OS symptom by affecting 19,2% of the cases.

The distribution of the main demographic and clinical patient characteristics in the observed groups SOA, SIA and SMA is presented in Table 2. Our results indicated that there were no significant differences (p>0,05) among the SOA, SIA and SMA groups regarding gender, mean age, nasal congestion, sneezing, itchy nose and runny nose. Additionally, our results indicated that there was a highly significant difference (p<0,001) in ocular redness, eye itching and tearing in the SIA and SOA patients and a significant difference (p<0,05) between the SIA and SMA groups.

Table 2: Patients demographic and clinical characteristics

	SIA (N=81)	SOA (N=63)	SMA (N=168)
Gender (males)	27 (33.33)	22 (34.92)	55 (32.74)
Mean age (±sd)	28.7± 10.1	27.23± 12.2	27.69± 11.9
Nasal congestion N (%)	81(100)	61(92.82)	161(95.83)
Sneezing N(%)	30(37.04)	23(36.51)	63(37.50)
Itching of nose N (%)	23(28.39)	20(31.74)	57(33.93)
Runny nose N (%)	19(23.46)	15(23.81)	38(22.62)
Ocular redness N(%)[a,b]	1(1.23)	12(19.05)	20(11.90)
Eye itching N(%)[a,b]	2(2.47)	19(30.16)	39(23.81)
Tearing N(%)[a,b]	0 (0.00)	6(9.52)	12(7.14)

SIA patients sensitised to indoor aeroallergens;
SOA patients sensitised to outdoor aeroallergens;
SMA patients sensitised to outdoor and indoor aeroallergens; N, number of subjects;
a Highly statistically significant difference between SIA and SOA (p<0,001).
b Statistically significant difference between SIA and SMA (p<0,05).

A significant finding was noted in the SOA and SMA groups and experience of OS. We found a highly significant presence of OS (p<0,001) (χ^2=29,747; df=1; p=0,000) in patients who were sensitised to outdoor aeroallergens. In the group sensitised to mixed aeroallergens, OS were positively correlated (p<0,05) (χ^2=10,085; df=1; p=0,002). Only three patients had OS in the group sensitised to indoor aeroallergens (not significant; p>0,05).

DISCUSSION

Allergic rhinitis represents a global health problem and has considerable prevalence, especially in children and young people (15,16). Half of the patients in this study were 12-25 years old, which is comparable with previous studies conducted elsewhere (17,18).

In this study, a predominance of females was observed, which is in agreement with other studies (19), but other studies reported a male (20) predominance and no gender predilection (21).

In our study, the existence of OS was not correlated with age. According to data from the literature (22), patients under 50 years of age have more frequent combined nasal and OS. This finding might be partly due to other ocular conditions that could develop later in life contributing to this result, such as tear film dysfunction, which appears to increase with age, whereas atopy decreases with age (23).

Most patients presented with sensitisation to weed pollen, followed by Dermatophagoides and Plumage, in our study. These data depend on the flora and climate of the region and environmental conditions (24-26).

Generally, "polysensitisation" means "more than one sensitisation" (i.e., anything other than monosensitisation). According to Jong et al. (27), the term "polysensitisation" describes 2 to 4 sensitisations, and "polysensitisation" describes 5 or more sensitisations. Clinical symptoms were more severe in polysensitised patients than in monosensitised patients (28). In our study, the majority were sensitized to 2-4 aeroallergens, which was similar to the results of recently published studies (29). Establishing mono- and polysensitisation may be clinically significant because polysensitisation is correlated with disease severity (9).

Allergic rhinitis is a complex and multifactorial IgE-mediated disorder that is associated with the epithelial accumulation of effector cells, such as mast cells, eosinophils and basophils, and with the formation and release of various inflammatory mediators that are responsible for the early symptoms of rhinitis, such as nasal itch, sneezing and

rhinorrhoea (7). Pathophysiologically, the disease is characterized by a two-phase process that involves an initial sensitization phase (allergen exposure resulting in IgE overexpression), with subsequent allergen exposure provoking an allergic response. Clinically, the allergic response can be divided into the following two phases: the early phase inflammatory response, which is initiated within minutes of re-exposure to the allergen and is primarily caused by mast cell degranulation, and the release of preformed mediators such as histamine and a newly generated different type of mediators. For the patient, the clinical manifestations are early symptoms of AR such as sneezing, itching and rhinorrhoea. Additionally, they stimulate the production, adhesion and infiltration into local tissue of circulating inflammatory cells such as eosinophils, basophils, monocytes, and lymphocytes (7,30). The late-phase inflammatory response begins 2–4 h after allergen exposure and involves the activated inflammatory cells which release further mediators, thus promoting local oedema and tissue damage and the continuation of the overall inflammatory process. Symptomatically, the late-phase allergic reaction is characterized by nasal congestion and obstruction (30).

All patients in this study had nasal symptoms, and nasal congestion was found in most patients. Nasal congestion is a symptom that characterises both the early and late phases of AR, and it often persists long after allergen exposure. The same observation was also reported in another study (31). Rhinorrhoea was also reported as the most frequent symptom, but the difference in the data was likely due to different methodological approaches and the different characteristics of the population and environmental milieu (including children younger than 12 and examined patients suffering from seasonal AR). Rhinorrhoea is common in small children and is commonly accompanied by viral and bacterial infections, particularly in children under five years of age, who were the majority in this study (32).

Allergic conjunctivitis is the typical conjunctival reaction in allergic rhinitis or following exposure to allergens. The pathophysiology underlying OS remains to be elucidated. The symptoms probably arise via a combination of mechanisms that include direct contact of the conjunctiva with natural pollen and reflex mechanisms originating in the nose. Pollen exposure can result from the direct transfer of pollen to the conjunctiva from the blowing of air or nasal secretions that contain the antigen up the nasolacrimal duct. In support of direct contact of pollen as a source of ocular symptoms in patients with allergic rhinitis, pollen can be washed out of the conjunctiva on windy days, although the amount is 10-fold less than the amount of pollen recovered simultaneously from the nose (33).

Ocular symptoms occur in a large proportion of patients with AR. The rate of OS found in this study (27,6%) is lower than the rate presented in the literature, which is higher than 40% (34-36). Traditionally, allergy investigations have focused on nasal symptoms, but recent studies have highlighted the prevalence and significance of OS. Evidence suggests that OS are particularly prevalent in seasonal AR sufferers (37) who were sensitised to outdoor aeroallergens (9). Despite previous opinions, OS are not only common but also distressing for sufferers (36).

The present study was performed to establish a correlation among the demographic characteristics in AR patients who were sensitised to various types of aeroallergens: SIA, SOA and SMA. There were no significant differences in regard to gender, age and nasal symptoms, which were equally represented in the observed groups. Our results indicate significant differences in the ocular redness, eye itching and tearing among the observed groups. These results indicate that the main risk factor for experience of OS is sensitisation to outdoor aeroallergens. Our results are similar to the results of recent studies (9,38).

Our present study demonstrated a lower presence of OS than did the data from the other studies, which may indicate that the majority of the patients with AR who also have OS either seek care from their general practitioner or do not recognise their symptoms to be a disease at all. Some of them also act as their own doctors. In AR sufferers, it is necessary to address all symptoms, especially OS, which are more common in patients who are sensitised to outdoor aeroallergens. These patients must be referred to an ophthalmologist for examination and adequate treatment because currently, the ophthalmologic examination is not part of the AR diagnostic protocol.

Conflict of Interest

There is no financial interest and no other conflict of interest.

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POTENTIAL PRO-INFLAMMATORY ROLE OF VEGF IN PATIENTS WITH CROHN'S DISEASE

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MOGUĆA PROZAPALJENSKA ULOGA VEGF KOD PACIJENATA SA KRONOVOM BOLEŠĆU

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ABSTRACT

The aim of this study was to investigate the expression patterns of p16, p53 and VEGF in affected tissue and serum levels of cytokines TNF-α, IL-6, TGF-β and IL-17 in patients with ulcerative colitis (UC) and fistulating Crohn's disease (CD). Serum levels of cytokines in patients with ulcerative colitis (n=24) and with Crohn's disease (n=7) were analysed by ELISA. In colonoscopically obtained biopsies, p16, p53 and vascular endothelial growth factor expression were evaluated by immunohistochemistry.

The results of this study clearly show the predominance of pro-inflammatory type 1 and 17 immune response in patients with CD compared to those with UC. We believe that altered p16 and p53 induce enhanced VEGF expression and implicates enhanced production of pro-inflammatory TNF-α and IL-6. TNF-α and IL-6 further facilitate development of type 1/17 immune response.

Key words: *ulcerative colitis, Crohn's disease, p16, p53, VEGF, cytokines*

SAŽETAK

Cilj ove studije je bio da se ispita ekspresija p16, p53 i VEGF u obolelom tkivu i serumske koncentracija citokina TNF-α, IL-6, TGF-β i IL-17 kod pacijenata sa ulceroznim kolitisom (UC) i fistulizirajućom Kronovom bolešću (CD). Serumske vrednosti citokina kod pacijenata sa ulceroznim kolitisom (n=24) i sa Kronovom bolešću (n=7) analizirane su ELISA metodom. U kolonoskopski dobijenim biopsijama, ekspresija p16, p53 i vaskularnog endotelnog faktora rasta određivana je imunohistohemijski.

Rezultati ovog istraživanja jasno pokazuju predominaciju proinflamatornih tip 1 i tip 17 imunskih odgovora kod pacijenata sa CD u poređenju sa pacijentima obolelim od UC. Mi verujemo da izmenjena ekspresija p16 i p53 indukuje pojačanu ekspresiju VEGF-a koja implikuje pojačanu sekreciju pro-inflamatornih citokina TNF-α i IL-6. TNF-α i IL-6 sledstveno facilitiraju razvoj Tip1/17 imunskog odgovora.

Ključne reči: *ulcerozni kolitis, Kronova bolest, p16, p53, VEGF, citokini*

ABBREVIATIONS

ABC-Avidin-Bioton peroxidase Complex
CD-Crohn's disease
CDK-Cyclin dependent kinase
CARD-Caspase activating recruitment domain
DNA- Deoxyribonucleic acid
ELISA-Enzyme Linked Immunosorbent Assay
IBD-Inflammatory bowel disease (IBD)
IFN-γ- Interferon-γ
IL-Interleukins
MAPK-Mitogen Activated Protein Kinase
NF-kB –Nuclear factor kappa light chain enhancer of activated B cells
NOD2-Nucleotide binding oligomerization domain containing protein 2
NK cells-Natural Killer Cells
VEGF- Vascular endothelial growth factor
TNF-α-Tumour necrosis factor alpha
TGF-β-Transforming growth factor beta
UC-Ulcerative colitis

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INTRODUCTION

The inflammatory bowel diseases (IBDs) are a group of inflammatory conditions of the colon and small intestine. IBDs include Crohn's disease (CD) and ulcerative colitis (UC) and are characterized by spontaneous remittances and relapses (1). The exact cause of IBDs remain unknown. Available evidence suggests that an abnormal immune response against the microorganisms of the intestinal flora is responsible for the diseases in genetically susceptible individuals (2). Ulcerative colitis is characterized by continuous inflammation of the intestinal lamina propria, starting from the rectum and potentially involving the whole colonic mucosa. At present, its pathogenesis is still unclear, but evidence suggests that the disease occurs in genetically susceptible subjects and is trigged by environmental factors, which lead to an exaggerated and uncontrolled immune response (3). Cytokines have a crucial role in the pathogenesis of UC, where they control multiple aspects of the inflammatory response. In particular, the imbalance between pro-inflammatory and anti-inflammatory cytokines that occurs in UC impedes the resolution of inflammation and instead leads to disease perpetuation and tissue destruction (4). Beside monocyte/macrophages, CD4+ helper T lymphocytes are major producers of cytokines and can be classified according to the type of cytokines they produce: Th1 (IFN-γ and TNF-α), Th2 (IL-4, IL-5 and IL-13), Th17 (IL-6 and IL-17) and Tregs (IL-10 and TGF-β) (5).

Crohn's disease, also known as Crohn syndrome and regional enteritis, is a type of inflammatory bowel disease (IBD) that may affect any part of the gastrointestinal tract from mouth to anus (6). Crohn's disease is caused by a combination of environmental, immune and bacterial factors in genetically susceptible individuals (7-9). It results in a chronic inflammatory disorder, in which the immune system attacks the gastrointestinal tract, with possible direction at microbial antigens (8-10). While Crohn's is an immune related disease, it does not appear to be an autoimmune disease (in that the immune system is not being triggered by the body itself) (11). The exact underlying immune problem is not clear; however, it may be an immunodeficient state (10-12). About half of the overall risk is related to genetics, with more than 70 genes found to be involved (13, 14). The first mutation found to be associated with Crohn's was a frameshift in the NOD2 gene (also known as the CARD15 gene) (15), followed by the discovery of point mutations (16). Certain characteristic features of pathology point towards Crohn's disease including a transmural pattern of inflammation, meaning the inflammation may span the entire depth of the intestinal wall (17).

Patients with IBDs are at increased risk for colon cancer (18-21). Many authors have reported p53 mutations in epithelium adjacent to dysplasia in IBDs (18-21). These alterations of p53 underlie the multistep process of oncogenesis (22). Vascular endothelial growth factor (VEGF) belongs to a family of platelet-derived growth factors and plays an important role in tumour growth and metastasis (23). Still, the role of VEGF in the modulation of immune responses remains unclear. The tumour suppressor p16INK4a belongs to the INK4 family of cyclin-dependent kinase (CDK) inhibitors (24). p16INK4a inhibits cell-cycle progression by preventing cyclin D–CDK 4/6 complex formation. p16INK4a inactivation by deletion, point mutation, or promoter methylation occurs frequently in most tumours (25). In addition to its role in cancer as an inhibitor of cell-cycle progression, p16 plays important role in the modulation of immune response (26). In response to DNA damage, wild-type p53 upregulates the p21 (WAF1/Cip1) protein, which is a general inhibitor of CDKs and contributes to G1 cell cycle arrest under these circumstances (27).

Therefore, the aim of this study was to investigate the expression patterns of p16, p53 and VEGF in affected tissue and serum levels of cytokines TNF-α, IFN-γ, IL-4, IL-6, IL-10 and IL-17 in patients with UC and Crohn's disease. We report higher values of pro-inflammatory cytokines in patients with Crohn's disease and positive staining of p16, p53 and VEGF. Expression of p53 is found to be a highly specific marker in differentiation between UC and Crohn's disease.

MATERIAL AND METHODS

Patients

From May 2011 until March 2013, patients were enrolled at the Center for Gastroenterology, Clinical Centre Kragujevac. All immunological measurements were conducted at the Center for Molecular Medicine and Stem Cell Research, Faculty of Medical Sciences, University of Kragujevac. All ethical approvals were obtained, and research was conducted in accordance with the regulations of Good Clinical and Laboratory Practices.

We investigated a total of 24 patients with ulcerative colitis (15 males and 9 females; mean age: 48.38 ± 17.94 years) and 7 patients with Crohn's disease (4 males and 3 females; mean age: 20.75 ± 1.71 years). The diagnoses of ulcerative colitis and Crohn's disease were based on endoscopic and histopathological criteria. The study did not include patients with inflammatory bowel disease who were previously treated with antibiotics, aminosalicylates, corticosteroids, immunosuppressive agents, or biological therapy. All subjects had a complete medical history, including physical examination, routine laboratory tests and diagnostic imaging (chest X-ray, abdominal ultrasound, abdominal computed tomography scan and endoscopy).

Assessment of serum level TNF-α, IL-6, IL-17 and TGF-β

Blood samples were obtained before application of therapy. The control group consisted of 37 healthy male and female blood donors at the Clinical Centre of Kragujevac. The control group was matched with the experimental groups on the basis of gender. Serum was separated, and

all serum samples were kept at -20°C before use. Serum levels of cytokines were measured as described before (28), using sensitive enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems Minneapolis, MN, USA for IL-6, TNF-α, TGF-β and IL-17), specific for human cytokines, according to the manufacturer's instructions.

Immunohistochemical study of VEGF, p16 and p53

Immunohistochemical staining was performed using the streptavidin-biotin technique, as previously described (29). Immunohistochemistry was performed on multiple endoscopically obtained colonic mucosal biopsy specimens collected from patients with ulcerative colitis and patients with Crohn's disease. Paraffin-embedded tissue samples were sectioned at 4-5 μm. Briefly, tissue samples were deparaffinised. Antigen retrieval was performed by microwave heating for 20 min in 10 mM sodium citrate buffer (pH 6.0). The sections were incubated with 3% hydrogen peroxide to block endogenous peroxidase activity and then incubated with mouse monoclonal antibodies against VEGF (ab16883, Abcam, Cambridge, UK, at a 1:200 dilution), p53 (ab17869-250, Abcam, Cambridge, UK, at a 1:200 dilution) and p16 (sc-81156, Santa Cruz Biotechnology, Santa Cruz, CA, USA, at a 1:50 dilution) for 60 min in a humidity chamber, followed by incubation with biotinylated secondary antibodies. The ABC (Avidin-Bioton peroxidase Complex) method was used for detection. The slides were examined by conventional light microscopy. Negative controls were treated identically, but with the primary antibodies omitted. Positive controls consisted of tissue known to contain the protein of interest. The VEGF, p16 and p53 stained sections were assessed semiquantitatively by two pathologists. The staining score was evaluated as the percentage of stained cells out of the total number of evaluated cells. The extent of each staining pattern of p16 and p53 was recorded as the number of positive nuclei per 250 cells. The same pattern was used for VEGF in cytoplasm. Percentage of positive cells was determined by counting 5 non overlapping microscopic fields at 400x magnification. Staining for p16, p53 and VEGF was defined as positive when >10% of the cells were stained and as negative when ≤ 10% of the cells were stained (30, 31).

Statistical analysis

Software package IBM SPSS Statistics 20 was used for the statistical analysis. Data are grouped and shown in tables and graphs. Methods of descriptive statistics were used in determining measures of central tendency and variability. The results are presented as the mean and standard deviation. Pearson's and Spearman's correlation coefficients were used for determining the strength and direction of correlation between the variables. A chi-squared test was used for determining the independence of variables. In determining statistically significant differences between the means of the two groups, Student's t-test for independent samples was used if the data were normally distributed, while a Mann-Whitney U-test was used for data that were not normally distributed. A One-Way ANOVA test was used for determining statistically relevant differences between the means of various groups of normally distributed data and a Kruskal-Wallis test was used for data that were not normally distributed. In determining the normality of data distribution, a Kolmogorov-Smirnov test and a Shapiro-Wilk test were used. Levene's test for equality of variances was used to determine the homogeneity of variances. This test determines if the results from two groups of data have equal variances. An ROC curve was used for the review of sensitivity and specificity. All statistical analyses in this paper were conducted with a confidence interval of 95%. The results of the statistical analysis were taken as statistically significant if the level of probability of null hypothesis was lower than 5%, that is to say, if the relevance of the test was $p<0.05$.

RESULTS

Higher frequency of patients with Crohn's disease had positive staining of p16, p53 and VEGF, in comparison to patients with UC

The expression patterns of p16, p53 and VEGF protein were assessed by immunohistochemistry on biopsy specimens. All patients with UC and Crohn's disease were classified on the basis of positive (>10% positive cells) or negative (≤10% positive cells) immunostaining of p16, p53 and VEGF, respectively. Only nuclear staining of p16 and p53 was scored. The frequency of patients with Crohn's disease that had positive staining for p16 and p53 was significantly higher when compared to UC subjects ($p=0.024$; $p=0.041$, respectively).

Biopsy specimens of all patients with Crohn's disease were p16 positive, while 83% of specimens from patients with Crohn's disease had positive p53 nuclear staining (Fig 1). VEGF expression was found to occur in the cytoplasm and cell membrane. Of specimens from patients with Crohn's disease, 82% had positive VEGF staining, with no significant difference between groups (UC vs CD; Fig 1).

Representative images of immunostaining for the mentioned markers examined in tissue of patients with ulcerative colitis and Crohn's disease are shown in Figure 2.

Serum concentrations of pro- and anti-inflammatory mediators and their ratios in UC patients and patients with Crohn's disease

Next, we examined the serum levels of pro-inflammatory (IL-4, IL-6, TNF-α, IFN-γ and IL-17) and anti-inflammatory mediators (IL-10) in patients with ulcerative colitis and Crohn's disease. Twenty-four patients with ulcerative colitis, 15 males and 9 females with a mean age of 48.38 ± 17.94, and seven patients with Crohn's disease, 4 males and 3 females

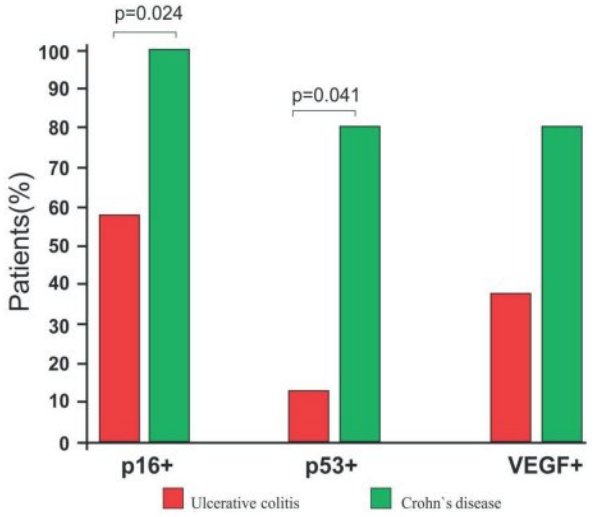

Figure 1. Expression of p16, p53 and VEGF in patients with Crohn's disease and ulcerative colitis. Higher percentages of Crohn's disease patients with positive staining of p16, p53 and VEGF in comparison with patients with UC. Patients with CD and UC were divided in two groups based on expression of p16, p53 and VEGF. Statistical significance was tested by a Mann–Whitney Rank Sum test or Independent Samples t-test, where appropriate.

with mean age 20.75 ± 1.71 years, were studied. We obtained a significantly higher serum level of TNF-α (p=0.032; Fig 3A, left panel) and IL-6 (p=0.039; Fig 3A, right panel) in patients with Crohn's disease, in comparison with the UC patients. It had been suggested that the ratio of counterregulatory cytokines could be a marker of disease progression. Therefore, we analysed ratios of pro- and anti-inflammatory cytokines and found a significantly higher value of IL-6/TGF-β ratio (p=0.045; Fig 3B, left panel) and IL-17/TGF-β ratio (p=0.024; Fig 3B, right panel) in patients with Crohn's disease compared to patients with UC.

Logistic regression analyses of p53 expression and TNF-α serum level in colorectal inflamed tissue

Binary logistic regression showed that higher expression of p53 strongly correlated with the presence of Crohn's disease. Analysis showed that p53 can be a valuable marker for differentiating Crohn's disease from UC (sensitivity 78.6%, specificity 95.2%, cut-off >0; Fig 4, right panel). Analysis also showed that TNF-α (sensitivity 78.7%, specificity 87.5%, Fig 5, left panel) can be a valuable marker for distinguishing UC and Crohn's disease. The optimal cut-off value estimated for TNF-α that allows discrimination between UC and Crohn's disease patients was 35 pg/ml. For this cut-off, we determined sensitivity to be 78.7% and specificity 87.5%.

DISCUSSION

In the present study, we found a predomination of pro-inflammatory cytokines in patients with Crohn's disease

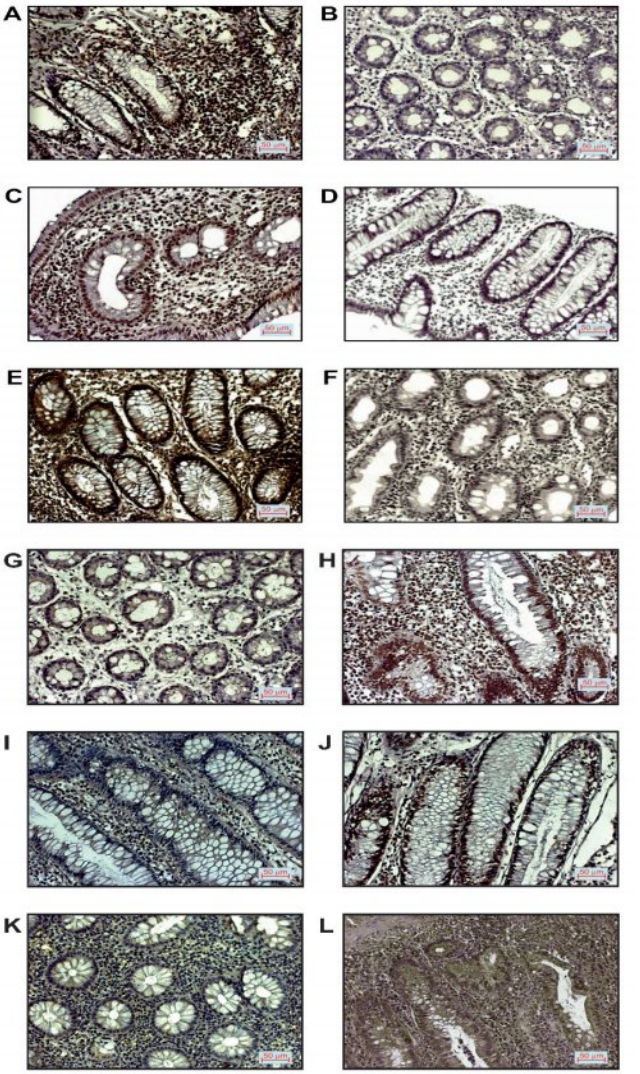

Figure 2. p16, p53 and VEGF expression in patients with UC and CD. Ulcerative colitis: A. Positive p16 staining (200x magnification) **B**. Negative p16 staining (200x magnification) **C**. Positive p53 staining (200x magnification) **D**. Negative p53 staining (200x magnification) **E**. Positive VEGF staining (200x magnification) **F**. Negative VEGF staining (200x magnification). **Crohn's disease: G**. Positive p16 staining (200x magnification) **H**. Negative p16 staining (200x magnification) **I**. Positive p53 staining (200x magnification) **J**. Negative p53 staining (200x magnification) **K**. Positive VEGF staining (200x magnification) **L**. Negative VEGF staining (200x magnification).

compared to patients with UC. Positive staining of p16, p53 and VEGF was detected in the mucosa of both groups, and its expression was significantly higher in Crohn's disease compared to ulcerative colitis. Furthermore, the expression of p53 and serum values of TNF-α higher than 35 pg/ml present a highly sensitive and specific marker in the differentiation between Crohn's disease and UC.

The type of immune response is different in CD and UC. CD is characterized by granuloma formation, while the hallmark of UC is infiltration of neutrophils with the destruction of the epithelium (32). Additionally, CD is dominantly followed by a Th1-type immune response (with

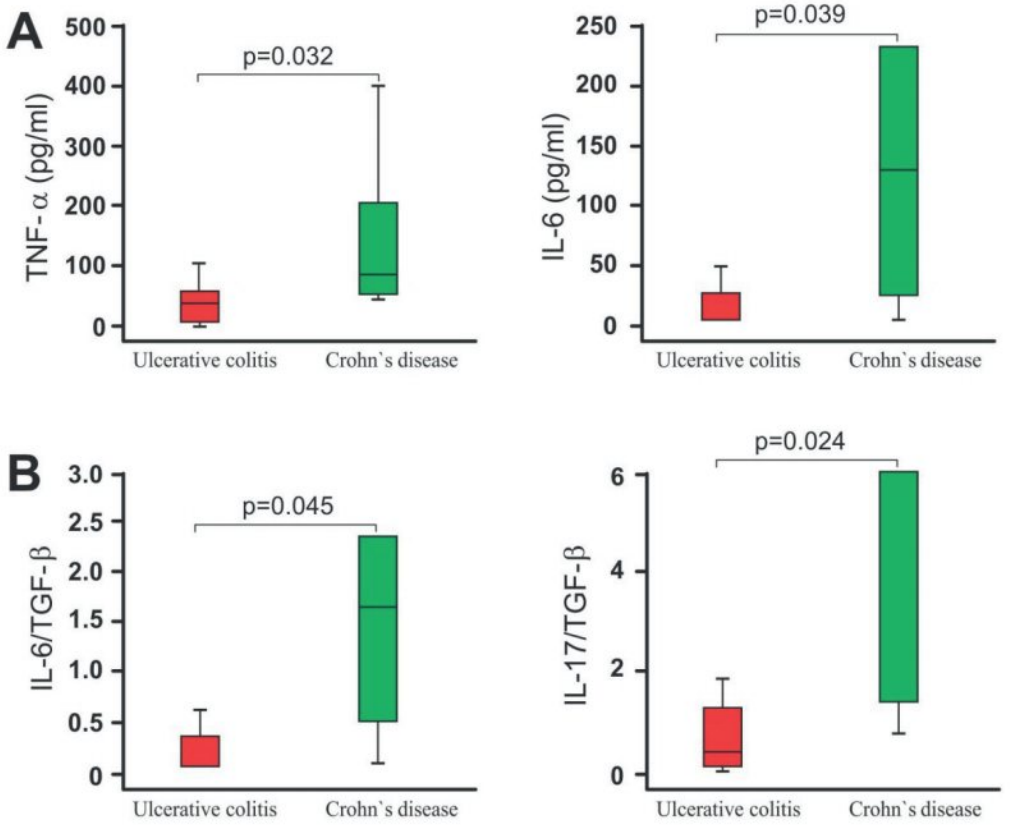

Figure 3. Serum values of mediators of inflammation in patients with UC and CD.
A. Higher serum concentration of TNF-α and IL-6 in patients with Crohn's disease.
B. Higher values of IL/6/TGF-β and IL-17/TGF-β ratios in patients with Crohn's disease. IL/6/TGF-β and IL-17/TGF-β ratio was evaluated for each patient, separately. Serum levels of all mentioned cytokines were determined by ELISA. Statistical significance was tested by a Mann–Whitney Rank Sum test or independent samples t-test, where appropriate.

the production of IL-2, IL-8, IL-12, IFN-γ and TNF-α) (32), while in UC, a Th2 type immune response dominates (production of IL-4, IL-5, IL-6, IL-10 and IL-13), which stimulates humoral immunity (32). In this study, we estimated significantly higher concentrations of TNF-α and IL-6 in the serum of patients with CD compared to those with UC (Fig 4A). The role of TNF-α in the pathogenesis of CD is well known (33). The high systemic values of TNF-α in patients with CD is in accordance with data from the literature (32-34). According to the aforementioned data, it may be assumed that the increased TNF-α in sera induces IL-6 production, with subsequent elevation of IL-6 in the serum of patients with CD. Increased concentrations of IL-6 in patients with CD, in comparison to UC, can also be explained by the fact that all patients had the fistulating form of CD. The fistulised form of CD can cause changes in colon microenvironment, with subsequent changes in the type of immune response (33).

Furthermore, we analysed ratios of contra-regulatory cytokines IL-6, IL-17 and TGF-β, and documented that patients with CD (compared to patients with UC) had significantly higher IL-6/TGF-β and IL-17/TGF-β ratios (Fig 4B). TGF-β is an inhibitory cytokine and the key regulator of immune homeostasis and inflammation (35). It prevents proliferation of leukocytes and their activation and takes a part in the inhibition and spread of the inflammatory process (35). Decreased activity of the TGF-β is considered to be responsible for the development of a variety of autoimmune diseases, including inflammatory bowel disease (35). TGF-β is a negative regulator of inflammation of the bowel mucosa (36) and also an important factor for the development of regulatory T lymphocytes (37). Regulatory T cells produce a large amount of TGF-β, which plays a significant role in the maintenance of peripheral immune tolerance and immunosuppression (38). Th17 cells are a sub-population of helper T lymphocytes which have been isolated for the first time from the peripheral blood of patients with CD. As a subclass of lymphocytes, which produce a large amount of IL-17 and IL-23 (39), it is believed that Th17 cells promote tissue destruction during inflammatory processes and are significant in the genesis and development of chronic inflammatory diseases (40). TGF-β, in combination with IL-6, directs the differentiation of naive T cells into Th17 cells (36, 40-43). Participation of TGF-β in the genesis of Th17 lymphocytes is quite surprising because TGF-β alone has an anti-inflammatory effect and is important for the generation of regulatory T lymphocytes (40). It has been assumed that there is a natural antagonism be-

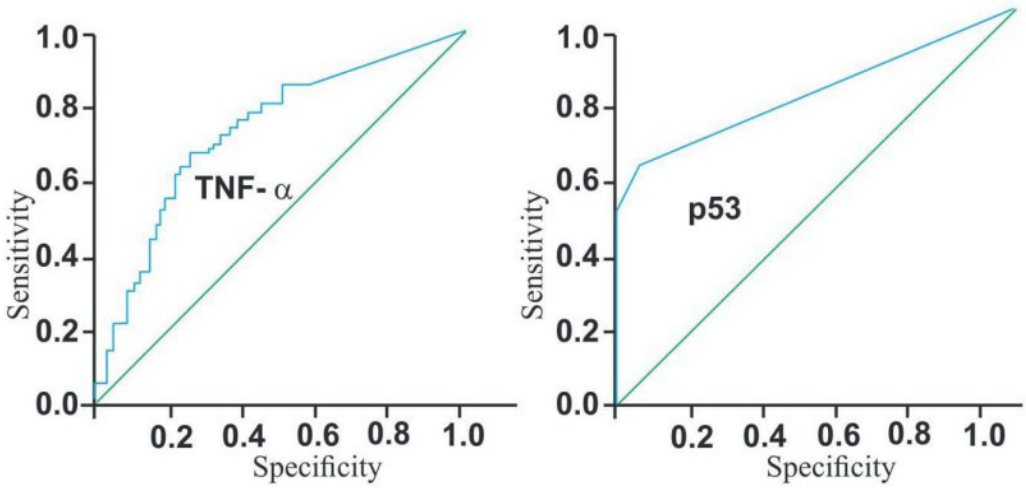

Figure 4. Specificity and sensitivity of TNF-α serum concentration and p53 expression.
The ROC curve illustrates the specificity and sensitivity of TNF-α serum concentration, comparing CD with UC (left panel) and specificity and sensitivity of p53 expression, comparing CD with UC (right panel).

tween Th17 and regulatory T cells, and in the absence of an inflammatory environment, the TGF-β produced will induce suppression of the effector T cells and the formation of regulatory T cells, which is important for maintenance of immuno-tolerance (37). In our study, elevated IL-6 in the sera of patients with CD may suppress formation of regulatory T cells and induce the development of a Th17 immune response (37). Additionally, increased production of pro-inflammatory cytokines, such as TNF-α, reduce the secretion of TGF-β (35). IL-17 mediates the pathogenesis of CD, and it is a potent inducer of local infiltrations with neutrophils (44). Additionally, IL-17 induces expression of other pro-inflammatory cytokines (TNF-α, IL-1, IL-6) (45), nuclear growth factor (NF-kB) and the MAPK kinases (46). IL-17 has an effect on fibroblasts and induces the secretion of metalloproteinases, which further contribute to tissue damage (47).

We also analysed expression of anti-oncogenes p16 and p53 and VEGF in affected tissue.

The percentage of patients with CD and a positive expression of p16, p53 and VEGF was higher than those with UC (Fig 1). Vascular proliferation in the submucosa is typical for CD (33). In this study, all subjects with CD had the highest histological grade of inflamed colonic mucosa (histological grade III), which was not the case with subjects with UC. In inflammatory bowel disease, higher expression of p16 and p53 genes correlates with a higher histological grade of inflamed mucosa (48, 49). We assume that a higher degree of histological damage of inflamed mucosa correlates with a higher alteration of p16 and p53 genes. Various studies have revealed that wild type p16 and p53 reduce angiogenesis via down regulation of VEGF (50-52). We proposed that altered p16 and p53 may induce VEGF overexpression and stimulate angiogenesis (29). Higher expression of anti-oncogenes p16 and p53 indicates a loss of function and facilitates neovascularization via VEGF. Our results suggest that enhanced systemic pro-inflammatory immune response correlates with local tissue angiogenesis, in patients with CD.

VEGF also has a role in inflammation and immunity, as a key mediator in recruitment of inflammatory cells and enhanced expression of co-stimulatory molecules on recruited and resident mononuclear cells, leading to up-regulation of the pro-inflammatory cytokines Th1 and Th17 (53, 54).

Finally, we assessed the expression of studied tissue markers and cytokines as useful markers in the assessment of CD. P53 expression showed a high sensitivity and specificity for CD in

differentiating from UC (Fig 4). We also envisioned the possible role of TNF-α as a biomarker in distinguishing CD from UC and showed that increased levels of TNF-α enhanced the likelihood of UC, compared to CRC (Fig 4).

The results of this study clearly show the predominance of pro-inflammatory types 1 and 17 of immune response in patients with CD compared to those with UC. We believe that altered p16 and p53 induce enhanced VEGF expression that implicates enhanced production of pro-inflammatory TNF-α and IL-6. TNF-α and IL-6 will further facilitate development of Type 1/17 immune response.

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Competing Interests

The authors have declared that no competing interests exists.

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BONE QUALITY ASSESSMENT OF DENTAL IMPLANT RECIPIENT SITES

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PROCENA KVALITETA KOSTI U LEŽIŠTIMA DENTALNIH IMPLANATA

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ABSTRACT

The term bone quality is not clearly defined and depends on many factors, such as bone density, bone vascularity, bone metabolism and other factors that may affect implant outcome. The assessment of bone volume and bone density is most common in planning the treatment of dental implants. Bone quality is an important predictor of primary implant stability, which influences the future implant osseointegration. Numerous classifications have been described for the evaluation of bone density. The most commonly used has been the one proposed by Lekholmu and Zarb. For the objective evaluation of bone density, conventional computed tomography (CT) or Cone Beam Computed tomography (CBCT), have been proposed. Both methods are reliable for the measurement of bone density, but preference is given to CBCT, due to the lower radiation doses, greater comfort for the patient and the lower prices. Pre-operatively defined bone density is a good indicator of the future success of implant therapy. In addition to the bone density, vascularity of the jawbone is an important factor of the quality of the bone for the osseointegration of dental implants. Laser Doppler is a simple method that can determine the vascularity of bone during implant insertion. The development of modern diagnostic methods for assessing the quantity and quality of the jawbone has enabled easier implant planning and has provided a secure outcome.

Key words: *Bone quality, implant stability, computed tomography, cone beam computed tomography, jawbone vascularity*

SAŽETAK

Kvalitet viličnih kostiju nije u potpunosti definisan pojam i zavisi od brojnih faktora kao što su: gustina, vaskularizacija, koštani metabolizam i drugi, koji mogu uticati na ishod implantne terapije. U kliničkoj praksi se najčešće procenjuju volumen i gustina kosti, prilikom planiranja terapije dentalnim implantima. Kvalitet kosti je važan prediktor primarne implantne stabilnosti, od koje zavisi buduća oseointegracija implanata. Za procenu gustine kosti su opisane brojne klasifikacije, od kojih se najčešće koristi subjektivna metoda po Lekholm-u i Zarb-u. Objektivna procena vrši se primenom konvencionalne kompjuterizovane tomografije (CT) ili kompjuterizoane tomografije konusnog zraka (CBCT). Obe metode su pouzdane za merenje gustine kosti, ali se prednost daje CBCT-u, zbog nižih doza zračenja, većeg komfora za pacijenta i manje cene. Vrednosti gustine kosti izmerene preoperativno su dobar pokazatelj budućeg uspeha implantne terapije. Osim gustine i vaskularizacija vilične kosti je veoma važan pokazatelj kvaliteta kosti od koga zavisi oseointegracija dentalnih implanata. Laser Dopler je jednostavna metoda kojom se može odrediti vaskularizacija kosti implantnog ležišta u toku ugradnje implanata. Razvojem savremenih dijagnostičkih metoda za procenu kvantiteta i kvaliteta viličnih kostiju, na mestu ugradnje budućeg implanta, omogućeno je lakše planiranje ugradnje implanata i obezbeđen bolji uspeh.

Ključne reči: *Kvalitet kosti, Implantna stabilnost, kompjuterizovana tomografija, kompjuterizovana tomografija konusnog zraka, vaskularizacija viličnih kostiju*

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INTRODUCTION

The insertion of dental implants has become an increasingly common procedure in the oral rehabilitation of partially and totally edentulous patients. This trend has certainly contributed to the positive results of numerous clinical studies regarding implant survival rates (1,2,3,4). The success of any implant procedure depends on a series of patient-related and procedure dependent parameters, including general health conditions, biocompatibility of the implant material, the features of the implant surface, the surgical procedure, and the quality and quantity of the local bone (5,6). Based on the literature data, the success rate of implants is higher in the lower jaw than the upper jaw (4,5,6,7). This discrepancy may arise from the bone conditions around the implants. It is evident that, when compared with the maxilla, the bone surrounding the implant has a better volume and quality in the mandible (6).

There is no clear definition of bone quality, but it is generally presented as the sum of all of the characteristics of bone that influence its resistance to fracture (8). Many authors define bone quality as equivalent to bone mineral density. This includes physiological and structural aspects and the degree of bone tissue mineralization (9,10,11). Aspects such as bone metabolism, cell turnover, maturation, intracellular matrix and vascularity were also emphasized. Although the clinical outcome of an implant is influenced by many factors, including the implant body, the skill of the surgeon, and the oral environment, the key factor for success is the primary stability at the implant placement. Some studies have demonstrated that the quality of the alveolar bone is the most important factor for achieving good primary stability (12,13).

Primary implant stability has been acknowledged as an essential criterion for later achievement of osseointegration (14). The primary stability could be increased with increased bone quality, which would improve the osseointegration and increase the survival probability of the dental implant. Poor bone quantity and especially poor bone quality are the main risk factors for implant failure using the standard protocol for implant insertion (15). The primary stability depends on the quality of the local bone, the implant geometry and the applied surgical techniques (16). By applying additional surgical techniques, such as the absence of threads at the implant site; the use of a profile borer at a reduced diameter; the use of a larger implant of greater diameter and length; and the presence of bicortical stabilization, can make for greater primary implant stability (17,18,19). Detailed preoperative analysis of the jawbone helps therapists in making decisions about the type of surgical procedure and the type of implants.

Bone density

Bone density seems to be of great importance not only in primary implant stability but also in the predictability for oral implant outcomes (10). The literature describes a large number of classifications and procedures for the determination of jawbone density (20,21,22). The most commonly used classification has been the one proposed by Lekholmu and Zarb (1985), based on the amount of cortical and trabecular bone shown in preoperative panoramic and cephalometric radiographs. They classified bone density as Q1 to Q4 according to the ratio of cortical bone to spongy bone (10) (table 1). This method provides information on bone density but is considered to be a subjective method (23). Misch suggested that computed tomography (CT) can be used for the objective quantification of direct density measurements of bone, expressed in Hounsfield units (HU) (table 1). HU represent the relative density of body tissues according to a calibrated grey-level scale.

CT bone density

The introduction of new radiographic procedures that allow 3D analysis of the jawbone significantly facilitated the work of therapists and ensures a better treatment outcome.

In a CT scan, HU is proportional to the degree of x-ray attenuation, and it is allocated to each pixel to show the image that represents the density of the tissue. This method for pre-operative quantitative and qualitative assessment of dental implant sites is objective and reliable. The dental literature has numerous studies on the usefulness of CT for assessing bone volume and morphology and on the relationship between CT values and primary implant stability (7,9,12,14,16,19). It has been shown that there is a strong correlation between the pre-operative bone density

Table 1. Bone classifications and bone densities in Hounsfield units (HU)

Lekholm and Zarb clasiffication			
Type1	Type2	Type3	Type4
Large homogenous cortical bone	Thick cortical layer surrounding a dense medullar bone	Thin cortical layer surrounding a dense medullar bone	Thin cortical layer surrounding a sparse medullar bone

Misch clasification				
D1	**D2**	**D3**	**D4**	**D5**
> 1250 HU	850 to 1250 HU	350 to 850 HU	150 to 350 HU	< 150 HU
Dense cortical bone	Thick dense to porous cortical bone on crest and coarse trabecular bone	Thin porous cortical bone on crest and fine trabecular bone within	Fine trabecular bone	Immature, non-mineralized bone

Table 2. Bone densities in HU in diferent jaw regions

Jawbone region	Turkyilmaz et al. (2007)	de Oliveira et al. (2008)	Fun et al. (2010)
Anterior mandible	945 ± 207	383 ± 243	530 ± 161
Anterior maxilla	716 ± 190	370 ± 176	516 ± 132
Posterior mandible	674 ± 227	306 ± 187	359 ± 150
Posterior maxilla	455 ± 122	255 ± 184	332 ± 136

values and the primary stability measured after implant insertion (5,6,11,14,16)

The available literature indicates that the implant location greatly affects the implant success, which is approximately 4% higher in the mandible than in the maxilla, and it is higher in the anterior region than in the posterior region (approximately 12% and 4% in the maxilla and mandible, respectively). This might be explained by the mean bone density being highest in the anterior mandible, followed by the anterior maxilla, posterior mandible, and posterior maxilla (7, 24,25) (table 2).

There is also a difference in bone density between females and males, which may be explained by the hormonal peculiarities in females and the generally higher bone mass in males (25).

HU derived by CT can be used as a diagnostic parameter to predict possible implant stability. Thus, preoperative assessment of bone densities by HU is very important for optimizing primary implant stability. The use of CT has continued to grow, although its systematic use in clinical practice has been limited by concerns about high radiation doses and the relatively high cost.

Cone beam CT bone density

In recent years, due to the need for less expensive image acquisition protocols and scanners with a lower radiation dose, cone beam computed tomography (CBCT) has become widely used for oral and maxillofacial imaging. CBCT is a new medical imaging technique that generates 3-D images at a lower cost and at a lower absorbed dose compared with conventional computed tomography. This imaging technique is based on a cone-shaped X-ray beam centred on a 2-D detector that performs one rotation around the object, producing a series of 2-D images. These images are re-constructed in 3-D using a modification of the original cone-beam algorithm developed by Feldkamp et al. in 1984 (26).

In recent years, CBCT has been used for preoperative diagnosis in implant treatments. CBCT is superior because of its high definition, reduction of the exposure dose, low cost, and usability compared with CT. With the use of the CBCT, the dimensional accuracy is also comparable with CT, but in contrast to CT; the grey density values of the CBCT images (voxel value [VV]) are not absolute. In CBCT, the degree of one x-ray attenuation is shown by a grey scale (voxel value) (27). Some studies have shown good correlation between HU and VV and suggest that CBCT can be used in pre-operative evaluation of jawbone density in planning for implants (28,29).

Many articles from the literature suggest that bone quality evaluated by CBCT has a high correlation with the primary stability of the implants (30,31,32). Hence, preoperative density value estimations by CBCT may allow clinicians to predict implant stability.

Bone vascularity

Bone vascularity is an important factor in the process of osseointegration. After implant site preparation and implant insertion, tissue repair requires the development of a vascular system for a complete healing process (33,34). The early phase of healing proceeds from haematoma formation to woven bone formation. The late phase of healing results in bone remodelling and the formation of new bone, leading to osseointegration of the implant (35,36).

For assessment of tissue vascularity at the level of microcirculation, laser Doppler Flowmetry (LDF) is an appropriate method (37).This method has been used for the detection of blood flow in oral mucosal, pulpal, muscular and gingival tissues (38,39,40,41,42,43,44).

Recent animal and clinical studies showed that LDF is a reliable method for bone vascularity assessment during implant insertion (45,46). The method is based on a phenomenon known as the Doppler Effect i.e., a change in the frequency of light upon reflection from blood cells in motion.

Using the laser Doppler device software, the electronic impulse is expressed in perfusion units (PU), representing the number of cells multiplied by their average speed. Because the red blood cells are the majority of the mobile cells in the tissue, this means that the perfusion units are the blood velocity in the tissue (47). In their clinical study, Kokovic et al. showed that there is a statistically significant correlation between LDF measured during implant insertion and the changing values of implant stability in the late phase of osseointegration of dental implants in posterior mandibles (48).

Dental implant therapy requires an accurate preoperative assessment of the patient's hard and soft tissues. Clinicians should understand the indications, applications, and limitations of different imaging techniques to obtain information while keeping radiographic risks to a minimum. The use of CBCT with interactive planning software appears to meet the standard of care required for planning dental implant therapy (49). Bone density assessment using CBCT is an efficient method and significantly correlated with implant stability parameters and the Lekholm and Zarb index (50).

It can be concluded that CT and CBCT scanning are useful tools, providing not only morphological information but also bone density data, enabling the evaluation of the adequacy of potential dental implant sites prior to

implant placement. Keeping in mind the advantages of CBCT over conventional CT, it has to be used for the preoperative evaluation of bone quality.

Vascularity at the implant site has been identified as an important factor for the successful outcome of dental implant treatment (51). LDF is a reliable method for the determination of bone vascularity prior to implant insertion and might determine future implant stability.

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EVALUATION OF POTENTIAL CYTOTOXIC EFFECTS OF HERBAL EXTRACTS

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EVALUACIJA BILJNIH EKSTRAKATA SA POTENCIJALNIM CITOTOKSIČNIM EFEKTOM

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Abstract

Herbal medicines have played an important role in treating different diseases since ancient times. Bioactive components of medicinal plants are a good starting point for discovering new drugs such as chemotherapeutics. Currently, there are four classes of plant-derived chemotherapeutic drugs used in clinical practice. However, to discover new potential cytotoxic molecules, the research effort on herbal extracts has not diminished. The aim of this review was to evaluate the chemical constituents of plants that possess cytotoxicity, the signalling pathways responsible for this effect, and the influence of solvent polarity on potential cytotoxic effect and to present the cytotoxic activity of selected herbal extracts. The polyphenolic, anthraquinon, diterpneoid, triterpenoid, flavonoid, betulinic acid and berberine content contributes to cytotoxicity of herbal extracts. The inhibitory effect on cancer cells viability could be a consequence of the non-apoptotic processes, such as cell cycle arrestment, and the apoptotic process in tumour cells through different signalling pathways. The influence of solvent polarity on potential cytotoxic effect of herbal extracts should not be ignored. In general, the best cytotoxic activity was found in nonpolar and moderately polar herbal extracts. The herbal extract with IC_{50} below 30 μg/ml could be considered a very strong cytotoxic agent. Considering that many antitumor drugs have been discovered from natural products, further research on plants and plant-derived chemicals may result in the discovery of potent anticancer agents.

Keywords: *cytotoxicity; herbal extracts; herbal chemical constituents, apoptosis.*

SAŽETAK

Biljni lekovi imaju značajnu ulogu u lečenju različitih bolesti od davnina. Boaktivna jedinjenja lekovitih biljaka mogu biti dobra osnova za otkriće novih lekova kao što su hemoterapeutici. Trenutno se u kliničkoj praksi koriste četiri klase hemoterapetika biljnog porekla, ali istraživanje biljnih ekstrakata sa ciljem otkrića novih potencijalno citotoksičnih molekula ne prestaje. Cilj ovog preglednog rada je bio procena hemijskih jedinjenja koja pokazuju citotoksični efekat, signalnih puteva odgovornih za ovakav efekat, uticaja polarnosti rastvarača na potencijalnu citotoksičnost i prikaz citotoksične aktivnosti odabranih biljnih ekstrakata. Sadržaj polifenolnih jedinjenja, antrahinona, diterpenoida, triterpenoida, flavonoida, betulinske kiseline i berberina doprinosi citotoksičnom efektu biljnih ekstrakata. Inhibicija vijabilnosti ćelija kancera može biti posledica neapoptotskih procesa, kao što je prekid diferencijacije ćelija i apoptotskog procesa posredstvom različitih signalnih puteva. Ne treba zanemariti ni uticaj polarnosti rastvarača na potencijalni citotoksični efekat biljnih ekstrakata, ali generalno se smatra da najbolju citotoksičnu aktivnost poseduju nepolarni i umereno polarni ekstrakti. Biljni ekstrakti čija je IC_{50} vrednost ispod 30 μg/ml se mogu smatrati snažnim citotoksičnim agensom. Imajuću u vidu da su mnogi antitumorski lekovi dobijeni iz prorodnih proizvoda, dalja istraživanja biljaka i njihovih hemijskih jedinjenja mogu rezultovati otkrićem potentnih antitumorskih agenasa.

Ključne reči: *citotoksičnost, biljni ekstrakti, hemijski sastav biljaka, apoptoza*

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INTRODUCTION

For centuries, herbal medicines played a very important role in treating different diseases as a part of the Indian ayurveda, the traditional Chinese and Greek medicines (1). Today, there are more than 20 000 plant species that are used in traditional medicines (2). Bioactive components of medicinal plants are a good starting point for discovering new drugs (3). For example, cardiac glycosides obtained from *Digitalis lanata* and *Digitalis purpurea* (*Plantaginaceae*) have been successfully used for treating heart failure and certain cardiac arrhythmia with no alternative among synthetic drugs (1). According to ethnomedicinal uses, some plant compounds are used in therapies for different widespread diseases, including cancer (4). Currently, there are four classes of plant-derived chemotherapeutic drugs that are used in clinical practice. These are vinca alkaloids (vinblastine, vincristine and vindesine), epipodophyllotoxins (etoposide and teniposide), taxanes (paclitaxel and docetaxel) and the camptothecin derivatives (camptotecin and irinotecan) (5). According to the American National Cancer Institute, the upper limit for the IC_{50} value, which indicates the promising plant material for further cytotoxic agent purification, is 30 μg/ml (6).

Chemotherapy is routinely used for cancer treatment, but the toxicity of chemotherapeutics on healthy cells of the human body is obvious (5). This is the reason for discovering new, natural origin, substances with potential cytostatic effect and less toxic side effects on the healthy cells (2). Therefore, the main advantage of herbal extracts compared to synthetic drugs is their lower toxicity. For example, it is well known that long-term anthracycline (doxorubicin) therapy causes severe complications such as cardiotoxicity and acute myelocytic leukaemia (7). However, repetitive treatment with natural anthracycline (aloin) did not produce oxidative stress-induced cardiotoxicity in the animal model (8). Another important problem related to chemotherapeutic treatment is its multi-drug resistance, which leads to the decreased effectiveness of anti-cancer drugs. Several plant derivatives from the flavonoid class demonstrated a synergistic effect with anti-cancer drugs and increased the accumulation of drug in resistant cancer cells (9). A number of studies indicated that quercetin and its derivatives act synergistically with doxorubicin and daunorubicin to overcome multi-drug resistance in cancer patients (9, 10). Nevertheless, research effort on herbal extracts to discover new bioactive molecules with potential cytotoxic effect has not diminished (11).

The aim of this review was to evaluate both the chemical constituents of plants that possess cytotoxicity and the signalling pathways responsible for this effect. The solvent polarity influence on the potential cytotoxic effect of herbal extracts was taken into account. Additionally, the cytotoxic activity of selected herbal extracts was discussed.

Chemical constituents that exhibit cytotoxicity

There is a wide range of phytochemical compound classes that may contribute to cytotoxic effect of the plant. Some of the most common classes are anthraquinons, polyphenolic compounds and triterpenoid saponins. The high content of polyphenolic compounds and anthraquinons is responsible for the antioxidant activity, which is associated with stronger cytotoxic activity (6, 12). Several mechanisms have been involved in the anticancer activity of anthraquinons. These include the intercalation of DNA, inhibition of DNA topoisomerase II, production of free radicals and subsequent cleavage of DNA (13, 14). Esmat et al. researched the cytotoxic effect mechanism of the constituent (anthraquinone – aloin) in different aloe varieties on human breast cancer cell lines. Aloin showed dose-dependent cytotoxic action by reducing the proportion of cells that undergo mitosis by inducing apoptosis, inhibiting topoII**α** protein expression and downregulating cyclin B1 protein expression in breast cancer cells without erbB-2 and topoII**α** coamplification. TopoII**α** is primarily produced in the late S phase and during the G2M phase of the cell cycle. Meanwhile, cyclin B1 is important in the G2M progression because its reduced expression results in the accumulation of inactive Cdk1-cyclin B1 kinase complex (15). It is concluded that anthraquinone (aloin) induced the apoptosis and cell cycle arrest at the G2M phase (7).

The antioxidant activity of polyphenolic compounds is partly responsible for the correlation between the increased polyphenol intake and the reduced cancer risk. Polyphenols have the ability to down-regulate oxidative and inflammatory signal cascades by inhibiting transcription factors, such as the nuclear factor-kappaB (NF-kB) and the activator protein-1 (AP-1), which are responsible for the expression of the reactive oxygen species (ROS)-induced inflammatory enzyme cascade (16 - 18). The natural polyphenolic compounds, such as curcumin, genistein, resveratrol and catechins, can inhibit the number of growth factors involved in carcinogenesis. Additionally, phenolics have the ability to arrest cells in the S/G2 transition phase, increase the number of cells in the G1/S phase and induce apoptosis. Apoptosis is, probably, the consequence of decreased expression of the major anti-apoptotic oncogene (Bcl-2) (19). Phenolic suppression of angiogenesis is the result of matrixmetalloproteinase (MMP) attenuation and inhibition of vascular endothelial growth factor (VEGF) and their Src kinases (16). Additionally, phenolic compounds can influence the reduced metastatic process by attenuating the expression of vascular adhesion molecules (19). Flavonoid (apigenin) induces apoptosis by increasing the phosphorylation of Her2/neu via the PI3/Akt kinase pathway. The subsequent proteosomal degradation in breast cancer cells that over-express this receptor/transcription factor suppresses tumour growth (20). The possible attenuation mechanism of tumour metastasis is down-expression of the circulating soluble adhesion molecules (ICAM-1, E-selectin and E-cadherin) (19).

The cytotoxic effect of most of the researched triterpenoid saponins was based on their ability to stimulate apoptotic process in tumour cells, usually through its intrinsic pathway. In addition, the non-apoptotic processes, such as cell cycle arrestment, autophagic cell death stimulation, metastasis inhibition and cytoskeleton disintegration, were involved in the cytotoxic activity (21). The cytotoxic activity of triterpenoid saponins isolated from different plant species has been reported for various cell lines, which include human cervix (HeLa), human hepatocyte (Hep-G2), fibrosarcoma (HT1080) and human promyelocytic leukaemia (HL-60) cell lines (22). There are several possible apoptosis mechanisms. One of them is the caspase-3-mediated apoptotic pathway of triterpenoid saponins isolated from *Anemone flaccida* and confirmed on the HeLa cell line (23). The cell death induced by the plant extracts via the classic caspase-3-dependent apoptosis follows one substrate of caspase 3, the poly (ADP-ribose) polymerase (PARP) protein that undergoes cleavage after receiving the apoptotic signal (24). Additionally, the triterpenoid saponin class showed antitumor activities via the COX-2/PGE2 signalling pathway. Flaccidoside II, isolated from *Anemone flaccida,* has the ability to suppress COX-2 on both the mRNA and protein levels as well as on the PGE_2 synthesis level (the product of COX-2). Considering that the increased levels of COX-2 and its product PGE_2 are identified in cancer cells as markers of tumour angiogenesis, the inhibition of the COX-2/PGE2 pathway may be one of the possible apoptotic mechanisms of triterpenoid saponins (23, 25). Triterpenoid saponin, tubeimoside 1, which was isolated from *Bolbostemma paniculatum* and applied to HeLa cells, induced the depletion of mitochondrial transmembrane potential and caused the activation of caspase-dependent apoptotic cell death (21). Additionally, the increased expression of GADD153/CHOP transcription factor, which is associated with growth arrest and apoptosis in the event of prolonged endoplasmic reticulum (ER) stress, was noticed (26). The saponin-enriched fraction, isolated from *Bupleurum kaoi*, showed cytotoxic activity on human non-small cell lung cancer (A549) via the extrinsic apoptosis pathway. The enhancement in Fas and its two ligand forms, membrane-bound Fas ligand and soluble Fas ligand, was observed (27).

Betulinic acid, an antitumor agent, is a natural pentacyclic triterpenoid, which activates the mitochondria-mediated intrinsic apoptosis pathway (9). It was detected that betulinic acid has the ability to induce mitochondrial outer membrane permeabilization as the initial step in the activation of the mitochondrial pathway (28). In intact cells, betulinic acid caused the release of apoptogenic factor, cytochrome c, in a caspase-independent and permeability transition pore-dependent manner. It was reported that betulinic acid treatment caused ROS generation in different cancer cells that were involved in initiating the mitochondrial membrane permeabilization (29, 30). This is associated with the activation of pro-apoptotic p38 and stress-activated protein kinases (SAPK)/Jun amino-terminal kinases (JNK) without change in the phosphorylation of extracellular signal-regulated kinases (ERK). Therefore, ROS act upstream of the ERK in the betulinic acid signalling pathway (28). Additionally, betulinic acid was marked as the activator of NF-κB transcription factor and the regulator of stress-induced transcriptional activation in numerous cancer cell lines (31).

Berberine, which is extracted from roots, rhizomes, stems and bark of different medicinal plants, has the ability to inhibit cellular growth in melanoma, hepatocellular carcinoma, breast, prostate, colon cancers and leukaemia (32 - 34). It was suggested that the death receptor pathway may be involved in the apoptotic pathway induced by berberine because it influenced the up-regulated mRNA and/or protein expressions of Fas, FasL, TNF-alpha, caspase-3 and down-regulation of pro-caspase-3. Additionally, there is evidence that berberine-induced apoptosis was linked with up-regulated expression of the tumour suppressor gene p53 and prohibitin (PHB) and decreased vimentin expression (35). The possible mechanisms of berberine-induced breast cancer cell toxicity and apoptosis are presented in Image 1.

Taxane, a naturally occuring diterpneoid, exhibits an antiproliferative activity against breast cancer cells (36). There is no well-defined apoptotic pathway of taxanes. However, one important characteristic is the down-regulation of Bcl-2 and/or up-regulation of p53 and p21/WAF-1 (37). Some compounds that were later developed into full anticancer agents originated from natural sources and are lignans (38). Some of the lignan derivatives have reached phases I and II of clinical trials as anti-cancer agents and include GP-11, NK-611, TOP-53, NPF and GL-331 (39). The new native lignan, vitexin 6, activates the Jun N-terminal kinase (JNK) pathway and leads to the inhibition of cancer cell proliferation. Vitexin 6 treatment increases JNK phosphorylation, which is followed by the upregulation of P-Bcl-2 and P-C-Jun expression (40).

Methods for evaluating the cytotoxic effect

There are several *in vitro* and *in vivo* methods described in the literature that are used for evaluating cytotoxic activity. The most frequently used methods are the neutral red method, the Brine shrimp lethality assay, and the MTT and XTT assays (41 – 44).

The influence of solvent polarity on potential cytotoxic effect of herbal extracts

Herbal extracts could be obtained using extraction with polar or nonpolar solvents. The most commonly used polar solvents are water, ethanol, methanol, and the water/ethanol or water/methanol mixtures in different ratios. The nonpolar solvents used in herbal extraction are acetone, chloroform, methylene chloride and petrol ether (45). The type of extracted constituent depends on solvent polarity and, thus, contributes to different cytotoxicity.

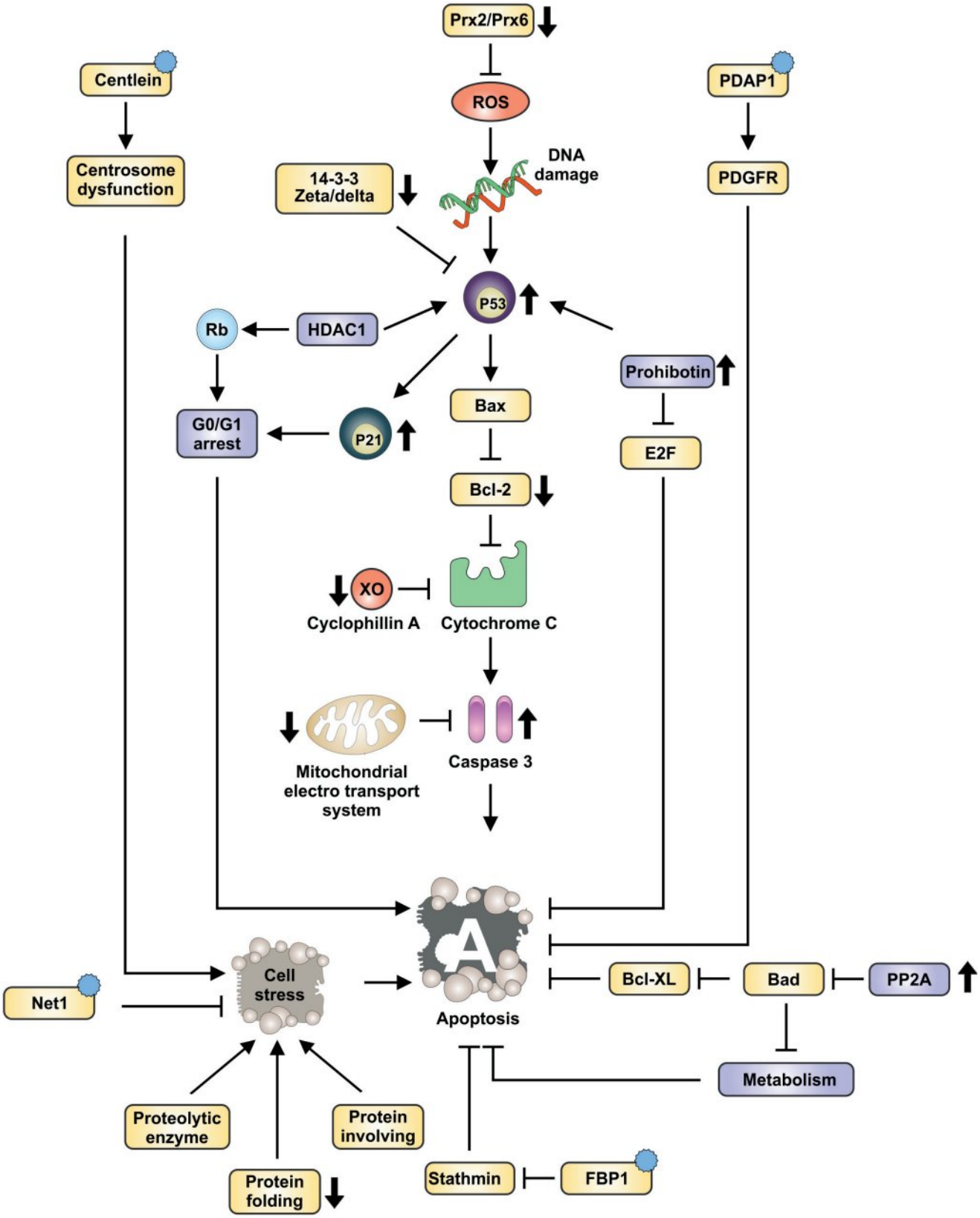

Image 1 - Mechanisms of berberine-induced breast cancer cell toxicity and apoptosis.

Even traditional herbal medicines are usually prepared as infusions or decoctions (water as an extraction solvent), and most of the aqueous extracts demonstrate a low cytotoxic activity (46, 47). The effect is usually observed by applying the extract in concentration above 250 μg/ml (46). However, components from the nonpolar extract possessed more clastogenic activity, which can be specifically targeted to destroy cancer cells (48). Additionally, the best cytotoxic activity was found in nonpolar and moderately polar herbal extracts (49). Data indicate that the majority of potent cytotoxic phytochemical compound classes can be extracted with nonpolar solvents. Triterpenes can be extracted with ether, petroleum ether and hexane, while flavonoids are usually detected in the chloroform and methanol extracts (50). Therefore, extraction with a nonpolar organic solvent is more appropriate for achieving a better cytotoxic effect.

Cytotoxic activity of the selected herbal extracts

Determination of cytotoxic activity of herbal extracts has become a prevalent research topic with numerous publications. A significant number of publications has been published in the last five years (2010 - 2014). The list of the investigated plant species for potential cytotoxic activity during this period is extensive: *Urtica dioica*, *Cucurbita pepo*, *Potentilla reptans*, *Struchium sparganophora*, *Sideritis scardica*, *Moringa oleifera*, *Cynometra cauliflora*, *Onosma paniculatum*, *Biophytum sensitivum*, *Ocimum viride*, *Terminalia arjuna*, *Garcinia indica*, *Acacia nilotica* and others (51, 2, 46, 38, 52 - 55, 11, 56-59). The cytotoxic effect of the extracts prepared from the selected medicinal plants will be discussed in this section.

Urticae folium (*Urtica dioica* L., *Urticaceae*) – the hydro-alcoholic extract (50:50) – was evaluated on HepG2 cells using the MTT assay in the presence of vinblastine as a positive control. The obtained EC_{50} value was 0.76±0.13 mg/mL. The authors concluded that the *Urticae folium* extract did not show a significant cytotoxic effect (51). This can be attributed to the extraction with a mixture of polar solvents and the absence of potent cytotoxic phytochemicals.

Solani fructus (*Solanum nigrum* L., *Solanaceae*) - hydro-alcoholic extracts with concentrations of 5, 25, 50, 100, 150 μg/ml were investigated on the HepG2 and CT26 (human colon) cancer cell lines in the presence of cisplatin and *Taxus baccata* extract as the positive control substances. The experiment was performed using the colonogenic assay. The IC_{50} values were 56.4±9.3 and 77.6±1.2 μg/ml for the HepG2 and CT26 cell lines, respectively. The *Solanum nigrum* extract could be a promising cytotoxic agent because of the obtained IC_{50} values that are similar and for the *Taxus baccata* extract, which is considered to be a common natural anticancer product (2). The cytotoxic activity of glycoprotein isolated from *Solanum nigrum* supports the fact that a single active compound usually possess a stronger activity compared with the entire extract, which is a mixture of all present compounds, the active and ballast material. Glycoprotein showed a significant cytotoxic and apoptotic effect at a concentration of 40 μg/ml after only 4 hours. It is suggested that the *Solanum nigrum* glycoprotein induces apoptosis via the mitochondrial apoptotic signal pathway. It caused the release of cytochrome c, stimulation of caspase-8, -9 and -3 activities, and the cleavage of poly(ADP-ribose)polymerase in HCT-116 cells (60). The cytotoxic activity of *Solanum nigrum* could be a consequence of the anthocyanidin presence in the fruits.

Potentillae rhizome and *herba* (*Potentilla reptans* L. *Rosaceae*) – in a previous work of the author, the cytotoxic activity of aqueous extracts for the concentration range of 100 – 800 μg/ml was evaluated on a mouse breast cancer cell line (4T1) using the MTT assay. The authors performed investigations with aqueous extracts because of the plant preparation method in its ethnopharmacological use (61). The results showed the dose-dependent low cytotoxic effect of *Potentilla reptans* extracts, which was in agreement with the literature data for the aqueous extracts activity (46). Flavonoids and phenol carboxylic acids are identified in the aerial parts of *Potentilla reptans* (62). These classes of compounds have shown the cytotoxic effect, but the limited extraction with water could be the reason for weak cytotoxic activity.

Struchii folium (*Struchium sparganophora* L., *Asteraceae*) – the methanol, aqueous and chloroform extracts were investigated. The extracts were used in concentrations of 10, 20, 40 and 80 μg/ml. The method used for the experiment was the *in vivo* test of mortality rate of tadpoles during 24 h. The aqueous extract applied at low concentrations (10 μg/ml and 20 μg/ml) showed a high cytotoxic effect. The chloroform extract showed the same cytotoxic effect as the aqueous extract, but at a higher concentration (80 μg/ml) (38). For the *in vivo* cytotoxicity testing of this plant, the aqueous extract showed a significant activity. This could be explained by the content of protocatechuic acid, p-coumaric acid and caffeic acid, which, because of their relative polarities, could be found in the aqueous fraction of the plant (63). The apoptosis mechanism has not yet been investigated.

Sideriti herba (Sideritis scardica L. *Lamiaceae*) – diethyl ether, ethyl acetate and *n*-butanol extracts were prepared. The extracts were used at a concentration range of 0 – 100 μg/ml, and the evaluation was conducted on mouse melanoma (B16) and promyelocitic leukaemia (HL-60) cell lines. The evaluation method used was the MTT assay. The diethyl ether extract showed a significant dose-dependent cytotoxicity on both cell lines at a concentration of 100 μg/ml. The most cytotoxic compounds in the diethyl ether extract of *Sideriti herba* were phenolics: luteolin, luteolin-7-o-β-glycoside, apigenin and apigenin-7-o-β-glycoside. There were two possible mechanisms involved in the cytotoxic activity of the *Sideriti herba* extract. The used cell types significantly increased their ROS production as a result of the extract treatment. Therefore, induction of the oxidative

stress might be involved in the cytotoxic activity. Additionally, the cell cycle block in the S/G2 M phase was observed in B16 cells after the extract treatment (52).

Moringae folium (*Moringa oleifera* L. *Moringaceae*) – the plant extraction was performed with a mixture of methanol and water with an 80:20 ratio. The experiment was performed on the Hela cell line using the MTT assay. Doxorubicin was used as the positive control substance. The authors applied the extract at very high concentrations (100, 200, 220, 240, 260, 280, 300, 320, 340, 360, 380, 400 and 500 μg/ml). A significant and concentration-dependent cytotoxic activity was observed at concentrations above 260 μg/ml (53). A high cytotoxic concentration obtained for the *Moringae folium* extract indicates that this plant did not show a significant cytotoxic activity. However, there is evidence that the aqueous extract of *Moringae folium* inhibits the NF-κB signalling pathway and increases the efficacy of chemotherapy in human pancreatic cancer cells. The nuclear factor kappa B plays a significant role in the resistance of pancreatic cancer cells to the apoptosis-based chemotherapy. An application of the investigated extract to the pancreatic cancer cells (Panc-1) caused expression down-regulation of the key NF-κB signalling pathway proteins and allowed sensitization of pancreatic cancer cells to chemotherapy. Additionally, the combined therapy of *Moringa folium* extract with cisplatin demonstrated a synergistic effect (64).

Cynometri fructus (*Cynometra cauliflora* L. *Fabaceae*) – the methanol extract with a concentration range of 5 - 30 μg/ml was applied to the HL-60 cell line. The cytotoxicity was determined using the colorimetric MTT assay, in the presence of vincristine sulphate as the cytotoxic control compound. The concentration of the extract that reduced 50% of the cell population was 0.9 μg/ml, which indicated that the methanol extract of *Cynometri fructus* possessed a very strong cytotoxic activity towards the HL-60 cells (54). *Cynometra cauliflora* contains phenolic compounds, flavonoids, triterpenoids and exhibits an antioxidant activity. The cytotoxic effect mechanism was not investigated, but it could be attributed to some signalling pathways described for its main constituents (65).

Onsomae radix (*Onosma paniculatum* L. *Boraginaceae*) – the cytotoxic activity of petrol ether extract with a concentration of 10 μg/ml was investigated on the human CCRF-CEM leukaemia, human breast (MDA-MB-231), glioblastoma (U251) and colon cancer (HCT 116) cell lines. The experiments were conducted using the XTT assay in the presence of vinblastine as the positive control. The viability of all cells treated with the petrol ether extract of *Onsomae radix* was strongly inhibited with a low extract concentration (10 μg/ml). The main compounds that express cytotoxicity belong to the naphthoquinone derivatives (66). The caspase-3-dependent apoptosis was detected in melanoma cells in response to the *Onosma paniculatum* extract (55). This is in agreement with the apoptosis pathway results for other naphthoquinone derivatives (67).

Cynarae flos (*Cynara cardunculus*, L. *Asteraceae*) – the n-hexane extract with five different concentrations (0.1-1 mg/ml) was applied to colorectal cancer cells (DLD1), and the cytotoxic effect was determined using the MTT assay. The *Cynarae flos* extract showed a dose-dependent inhibitory effect on the viability of DLD1 cells. The authors confirmed that this extract inhibited cell proliferation and induced the apoptotic pathway on DLD1 cells. Furthermore, the pro-apoptotic (BAX) gene expression and a cell cycle inhibitor (p21) were induced, while the anti-apoptotic BCL-2 gene expression was reduced in the extract's presence. However, a BAX/BCL-2 ratio increment was detected, which implied that the *Cynarae flos* extract had the ability to induce the intrinsic apoptotic pathway (68). Additionally, the phytochemical composition, which includes polyphenolic compounds, inhibits angiogenesis related to cancer (69).

Dilleniae radix (*Dillenia suffruticosa*, L. *Dilleniaceae*) – the cytotoxic effect of dichloromethane extract at the maximum concentration of 100 μg/ml was applied to the breast cancer cell line (MCF-7). The experiment was conducted using the MTT assay in the presence of tamoxifen as the positive control. The extract showed the time- and dose-dependent strong cytotoxic activity with an IC_{50} value of 15.5 ± 0.5 μg/mL after 72 hours of exposure. This significant result indicates that the *Dilleniae radix* extract has a potential for developing a new cytotoxic agent. In addition, the authors explained how the investigated extract exhibited the cytotoxic effect. The dichloromethane extract of *Dilleniae radix* induced the G0/G1 and G2/M phase cell cycle arrest and apoptosis in caspase-3 deficient MCF-7 cells probably via the up-regulation of NF-κB, JNK1 and down-regulation of the anti-apoptotic genes AKT1 and ERK1 (70). Additionally, other studies confirmed the signalling pathway responsible for cytotoxicity of *Dillenia suffruticosa* extract on breast cancer cells (71, 72). Qualitative phytochemical screening of *Dillenia suffruticosa* extracts indicated the presence of saponins, triterpenes, sterols, and polyphenolic compounds, which contribute to the cytotoxic activities (72).

Euphorbiae herba (*Euphorbia platyphyllos* L. *Euphorbiaceae*) – the extracts were prepared using four solvents with different polarity and applied with a concentration range of 10 to 300 μg/ml on the MCF-7 cell line. The trypan blue exclusion method was used for cytotoxicity evaluation. Unusually, the water extracts obtained using two different methods showed the lowest IC_{50} among all tested extracts. The IC_{50} values for infusion and decoction of *Euphorbiae herba* were 38.29 ± 0.57 and 27.79 ± 0.58, respectively. Therefore, decoction with an IC_{50} value lower than 30 μg/ml could be the potential source for obtaining a potent cytotoxic agent. It was shown that all *Euphorbiae herba* extracts induce significant DNA damage in MCF-7 cells. The direct interaction between a DNA-reactive agent and DNA is one of several pathways that leads to primary DNA damage. The *Euphorbia platyphyllos* content of flavonoids, which induces DNA damage, could be the reason

for cytotoxic activity. Additionally, the presence of jatrophane diterpenes might contribute to cytotoxicity of *Euphorbia platyphyllos* extracts (73).

Flueggea herba (*Flueggea leucopyrus*, L. *Phyllanthaceae*) – the aqueous extract cytotoxic effect was evaluated on human endometrial carcinoma cells (AN3CA) using the MTT assay, in the presence of thymoquinone as the positive control substance. The decoction exhibited a significant dose-dependent cytotoxicity with low IC_{50} values of 22.09 and 14.60 μg/mL at 24 and 48 h post-incubation, respectively (74). However, this result could not be considered as favourable because the extract was administered at a high concentration (approximately 400 μg/mL). *Flueggea leucopyrus* contains polyphenols, flavonoids and terpenoids, which could influence the cytotoxic effect (75). The apoptosis of AN3CA cells was improved using enhanced DNA fragmentation and caspase 3 and 9 activities, as well as the moderately increased radical scavenging activity (74).

Hydrangeae folium, (*Hydrangea angustipetala*, L. *Saxifragaceae*) – the extracts were prepared using extraction with 70% acetone and 50% ethanol. The cytotoxic effect was observed on the human gastric carcinoma cell lines (AGS and SNU-1) using the MTT test and also *in vivo* on the mouse lymphoid macrophage (P-388D1) cells transplanted intraperitoneally into the CDF1 male mice. The MTT assay was performed in the presence of doxorubicin. The acetone extract showed higher cytotoxicity than the ethanol extract, with an IC_{50} of 21.43 μg/ml. However, the two isolated active compounds (febrifugine and trans-3-p-coumaroylquinic acid) from the ethanol extract showed much lower IC_{50} values and a strong cytotoxic effect. The cytotoxicity of *Hydrangea angustipetala* extracts could be explained through decrease in PARP and pro-caspase 3 of the AGS and SNU-1 cells. The *in vivo* experiment showed that the *Hydrangea angustipetala* extract prolonged the survival days of the P-388D1- CDF1 bearing mice (76).

Picralimae semen, (*Picralima nitida* L. *Apocynaceae*) - aqueous, methanol, ethylacetate and hexane extracts were evaluated for cytotoxic activity *in vivo*, using the brine shrimp lethality assay. The extracts were applied at concentrations of 10, 100 and 1000 μg/ml. The hexane extract showed the strongest cytotoxic activity and the lowest LC_{50} value. The content of saponins, tannins, flavonoids and anthraquinones could be responsible for the expressed cytotoxicity (77).

Anemone rhizome, (*Anemone flaccida*, L. Ranunculaceae) - the subject of this research was not to investigate the cytotoxic effect of *Anemone rhizome* extract, but of the isolated triterpenoid saponins using the MTT test. The isolated constituents were applied to the human hepatocellular liver carcinoma (HepG2) and human hepatoma (BEL-7402) cell lines at a concentration range of 2.5 to 40 μg/ml. All tested substances showed a strong cytotoxic activity, considering the fact that all obtained IC_{50} values were below 30 μg/ml. The mechanism responsible for the cytotoxic effect of *Anemone flaccida* was inhibition of the COX-2/PGE2 pathway because this is confirmed for its main constituents, triterpenoid saponins (23).

CONCLUSION

This paper presents a review of the importance of investigations of herbal extracts to obtain natural products that potentially have a cytotoxic effect. According to the American National Cancer Institute, the herbal extract with IC_{50} lower than 30 μg/ml could be considered as a very strong cytotoxic agent. The majority of extracts presented in this review showed a significant cytotoxic effect, but only some of them are interesting for future research for developing an anticancer agent. An appropriate solvent for plant extraction should be chosen according to the phytochemical composition. In general, the best cytotoxic activity was found in the nonpolar and moderately polar herbal extracts. It was confirmed that the phytochemical compound classes, which contribute to cytotoxicity, have the ability to induce apoptosis using different signalling pathways.

This research topic is not of significant interest in Serbia, although our country is a notable source of medicinal plants. *Potentilla reptans* L. and *Sideritis scardica* L. that grow in Serbia are the only two species investigated for cytotoxicity by the domestic scientists. In generally, more research on plants and plant-derived chemicals is necessary to discover potent anticancer agents.

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ENDOMETRIAL INTRAEPITHELIAL NEOPLASIA (EIN) IN AN ENDOMETRIAL POLYP

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ENDOMETRIJALNA INTRAEPITELNA NEOPLAZIJA (EIN) U ENDOMETRIJALNOM POLIPU

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ABSTRACT

Endometrial intraepithelial neoplasia (EIN) is a monoclonal neoplastic cell proliferation of the endometrium associated with a significantly increased risk of endometrioid endometrial adenocarcinoma. We herein present the case of a 58-year-old female patient who underwent a hysterectomy with bilateral salpingo-oophorectomy because of the existence of endometrial intraepithelial neoplasia in an endometrial polyp. The patient had irregular uterine bleeding, which lasted 10 days. An endometrial polyp was diagnosed by ultrasound examination. The polyp was located in the isthmus of the uterus, on the back wall, and measured 32 mm x 25 mm. The patient underwent fractional dilation and curettage, and the specimens were subjected to a histopathological examination. The histopathological findings were EIN, endometrioid type, a focus of which was found within the endometrial polyps, as well as the endometrial polyp and proliferative endometrium. The endocervical tissue was normal. Given the age of the patient and the histopathological findings, she underwent a total abdominal hysterectomy with bilateral salpingo-oophorectomy. The final histopathological findings were EIN, endometrioid type with a focus found within the endometrial polyp; endometrial polyp; simple hyperplasia; chronic inflammation of the uterine cervix; hyperkeratosis of the cervical squamous epithelium; and cervicitis chronica. There was also hydrosalpinx of the left fallopian tube, and cystic follicles in the left ovary. There was no significant morphological change in the right ovary or fallopian tube. The surgical and postoperative course were normal. The patient was sent home on the fifth postoperative day in good general condition. A check-up performed one month after surgery showed normal findings.

Keywords: *endometrial intraepithelial neoplasia, endometrial polyp, adenocarcinoma*

SAŽETAK

Endometrijalna intraepitelna neoplazija (EIN) je monoklonalno neoplastično umnožavanje ćelija endometrijuma, sa znatno povećanim rizikom za razvoj endometrioidnog endometrijalnog adenokarcinoma. Prikazujemo pacijentkinju staru 58 godina kod koje je urađena histerektomija sa obostranom adneksektomijom a zbog postojanja endometrijalne intraepitelne neoplazije u endometrijalnom polipu. Pacijentkinja je imala neuredno krvavljenje iz materice koje je trajalo 10 dana. Ultrazvučnim pregledom je dijagnostikovan endometrijalni polip. Polip je bio lokalizovan u istmičnom delu materice, na zadnjem zidu, dimenzija 32x25mm. Kod pacijentkinje je urađena frakcionirana eksplorativna kiretaža i kiretman je poslat na patohistološki pregled. Patohistološki nalaz je bio: EIN, endometrioidnitip, višefokusa u okviru polipaendometrijuma. Polypusendometrii. Proliferativniendometrium. Endocervikalno tkivo običnih osobina. S obzirom na godine života pacijentkinje i patohistološki nalaz, pacijentkinja je podvrgnuta operaciji. Urađena je klasična abdominalna histerektomija sa obostranom adneksektomijom. Uterussaadneksimaje poslat na patohistološki pregled. Patohistološki nalaz je bio: Polypusendometrii. EINendometrioidnitip, nađeno je više fokusa u okviru endometrijalnog polipa. Hyperplasia endometrii simplex. Cervicitis chronica. Hyperkeratosis epithelii squamosi cervicis uteri. Metaplasia squamosa immatura endocervicis. Folliculi cystici ovarii sinistri. Hydrosalpinx et salpingitis chronica sinistri. Cystes paraovariales sinistri. Na isečcima iz desnog jajnika i jajovoda nisu nađene bitnije patohistološke promene. Operativni i postoperativni tok su protekli uredno. Pacijentkinja je otpuštana kući peti postoperativni dan, dobrog opšteg stanja. Kontrolni pregled mesec dana nakon operacije je bio uredan.

Ključne reči: *endometrijalna intraepitelna neoplazija, polip endometrijuma, adenokarcinom.*

ABBREVIATIONS

EIN – Endometrial Intraepithelial Neoplasia; **RI** - Resistance Index

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INTRODUCTION

Endometrial intraepithelial neoplasia (EIN) is a monoclonal proliferation of the endometrial glands, which is considered to be a premalignant condition due to its strong association with concurrent and/or subsequent endometrioid adenocarcinoma of the endometrium [1,2]. The risk factors for the development of EIN include exposure to oestrogens without opposing progestins, obesity, diabetes, and rare hereditary conditions. Protective factors include the use of combined oral contraceptive pills with low-dose oestrogen and progestin. EIN is a rare lesion, seen in only 1.4% of endometrial biopsy specimens [3]. A 45-fold increased risk of developing endometrial cancer has been reported in EIN positive patients [4]. Overall, 17.1% of women with EIN had carcinoma and 34.9% had either carcinoma or persistent EIN [5]. In women with EIN lesions, endometrial polyps in biopsy samples were encountered in 43.3 % of cases [3]. Depending on the population, the prevalence of endometrial polyps ranges from 8-35% [6, 7]. The prevalence of malignant and premalignant lesions in endometrial polyps rages from 0.5% to 4.8% [8, 9, 10]. The usual treatment for EIN is surgery *i.e.,* hysterectomy. In the case of younger women in whom fertility needs to be preserved, EIN can be treated with oral progestins or the application of hormonal intrauterine devices [4].

CASE REPORT

A 48-year-old perimenopausal woman was admitted to the Gynaecology and Obstetrics Clinic "Narodni Front" in Belgrade due to prolonged abnormal uterine bleeding and an endometrial polyp, which was diagnosed by ultrasound examination. The patient had irregular uterine bleeding that had started ten days earlier. Two months before she was admitted to our clinic, the patient had regular menstruation. She had a normal body weight with a body mass index of 23 kg/m^2. Her blood pressure and glucose levels were within the normal limits. There were no data on the existence of malignancy in her family history. She had undergone regular gynaecological check-ups. In terms of her obstetric history, she had experienced one birth and one miscarriage. Her menstrual cycles were previously normal. Three years earlier, the patient had undergone dilation and curettage due to prolonged uterine bleeding. The histopathological findings at that time were proliferative endometrium and a cervical polyp. The patient had again undergone fractional dilation and curettage due to prolonged abnormal uterine bleeding one year before visiting out clinic. The histopathological findings were simple hyperplasia and endometrial polyp.

After admission to our clinic, the patient underwent a gynaecological examination, colour Doppler ultrasound examination and a laboratory analysis. In the gynaecological examination, we observed that the uterine cervix was 2 cm long and closed. The uterus was in the normal position but was slightly higher than normal, had a firm consistency, and was mobile and insensitive. The right adnexa was free and insensitive, and the left adnexa was thickened and palpation-insensitive. A transvaginal colour Doppler ultrasound examination revealed that the uterine body was 80 mm x 45 mm x 47 mm, whereas the endometrial thickness was 11 mm. In the uterine isthmus, on the back wall, an endometrial polyp of 35 mm x 25 mm was found (**Figure 1**). The size of the right ovary was 28 mm x 20 mm, with a homogeneous texture. The size of the left ovary was 35 mm x 30 mm, with a unilocular cystic formation of 25 mm x 20 mm filled with transonic contents. Near the left ovary was a visibly expanded terminal portion of the left fallopian tube filled with transonic contents, measuring 30 mm x 20 mm. Normal blood flow was registered by colour Doppler ultrasound in the large blood vessels of the uterus and ovaries. In the central polyp blood vessel, the flow was registered to have a resistance index (RI) of 0.43, and the flow in the peripheral vessel was 0.65. The results of the laboratory analysis and biochemical tests were normal. The patient underwent fractional dilation and curettage.

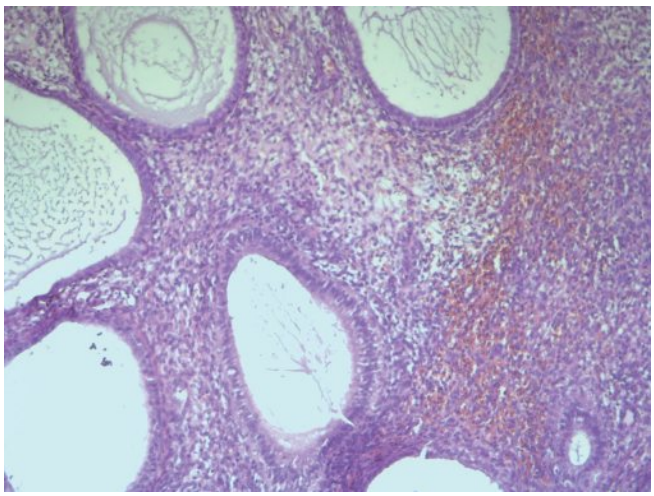

Figure 1. Endometrial polyp, 10X, hematoxylin and eosin stain

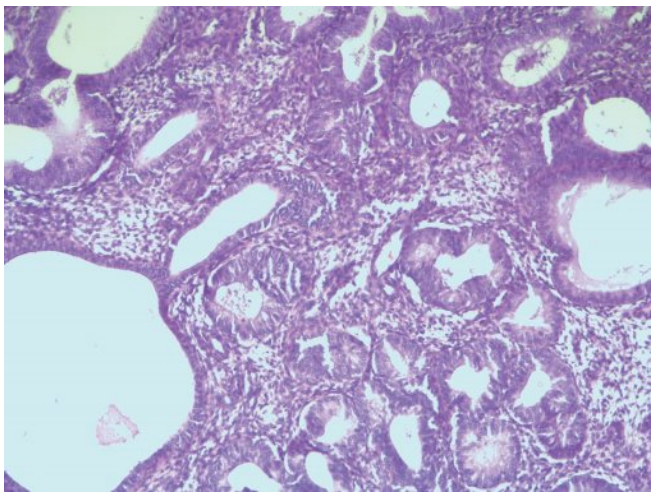

Figure 2. Endometrial intraepithelial neoplasia in the endometrial polyp, 10X, hematoxylin and eosin stain.

The specimens from the cervical canal and uterine cavity were sent for histopathological examination. The histopathological findings were EIN, endometrioid type, with a focus within the endometrial polyp (**Figure 2**), endometrial polyp, and proliferative endometrium. The endocervical tissue was normal.

Given the age of the patient and the histopathological findings, it was decided that surgery should be performed. Total abdominal hysterectomy with bilateral salpingo-oophorectomy was performed, and the uterus with adnexa was sent for a histopathological examination. The final histopathological findings were: EIN, endometrioid type, with a focus found within the endometrial polyp; endometrial polyp; simple hyperplasia; chronic inflammation of the uterine cervix; hyperkeratosis of the cervical squamous epithelium; hydrosalpinx of the left fallopian tube; and cystic follicles in the left ovary. No significant morphological changes were found in the right ovary and fallopian tube. The intraoperative and postoperative course were normal. The patient was sent home on the fifth postoperative day in good general condition. A check-up performed one month after surgery showed normal findings.

DISCUSSION

Endometrial intraepithelial neoplasia (EIN) is a monoclonal premalignant endometrial glandular lesion that precedes the development of endometrioid-type endometrial adenocarcinoma [11,12, 13]. The average age of patients at diagnosis was 55 years [6]. Our present patient was 48 years old and in a perimenopausal state with irregular uterine bleeding and endometrial polyps. The patient underwent fractional dilation and curettage. The histopathological examination showed the existence of EIN in the endometrial polyp**.** A classification system for endometrioid intraepithelial neoplasia was introduced in 2000 by the International Endometrial Collaborative Group and defines three disease categories: benign hyperplasia, EIN and cancer [1, 14]. EIN is not divided into subgroups. It is important to distinguish it from earlier phases of latent premalignant disease and endometrial carcinoma. EIN represents premalignant changes that can withdraw, persist or progress into invasion. EIN is a rare lesion, seen in only 1.4% of endometrial biopsy specimens [3]. Overall, 17.1% of women with EIN had carcinoma and 34.9% had either carcinoma or persistent EIN [5]. The risk factors for the development of EIN include exposure to oestrogens without opposition by progestins, obesity and diabetes, and a significant role is played by heredity. The protective factors have been suggested to include the use of combined oral contraceptives with low doses of oestrogen and progestin.

In order to make the diagnosis of EIN, five criteria must be met in a single fragment, including architectural gland crowding, altered cytology, a minimum size of 1 mm, exclusion of carcinoma, and exclusion of mimics [11]. The diagnosis of EIN can be summarized as a focus of clustered endometrial glands exceeding a gland to stroma ratio of 1:1, which have altered cytology from the background endometrium, and which comprise a sufficient volume of 1 mm [15]. The diagnosis of EIN is associated with a 27% likelihood of having "concurrent" adenocarcinoma within one year and carries a 45-fold increased risk for a future diagnosis of adenocarcinoma after one year [1]. EIN and benign simplex hyperplasia often appear together but have different histology. EIN is a monoclonal endometrial proliferation, while hyperplasia represents a polyclonal endometrial proliferation.

A polyp is a benign endometrial lesion that represents nodular protrusions above the endometrial surface, consisting of irregularly distributed endometrial glands and stroma [16]. Polyps can be found in women of all ages but are most common in women between 40 and 50 years of age. The incidence of endometrial polyps ranges from 10% to 40% in women with abnormal uterine bleeding [8]. The aetiology of endometrial polyps is believed to be related to oestrogen stimulation. Asymptomatic polyps are often incidentally found during transvaginal ultrasound investigations [17]. The symptoms of endometrial polyps include irregular menstrual bleeding, bleeding between menstrual periods, excessively heavy menstrual bleeding and vaginal bleeding after menopause [6,18]. Postmenopausal women with endometrial polyps have an increased risk of malignancy compared with premenopausal women with endometrial polyps [19]. The prevalence of premalignant or malignant polyps was 5.42% in postmenopausal women compared with 1.7% in reproductive-age women. The prevalence of endometrial neoplasia within the polyps in women with symptomatic bleeding is 4.15% compared with 2.16% for those without bleeding. Among symptomatic postmenopausal women with endometrial polyps, 4.47% had a malignant polyps in comparison to 1.51% of asymptomatic postmenopausal women [20].

In women with EIN lesions, endometrial polyps are present in 43.3% of biopsy samples of the endometrium, whereas in women without EIN lesions, endometrial polyps are diagnosed in 12.9% of cases [3]. Hysterectomy is usually the treatment of choice if a woman has decided not to have any more children. Young patients wishing to preserve their fertility and women who are poor surgical candidates may be treated with oral progestins or hormonal intrauterine devices [4, 21]. Treatment with systemic progestins can successfully ablate up to 90% of endometrial precancers in young women [22]. Progestin therapy for EIN is often accompanied by nuclear shrinkage in neoplastic glands [23].

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CLINICAL PRESENTATION OF THE ABUSE OF INSULIN: HYPOGLYCAEMIC COMA AND ASPIRATION PNEUMONIA IN NON-PROFESSIONAL BODYBUILDERS

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KLINIČKA PREZENTACIJA ZLOUPOTREBE INSULINA: HIPOGLIKEMIJSKA KOMA I ASPIRACIONA PNEUMONIJA KOD NEPROFESIONALNOG BODIBILDERA

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ABSTRACT

The use of medications that improve the physical performance of an individual represents a very serious worldwide health problem. The abuse of these medications is increasing. Herein, we describe a patient, at the age of 20, who was hospitalized due to loss of consciousness and difficulty breathing. At admission, the patient was unconsciousness, tahi-dyspnoic, and had a pale complexion and an athletic build. In gas analyses, extremely low saturation was observed, followed by acidosis, heavy hypoxia with normocapnia, higher lactates, hypocalcaemia and severe hypoglycaemia. The patient was treated with a hypertonic solution of glucose and intubated, with the aspiration of sanious content from the respiratory tract. After treatment, the patient woke from coma but was very confused. In the first 6 hours of hospitalization, severe hypoglycaemia occurred several times, despite the continuous administration of glucose. Due to the growth of inflammatory syndrome since the first day of hospitalization, the patient was kept in the hospital for treatment along with the administration of antibiotic treatment. On the fourth day of hospitalization, the patient stated that for the last year, he had been taking testosterone at a dose of 1 g a week, as well as tamoxifen pills and 15 i.j. of fast-acting human insulin (Actrapid®) daily for their combined anabolic effect. Hypoglycaemic coma, caused by the abuse of insulin, represents a severe complication in patients, which can be followed by confusion, a slowed thinking process, the weakening of cognitive functions and even death. It is necessary to invest great efforts into the prevention of the purchase of these medications via the Internet or on the black market for purposes of abuse in order to prevent such serious and life-threatening complications.

Key words: *bodybuilding, testosterone abuse, insulin abuse, hypoglycaemic coma*

SAŽETAK

Upotreba lekova koji poboljšavaju fizičke performance pojedinca predstavlja jako veliki zdravstveni problem u svetu. Zloupotreba ovih lekova je u velikom porastu. Prikazali smo pacijenta, starosti 20 godina, hospitalizovanog zbog gubitka svesti i otežanog disanja. Na prijemu pacijent je bio bez svesti, tahi-dispnoican, blede koze, atletske konstitucije. U gasnim analizama viđena je jako niska saturacija praćena acidozom, teška hipoksija sa normokapnijom, povišeni laktati, hipokalcemiju i teška hipoglikemija. Pacijent tetiran hipertonim rasvorom glukoze, intubiran, sa aspiracijom sukrvičavog sadržaja iz disajnih puteva. Nakon tretmana pacijent se budi iz kome, postaje budan ali jako konfuzan. U prvih 6h hospitalizacije više puta dolazi do pojave teške hipoglikemije i pored kontinuirane administracije glukoze. Zbog rasta zapaljenskog sindroma, od prvog dana hospitalizacije, pacijent je zadrzan na bolničkom lečenju uz ordiniranje antibiotske terapije. Četvrtog dana hospitalizacije pacijent navodi da poslednjih godinu dana koristi testosteron u dozi od 1g nedeljno, tablete tamoxifena kao i 15 i.j. brzodelujućeg kristalnog humanog insulina dnevno (Actrapid®), radi zajednickog anabolickog efekta. Neželjeni efekti preparata koji imaju anabolički efekat mogu biti jako opasni. Hipoglikemijska koma, izazvana zloupotrebom insulina, predstavlja tešku komplikaciju po pacijenta, koja može biti praćena, konfuzijom, usporenošću misaonog toka, slabljenjem kognitivnih funkcija ali i smrtnim ishodom. U budućnosti je potrebno uložiti jako velike napore u cilju onemogućavanja nabavke lekova sa ciljem zloupotrebe, preko interneta, na crnoj berzi, sa ciljem sprečavanja ovako teških, po život opasnih komplikacija.

Ključne reči: *bodybuilding, zloupotreba testosterona, zloupotreba insulina, hypoglikemijska koma.*

ABBREVIATIONS

AAS - anabolic androgen steroids
FSH - follicle-stimulating hormone
GnRH - gonadotropin-releasing hormone
GH - growth hormone
LH - luteinizing hormone
MV - mechanical ventilation

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INTRODUCTION

The use of medications that improve the physical performance of individuals represents a very serious worldwide health problem. The number of sportsmen in semi-professional as well as in popular sports self-administering ergogenic pharmacological agents continues to be an issue. Many athletes use anabolic-androgenic steroids (AAS) to obtain a well-trained, athletic, and healthy-looking body (1). The abuse of these medications is rapidly increasing. The greatest abuse of these medications is in non-professional athletes, especially bodybuilders. The most frequent age that these medications are abused is between 15 and 35 years (2). Professional athletes have a clearly defined plan and program to achieve their goals. In contrast, young people who are involved in sports or just starting to exercise abuse performance-enhancing drugs as a shortcut to their goal. The abuse of these substances is more common in men (3). The most significant factors that influence the decision to abuse ASS are the following: personality, potential side effects, benefit, social influence, and morality. (4, 5) These drugs are purchased via the Internet or on the black market (6, 7).

CASE REPORT

A patient, aged 20, was hospitalized due to the loss of consciousness and difficulty breathing. The data were heteroanamnestically obtained from the father. The loss of consciousness occurred on the day of admission, 5 hours before hospitalization, when the patient complained about hunger, after which he lost consciousness and started breathing heavily. The patient recently used an insulin preparation on his own initiative, for the purpose of gaining muscle mass. At admission, the patient was unconsciousness, tachypneic, dyspneic, had a pale complexion and an athletic build, with body height=185 cm, body weight=104 kg, and BMI=30.72 kg/m^2. The head and neck were of usual configuration and shape; the patient displayed dilated pupils, medially located bulbi, no neck rigidity, and stretch marks in the shoulder area. In the lungs there was intensified respiratory murmur, with the presence of a low-toned whistle; heart activity was rhythmic, with clear tones; TA=105/55 mmHg. At the level of the thorax, the abdomen did not displays a peritoneal reaction. An electrocardiogram indicated 112 beats/min, without pathologic changes. Gas analyses (arterial blood) showed very low saturation of 67%, acidosis at pH 7.33, severe hypoxia with normocapnia at pO2 4.9, pCO2 4.7, bicarbonates at the lower limit of 18.6, hypocalcaemia with ionized Ca 0.99, a limited value of potassium at 3.6, lactates of 8.6, an Hct of 56% and severe hypoglycaemia at 1.6 mmol/L.

Upon admission, the patient was treated with 100 ml of 50% glucose and intubated, with the aspiration of sanious content from the respiratory tract. Due to respiratory insufficiency and coma, the patient was sedated, intubated and put on mechanical ventilation. An urgent endocranial CT was performed to exclude neurologic diseases and was found to be normal. After treatment with a hypertonic solution of glucose, the patient woke from coma but was confused. In the first 6 hours of hospitalization, severe hypoglycaemia occurred several times (two times, 2.0 mmol/l), despite the continuous administration of glucose solution. An RTG of the lungs showed the presence of a blotchy confluent shadow on the right side in the upper and middle lung areas. As the increase in inflammatory syndrome markers from the first day of hospitalization **(Table 1)**, given the context of the RTG findings in the lungs, could be explained by the occurrence of aspiration pneumonia, the patient was medically treated via the administration of antibiotic therapy. On the fifth day of hospitalization, the patient was extubated and taken off the mechanical breathing apparatus **(Table 2)**. The confusion and bradypsychia of the patient remained until the fourth day of hospitalization, after which the patient reconstructed the events before the loss of consciousness. He stated that for the last year, during training cycles, he had been taking testosterone at a

Table 1. The first laboratory analysis

Analyses	Value	Reference value	Analyses	Value	Reference value
Le	19.4	3.0-10.0x109/L	Amylase S	178	28-104U/L
Er	5.11	4.35-5.72x1012/L	CK	915	0-170U/L
Hgb	147	138-175 g/L	CK MB	59.2	0-25U/L
Hct	0.426	0.415-0.530	Troponin I	0.127	0-0.04 µg/L
Tr	383	135-450x109/L	proBNP	21	0-125pg/ml
APTT	20.8	25-35s	Ca	1,96	2.0-2.65 mmol/L
INR	1.336	0.9-1.1	K	4.2	3.5-5.3 mmol/L
D Dimer	7206.64	0-500 ng/ml	Na	138	137-147 mmol/L
Urea	2.5	3.0-8.0 mmol/L	CRP	80	0-5.0 mg/L
Creatinine	102	49-106 µmol/L	Pct	0.049	0-0.5 ng/ml
AST	38	0-40IU/L	Albumin	30	35-52 g/L
ALT	35	0-40IU/L	HbA1c	5.1%	4.0-7.0%

Table 2. Gas analyses during hospitalization

Gas analyses	Day I	Day II	Day III	Day IV	Day V	Reference value
pH	7.33	7.46	7.45	7.42	7.47	7.35-7.45
pCO_2	4.70	4.9	5.9	5.9	5.3	<6.0
pO_2	4.9	13.7	16.7	13.6	9.9	>10.7
lactates	8.6	1.4	1.4	3.6	1.0	<2.2
HCO_3	18.6	27.1	29.7	27.5	28.8	18.0-23
Saturation O_2	67%	98%	99%	98%	96%	>95%
% O_2 in air	21%	MV - 50%	MV – 50%	MV – 35%	21%	

dose of 1 g a week, as well as 20 mg tamoxifen pills and 15 i.j. of fast-acting regular human insulin (Actrapid®) daily for their combined anabolic effect. The information about this effect of the medications was found on the Internet.

DISCUSSION

Doping in sport has a very long history. Every year, the Medical Committee of the International Olympic Committee issues a publication related to prohibited substances in sport. A large number of people use medications for the purpose of increased combined effects. In addition to the most frequently used anabolic androgen steroids (AAS), other preparations of growth hormone (GH), insulin, 5-α-reductase blockers and luteinizing hormone (LH) are also used. These hormones act synergistically and have an anabolic effect. Anabolism is defined as the state in which there is a positive balance of nitrogenous substances in the organism, and whether there is a stimulation of the protein synthesis in the organism or a decrease in the degradation of already-existing proteins in the organism.

Anabolic androgen steroids

Testosterone is a hormone with multiple physiologic, especially reproductive and metabolic, functions. It is the primary sex hormone in males, with androgenic and anabolic effects. Androstenedione and dehydroepiandrosterone have effects similar to those of testosterone. These hormones are mutually called AAS (8). These are relatively small molecules, and by passive diffusion, they reach all the cells in an organism, where they encourage gene transcription and lead to iRNK production. The enzyme 5 α-reductase has a significant role in the conversion of androgens and in the production of female sex hormones in males (9). Supraphysiological doses of AAS lead to a gain in muscular mass and strength, even with only occasional training. The anabolic effect is achieved by the binding of glyco-corticoid receptors and the prevention of the glyco-corticoid catabolic effect (10).

The most common case of abuse is the use of AAS in cycles of several weeks with short pauses in between. The effect of such AAS use is a decrease in the concentrations of thyroxin, cortisol, sex hormones and growth hormones (11). Overall, the use of AAS in supraphysiological doses leads to an increase in muscular mass and the strength of the muscles, a reduction in muscle damage, an increase in protein synthesis, an increase in lipolysis, a decrease in the percentage of body fat, an increase in bone density, an increase in the process of erythropoiesis, increases in the values of haemoglobin and haematocrit and an increase in glycogen reserves (12). Adverse effects of abuse are present in numerous organic systems. In the cardiovascular system, they intensify the occurrence of the prothrombogenic state, cause vasospasm in the blood vessels and have direct toxic effects on the myocardium. Frequent findings of AAS abuse are also eccentric hypertrophy of the left ventricle with diastolic dysfunction, arrhythmia and the frequent occurrence of ischemic events (13, 14). The most frequently observed types of liver damage are cholestasis, hepatitis and a disorder of the synthesis of coagulation factors (15, 16). Supraphysiological doses of AAS are hazardous to the endocrine and reproductive systems. Long-term use leads to the decreased production of testosterone, sex hormones, and proteins that bind sex hormones, as well as decreases in testicle size, sperm cell mobility disorders, decreases in fertility, changes in libido and the occurrence of gynaecomastia. The effects can sometimes be reversible. Gynaecomastia often develops with AAS abuse, and occurs as a consequence of the increased concentration of oestrogen that is produced by the endogen conversion of AAS and leads to breast enlargement in men (17). Changes in muscular-skeletal system are characterized by short periods of growth, frequent tearing of the ligaments and tendons, muscle damage and rhabdomyolysis. The adverse effects on the CNS can be various, from increased aggression towards others, behavioural disorders, manic behaviour, an inclination to violence, and the occurrence of psychoses, paranoia and hallucinations as well as depression and sleep disorders (17,18).

Blockage of 5 α-reductase

One of the additional ways by which the additional leap in testosterone concentration is achieved is by the induction of high concentrations of gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH) secretion.

Direct stimulation, via the administration of LH, is typically very complicated and carries huge risks of decreased testosterone secretion due to irregular use of the preparations; hence, many bodybuilders resort to an indirect stimulation of LH and testosterone production (19), which can be achieved in several ways: by applying neurotransmitters, neuropeptides, blockers of negative feedback mechanisms or blockers of oestrogen. The use of these medications leads to the increased production of endogenous LH via the manipulation of the physiological regulatory systems that are responsible for pulsatile hypothalamic GnRH secretion (19, 20). The aromatization of testosterone represents an irreversible process, and the blockage of this process leads to the blockage of the effect of oestrogen on an organism. With the blockage of oestrogen receptors in the hypothalamus by medication, the activation of the feedback mechanism occurs, increasing the secretion of sex hormones of the pituitary gland and consequently testosterone up to as much as 40% (21, 22). More efficient medications that act via this mechanism can lead to significant increases in muscular mass and strength. There are no significant clinical indications for the use of these medications, except in the treatment of gynaecomastia and older men with prostate carcinoma (23). Bodybuilders primarily use the effect of these medications to restart the secretion of sex hormones of the pituitary gland, which was suppressed by extremely high values of testosterone during the training cycle.

Growth hormones and Insulin-like Growth Factor 1

The abuse of growth hormones presents another health problem. GH acts synergistically with other hormones in an anabolic manner and leads to an increase in muscle mass (24). The main effect is achieved by the increased entry of amino acids into the cells and the increased synthesis of proteins. For the full effect of GH, a certain concentration of oestrogen is necessary, which is produced by peripheral conversion (25). The use of aromatization inhibitors leads to the increase of GnRH, LH, follicular stimulating hormone (FSH) and testosterone concentrations but also results in the decreased effect of insulin-like growth factor 1; hence, inhibitors are primarily used to start the secretion of sex hormones of the pituitary gland.

Insulin

The effects that insulin has on cells have led to it being abused by sportsmen. Insulin enables the easier entry of glucose into the cells, so a larger amount of glucose enters the cells than is currently necessary for the process of cell respiration, along with the formation of an energy depot in the form of glycogen. The increase in the energy depot has an important role in muscle recovery after a load. There are opinions that insulin also increases the size of the muscle itself. It is considered that this effect is primarily attributed to fast-acting insulin, which prevents the degradation of the existing proteins in the muscle itself, whereas GH is primarily responsible for the synthesis of new proteins in the muscle (26).

Physical activity presents a risk of hypoglycaemia when administering fast-acting insulin before training, which additionally increases the probability of the occurrence of hypoglycaemic episodes. Severe hypoglycaemic episodes, almost as a rule, are followed by the loss of consciousness. Injuries caused by the loss of consciousness can also be very serious for the patient. Hypoglycaemic episodes can lead to serious brain damage and increase patient mortality, especially if they last for an extended period of time. The administration of insulin to people who are not educated carries a high risk for the development of severe complications, and the abuse of this medication can be life threatening (27, 28).

CONCLUSION

The abuse of medications in sport represents a very serious health problem. Large numbers of amateur athletes use prohibited means to achieve better results. The adverse effects of medications that are used can be very dangerous for the health of patients. Hypoglycaemic coma, iatrogenically caused by the abuse of insulin, represents a very serious complication, which can be followed by confusion, a slowed thinking process, loss of cognitive function, and also death. It is important to invest significant effort into the prevention of the purchase of medications for the purpose of the abuse via Internet, on the black market or in gyms in order to prevent these serious, life-threatening complications.

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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.

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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.

3. Article

a. Journal Article (one author)

Format:

Author. (Year of publication). Article title. *Journal Title*. *Volume* (issue), range of pages. DOI.

Example:

Nikora, V. (2006). Hydrodynamics of aquatic ecosystems: spatial-averaging perspective. *Acta Geophysica*, 55(1), 3-10. DOI: 10.2478/s11600-006-0043-6.

b. Journal Article (two or more authors)

Format:

Author1, Author2 & Author3. (Year of publication). Article title. *Journal Title*. *Volume* (issue), range of pages. DOI.

Example:

Cudak, M. & Karcz J. (2006). Momentum transfer in an agitated vessel with off-centred impellers. *Chem. Pap.* 60(5), 375-380. DOI: 10.2478/s11696-006-0068-y.

c. Journal article from an online database

Format:

Author(s). (Year of publication). Article title [Electronic version]. *Journal Title. Volume* (issue), range of pages. Retrieved date of access, from name of database. DOI.

Example:

Czajgucki Z., Zimecki M. & Andruszkiewicz R. (2006, December). The immunoregulatory effects of edeine analogues in mice [Abstract]. *Cell. Mol. Biol. Lett.* 12(3), 149-161. Retrieved December 6, 2006, from PubMed database on the World Wide Web: http://www.pubmed.gov. DOI: 10.2478/s11658-006-0061-z.

d. Newspaper article (no author)

Format:

Article title. (Publication date). Journal Title. page.

Example:

Amazing Amazon region. (1989, January 12). New York Times, p. D11.

e. Encyclopedia article

Format:

Author. (Year of publication). Article title. In Encyclopedia title (volume number, pages). Place of publication: Encyclopedia name.

Example:

Bergmann, P. G. (1993). Relativity. In The new encyclopedia britannica (Vol. 26, pp. 501-508). Chicago: Encyclopedia Britannica.

4. Other formats

a. Web page

Format:

Author/Sponsor. (last update or copyright date). *Title*. Retrieved date of access, from URL.

Example:

Walker, J. (1996, August). *APA-style citations of electronic resources*. Retrieved November 21, 2001, from http://www.cas.usf.edu/english/walker/apa.html

b. Lecture note

Format:

Author(s). (Date of presentation). *Lecture title*. Lecture notes distributed in the unit, at the name of the teaching organisation, the location.

Example:

Liffers, M. (2006, August 30). *Finding information in the library*. Lecture notes distributed in the unit Functional Anatomy and Sports Performance 1102, University of Western Australia, Crawley, Western Australia.

c. Patent

Format:

Author. (Year). Patent number. The location. Issue body.

Example:

Smith, I. M. (1988). U.S. Patent No. 123,445. Washington, D.C.: U.S. Patent and Trademark Office.

d. Standard

Format:

Issue body. (Year). Standard name. Standard number. The location.

Example:

Standards Association of Australia. (1997). Australian standard: Pressure equipment manufacture. AS4458-1997. North Sydney.

e. Video

Format:

Producer, P. P. (Producer), & Director, D.D. (Director). (Date of publication). Title of motion picture [Motion picture]. Country of origin: Studio or distributor.

Example:

Zhang, Y. (Producer/Director). (2000). Not one less [Motion Picture].China: Columbia Pictures Industries, Inc.

f. Audio recording

Format:

Songwriter, W. W. (Date of copyright). Title of song [Recorded by artist if different from song writer]. On Title of album [Medium of recording]. Location: Label. (Recording date if different from copyright date).

Example:

Taupin, B. (1975). Someone saved my life tonight [Recorded by Elton John]. On Captain fantastic and the brown dirt cowboy [CD]. London: Big Pig Music Limited.

g. Mailing list

Format:

Author. (Exact date of posting). Subject line of message. Message posted to followed by name of mailing list, archived at followed by address for the archived version of the message

Example:

Hammond, T. (2000, November 20). YAHC: Handle Parameters, DOI Genres, etc. Message posted to Ref-Links electronic mailing list, archived at http://www.doi.org/mail-archive/ref-link/msg00088.html

h. Computer software

Format:

Author(s). (Year). Title [computer software]. The location: Company.

Example:

Ludwig, T. (2002). PsychInquiry [computer software]. New York: Worth.

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Article in a journal:

Lippke, S., & Ziegelmann, J. (2006). Understanding and modelling health behaviour change: The multi-stage model of health behaviour change. *Journal of Health Psychology, 11*(1), 37-50,
DOI:10.2478/s11533-007-0023-3.

Book:

Jones, E., Farina, A., Hastorf, A., Markus, H., Miller, D., & Scott, R. (1984). *Social stigma: The psychology of marked relationships*. New York: W. H. Freeman.

Chicago style[2]

Article in a journal:

Spitzer, Steven. Review of *The Limits of Law Enforcement*, by Hans Zeisel. *American Journal of Sociology* 91 (1985): 726-29; DOI:10.2478/s11533-007-0023-3.

Book:

Lloyd, Donald A., and Harry R. Warfel. *American English and Its Cultural Setting*. New York: Alfred A. Knopf, 1956.

Harvard style[3]

Article in a journal:

Conley, TG & Galenson, DW 1998, 'Nativity and wealth in mid-nineteenth century cities', *Journal of Economic History*, vol. 58, no. 2, pp. 468-493, DOI:10.2478/s11533-007-0023-3.

Book:

Hodgson, A 1998, *Accounting theory*, John Wiley & Sons, Brisbane.

Oxford style[4]

Article in a journal:

KHOO, G.K. Accounting for leases. *The Chartered Accountant in Australia*, 46(5): Nov. 1975: 19-23: DOI:10.2478/s11533-007-0023-3.

Book:

GIBBS, Graham. *Teaching students to learn: a student-centred approach*. Milton Keynes, Open University Press, 1981.

MLA style[5]

Article in a journal:

Joyce, Michael. "On the Birthday of the Stranger (in Memory of John Hawkes)." Evergreen Review 5 Mar. 1999. 12 May 1999 http://www.evergreenreview.com/102/evexcite/joyce/nojoyce.html. DOI:10.2478/s11533-007-0023-3.

Book:

Bird, Isabella L. A Lady's Life in the Rocky Mountains. New York, 1881. Victorian Women Writers Project. Ed. Perry Willett. 27 May 1999. Indiana U. 4 Oct. 1999

IEE style[6]

Article in a journal:

I.E. Sutherland, R.F. Sproull, and R.A. Schumaker, "A Characterization of 10 Hidden-Surface Algorithms," *ACM Computing Surveys*, Mar. 1974, pp. 1-55, DOI:10.2478/s11533-007-0023-3.

Book:

W.M. Newman and R.F. Sproull, *Principles of Interactive Computer Graphics*, McGraw-Hill, 1979, p. 402.

Vancouver style[7]

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.

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