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THE ROLE OF DIETARY POLYUNSATURATED FATTY ACIDS IN INFLAMMATION

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ULOGA POLINEZASIĆENIH MASNIH KISELINA U INFLAMACIJI

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ABSTRACT

Low-grade systemic inflammation is at the base of the most chronic non-communicable diseases, which are reaching epidemic proportions worldwide. Key players in the regulation of inflammation are n-6 and n-3 polyunsaturated fatty acids (PUFAs), in particular arachidonic acid (n-6) and eicosapentaenoic acid (n-3). They are precursors of eicosanoids - signaling molecules involved in modulating the intensity and duration of inflammatory responses. Eicosanoids derived from n-6 PUFAs have proinflammatory actions, while those derived from n-3 PUFAs act anti-inflammatory. Therefore, dietary intake of n-6 and n-3 PUFAs, as well as their ratio, could markedly affect the pathogenesis and manifestation of many chronic diseases associated with low-grade inflammation. This review will focus on the relationship between dietary PUFAs and inflammation, with reference to PUFAs status in plasma phospholipids in Serbian population.

Keywords: *Polyunsaturated fatty acids, inflammation, arachidonic acid, eicosapentaenoic acid, eicosanoids, cytokines*

SAŽETAK

U osnovi većine hroničnih nezaraznih bolesti, koje širom sveta dostižu epidemijske razmere, leži „tiha“ inflamacija. Ključni igrač u regulaciji inflamacije su polinezasićene masne kiseline (PNMK) n-6 i n-3 familije, posebno arahidonska (n-6) i eikozapentaenska kiselina (n-3). One su prekursori eikozanoida – signalnih molekula koji su uključeni u intenzitet i trajanje inflamatornog odgovora. Eikozanoidi poreklom od n-6 PNMK deluju proinflamatorno, dok oni poreklom od n-3 PNMK imaju anti-inflamatorno dejstvo. Zbog toga dijetarni unos n-6 i n-3 PNMK, kao i njihov odnos, može značajno da utiče na patogenezu i manifestaciju mnogih hroničnih oboljenja koja su povezana sa „tihom“ inflamacijom. Ovaj pregledni rad se bavi vezom između unosa PNMK i inflamacije, sa osvrtom na status n-3 i n-6 masnih kiselina u fosfolipidima plazme u populaciji u Srbiji.

Ključne reči: *Polinezasićene masne kiseline, inflamacija, arahidonska kiselina, eiokozapentaenska kiselina, eikozanoidi, citokini*

ABBREVIATIONS

AA - arachidonic acid
ALA - alpha-linolenic acid
CRP - C-reactive protein
DGLA - dihomo-γ-linolenic acid
DHA - docosahexaenoic acid
DPA - docosapentaenoic acid
DTA - docosatetraenoic acid
EPA - eicosapentaenoic acid
IL - interleukine
LA - linoleic acid
LT – leukotrienes
NFκB - nuclear factor κ B
PG - prostaglandins
PPAR - peroxisome proliferation activator receptor
PUFAs - polyunsaturated fatty acids
TX - thromboxanes
TNF-α - tumor necrosis factor-α
WHO - World Health Organisation

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Chronic non-communicable diseases, which include cardiovascular disease, type 2 diabetes, cancer and chronic respiratory conditions, are steadily increasing and the major cause of death worldwide. A key player in the pathogenesis of most non-communicable diseases is low-grade inflammation, characterized by increased systemic levels of some cytokines and C-reactive protein (CRP). Low-grade systemic inflammation has been associated with obesity, atherosclerosis and type 2 diabetes (1). Several factors may cause chronic inflammation, but the most important ones are unhealthy diet and sedentary lifestyle (2). According to the World Health Organization (WHO) the major nutritive risk factors for chronic diseases are high energy intake, high intake of total fats, saturated and trans fatty acids, cholesterol, refined carbohydrates and alcohol, with low intake of dietary fibers, antioxidants and n-3 polyunsaturated fatty acids (PUFAs) (3).

PUFAs are vitally important constituents of cell membranes which maintain membrane fluidity, assure the optimal environment for function of membrane proteins and regulate many cellular functions (4). There are two principal series of PUFAs: the n-3 and the n-6 families. They are physiologically and metabolically distinct and have different effects on human health. Precursors for synthesis of long-chain PUFAs are the 18-carbon fatty acids: linoleic acid (18:2n-6, LA) and alpha-linolenic acid (18:3n-3, ALA). They are essential fatty acids, which cannot be synthesized by mammals and therefore have to be obtained from the diet (5). Once ingested, LA can be converted to other n-6 PUFAs, and ALA to n-3 PUFAs by the relevant desaturase and elongase enzymes. As a consequence, the relative dietary amounts of LA and ALA determine the relative cellular content of n-3 and n-6 PUFAs. Although the ratio of n-6/n-3 PUFAs in the diet should not exceed 4, this ratio is typically 15-20 in the Western diet (6). This predominance of n-6 fat is due to the abundance in the diet of LA, which is present in high concentrations in soy, corn, safflower, and sunflower oils and in products made from these oils. By contrast, there is a low intake of n-3 PUFAs: ALA, which is present in leafy green vegetables, some nuts and in linseed and canola oils, as well as eicosapentaenoic (20:5n-3, EPA) and docosahexaenoic acid (22:6n-6, DHA) which are found in cold water fish (5). Since the most often consumed oil in Serbia is sunflower oil, with low intake of sea food and linseed oil (7), it can be assumed that the n-6-/n-3 ratio in the diet is very high.

LIPID AND PEPTIDE INFLAMMATORY MEDIATORS

The clinical signs of inflammation, such as pain, redness and swelling, result from the release of lipid (eicosanoids) and peptide (cytokines) inflammatory mediators . In response to trauma or an acute infection by pathogens, the level of cytokines in the circulation will increase. The first cytokines as they appear are tumor necrosis factor-α (TNF-α), interleukines IL-1β, IL-6, IL-1 receptor antagonist (IL-1ra), soluble TNF-α-receptors (sTNF-R), and IL-10 (2). Concentration of acute phase proteins from liver, such as CRP, rises as well. The elevation of cytokines in chronic low-grade inflammation is markedly lower than in an acute response: it is usually 2-3 fold higher than physiological levels (8). Nevertheless, recent studies have shown that plasma levels of IL-6, TNF-α, and in particular CRP, can predict the risk of myocardial infarction (9-11). The stimuli for the production of cytokines in chronic low-grade inflammation are still unknown, but the role of nutritional factor could not be excluded. Furthermore, the adipose tissue has been assumed as an origin of TNF-α in low-grade systemic inflammation (12). High levels of circulating TNF-α were found in people with type 2 diabetes, and its direct role in insulin resistance and metabolic syndrome was documented in several studies (13, 14). In contrast to TNF-α, *in vivo* studies provide little evidence on the involvement of IL-6 in metabolic syndrome. Moreover, IL-6 is classified as both pro- and anti-inflammatory cytokine, and its concentration is the first to increase in response to exercise, followed by increased levels of well-known anti-inflammatory cytokines IL-1ra and IL-10 (15).

A series of findings showed that production of TNF-α and other cytokines can be modified by eicosanoids. Eicosanoids, which includes prostaglandins (PG), leukotrienes (LT) and thromboxanes (TX), are signaling molecules generated from 20 carbon PUFAs, mainly arachidonic acid (AA), by the metabolic processes summarized in Figure 1. The essential proinflammatory eicosanoids PGE_2 and LTB_4 are derived from AA via cyclooxigenase and lipoxigenase-5 enzymatic pathways (16). However, EPA as the n-3 homologue of AA competes for the same enzymes and thus inhibit AA metabolism and suppress the generation of the n-6 inflammatory mediators (5). The relation between eicosanoids and cytokines depends on the type of mediators. Human monocytes exposed to leukotrienes (*e.g.* LTB_4) release high amounts of TNF-α (17). On the contrary, addition of PGE_2 strongly inhibited the release of TNF-α by macrophages (18). An enhancing role of leukotrienes on production of IL-1 and IL-6 was also advocated (19). The n-3 derived eicosanoids (Fig. 1) have less inflammatory or even anti-inflammatory effects; hence increasing dietary n-3 PUFAs would shift the balance of the eicosanoids formed to a less potent mixture in causing biological response. For instance, fish oil supplementation of the human diet leads to increased production of LTB_5, LTE_5, PGE_3 and 5-hydroxyeicosapentaenoic acid by inflammatory cells (20). The functional significance of this is that LTB_5 formed from EPA is 10–100-fold less potent as a neutrophil chemotactic agent than LTB_4 derived from AA (21), while PGE_3 is a less potent inducer of cyclooxygenase-2 gene expression in fibroblasts, and of IL-6 production by macrophages than PGE_2 (22). The third 20-carbon PUFA - dihomo-γ-linolenic acid (DGLA) belongs to the n-6 family, but inspite of this, DGLA derived eicosanoids (such as PG

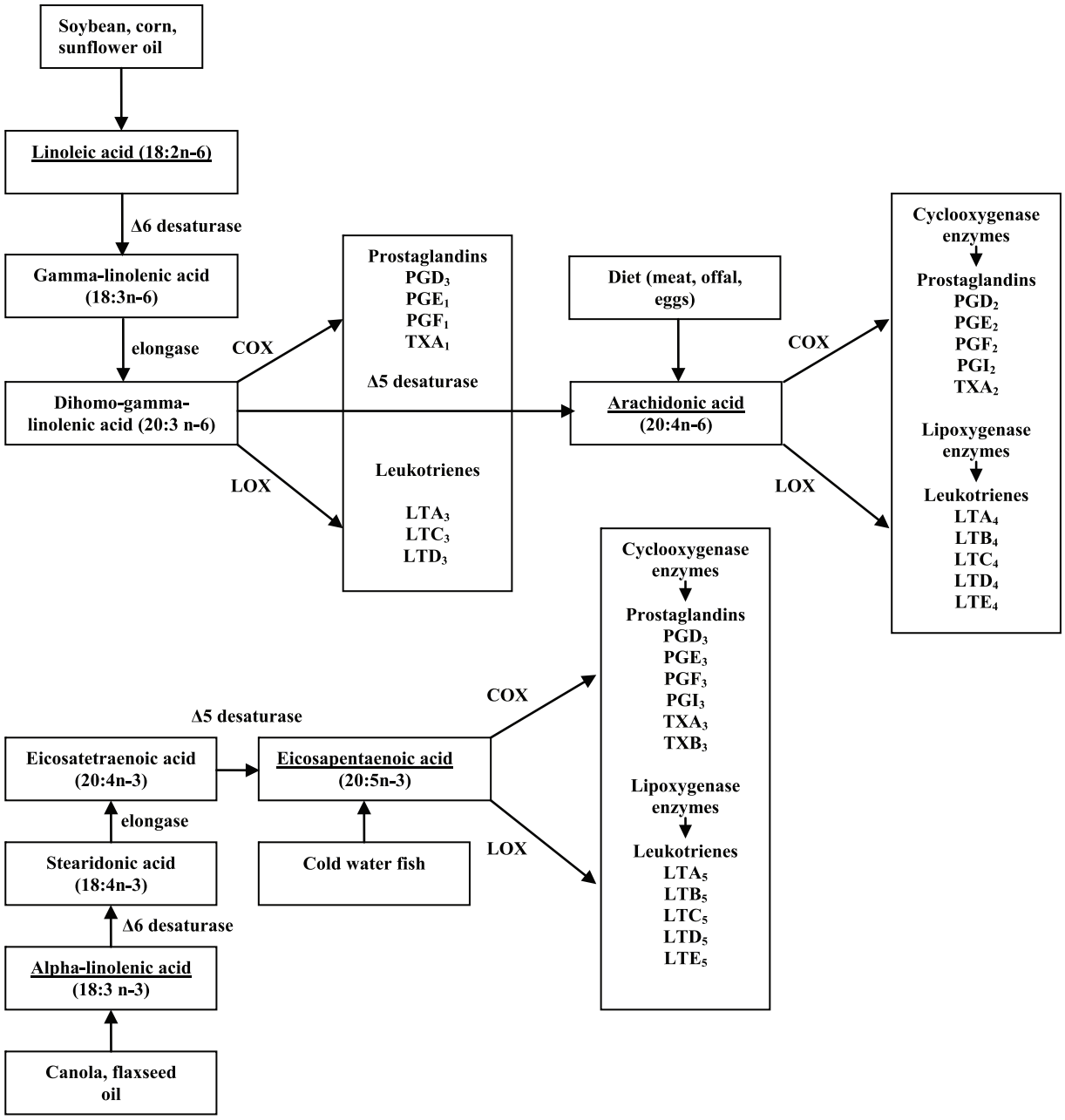

Figure 1. Dietary sources and simplified metabolism of n-3 and n-6 PUFAs
COX- cyclooxygenase, LOX-lipoxygenase (Source: Vučić V, Ristić-Medić D. Eicosapentaenoic Acid: The Role in Malignant Diseases. In Eicosapentaenoic Acid: Sources, Health Effects and Role in Disease Prevention. Editors: Theodore G. Bradley and Francisco P. Vargas. ISBN: 978-1-62257-480-3 Nova Science Publishers, Inc. pp 99-116, 2012. with written permission from the publisher).

series 1) have lower biological activity that its AA derived homologues. Similar to EPA, DGLA may reduce the production and efficacy of AA derivatives.

Besides direct effect on eicosanoids synthesis, EPA reduces the production of pro-inflammatory cytokines IL-1 and IL-6, as well as TNF-α and –β in response to an inflammatory stimulus (23). In a community-based sample Ferrucci et al. have found (24) that total n-3 PUFAs in plasma were independently associated with lower levels of proinflammatory markers (IL-6, IL-1ra, TNF-α, CRP) and higher levels of anti-inflammatory markers (soluble IL-6r, IL-10, TGF–β) independently on confounders. The mechanisms responsible for the modulation of cytokine production by EPA and/or DHA remain unclear, although this mechanism could include suppression of eicosanoid production by n-3 PUFAs, based on the link between eicosanoids and cytokines.

Low-grade chronic inflammation is characterized by the same inflammatory mediators as an acute infection; however their levels are lower and the biologic response to a given cytokine may differ between acute and chronic inflammatory states. Multiple mechanisms may account for

this variability, including differences in plasma cytokine/eicosanoid concentration, presence and concentration of soluble cytokine receptors and receptor antagonists, balance of pro- versus anti-inflammatory cytokine networks, and presence of bacterial cell wall products (25).

PUFAs STATUS IN SERBIAN POPULATION

Since AA and EPA are crucial precursors for biosynthesis of eicosanoids, they have important roles in inflammation. Arachidonic acid is the most important fatty acid in production of inflammatory mediators. Although it can be ingested from meat, eggs or dairy products, AA is mostly produced from its precursor LA. Namely, comparison of fatty acid composition in vegetarians and omnivorous humans showed relatively small differences in AA level in plasma phospholipids (12.8% vs. 11.1%) and cholesteryl esters (7.8% vs. 6.5%), indicating that animal products in the diet increase the percentage of AA moderately (26).

In addition to reduced dietary intake of n-6 PUFAs, AA level in circulation can be decreased by an increased intake of EPA. The richest food sources of EPA are oily fish (tuna, salmon, mackerel, herring, and sardine) and fish oil (27). One fish oil capsule (1g) provides about 180 mg EPA+ 120 mg DHA, while one oily fish meal can provide between 1.5 and 3.5 g EPA+DHA (27). Another way to obtain n-3 PUFA is metabolism of ALA to its longer chain and more unsaturated forms, including EPA, docosapentaenoic acid (DPAn-3, 22:5n-3) and DHA. However, the efficacy of this conversion is not very high and depends on the intake of LA, since both ALA and LA compete for the same elongase/desaturase enzymes. Even though ALA is the preferred substrate, it is the most rapidly oxidized among all dietary PUFAs studied in humans; therefore conversion of dietary ALA to EPA is limited. Accordingly, Brenna et al. reported that supplementation of the diet with high amounts of ALA led to small but significant increases in EPA and DPA, that was ascribed to increased oxidation of ALA, while supplementation with preformed EPA was approximately 15-fold more efficacious in this regard (28).

In countries with low dietary intake of oily fish, levels of n-3 PUFAs in plasma phospholipids are generally low. Together with high dietary intake of LA from sunflower oil in our population, the n-6/n-3 ratio became unfavorable. Proportions of plasma n-3 and n-6 PUFAs in apparently healthy Serbian population with regard to their gender and age, are summarized in Table 1.

As it can be seen from Table 1, there are small differences in plasma phospholipids PUFAs among healthy Serbian people. Proportions of LA, AA and n-6 PUFAs are high, and levels of all n-3 PUFAs are very low; thus the n-6/n-3 ratio is very high as well. A particularly high n-6/n-3 ratio was found in young males (29), while the lowest ratio was reported in middle-age population (32). In comparison, proportion of LA and AA in apparently healthy population from UK and Canada showed similar results as the studies from our country, but percentage of EPA ranged 1.2-1.5% of total fatty acids (33, 34), unlike 0.3-0.4% in Serbia. Thus the n-6/n-3 ratio in these studies ranged 4.95-5.50, while in our country it exceeded 10.

ROLE AND STATUS OF PUFAs IN LOW-GRADE INFLAMMATION

A number of studies have investigated possible mechanisms in PUFAs modulation of the inflammatory response, but the data are often inconsistent. Beneficial anti-inflammatory effects of fish oil supplementation have been found in patients with active inflammation diseases such as rheumatoid arthritis and Crohn's disease (4), but also in patients with cancers (35) or cardiovascular disease (36). Since IL-1 and TNF are principal mediators of inflammation, reduced

Gender/age (y)	M/ 24.4± 3.4 (n=19) (29)	MF/57 (19-74) (n=29) (30)	MF/60 (54-68) (n=15) (23)	F/ 23.67± 1.56 (n=14) (31)	MF/55 (44-62) (n=29) (32)
LA	26.72±2.54	25.9±3.5	28.39±2.81	27.89±2.48	26.47 ± 2.78
DGLA	2.96±0.72	2.7±1.0	2.71±0.84	2.66±0.68	2.42 ± 0.71
AA	12.74±2.14	11.2±2.0	12.41±2.20	10.96±1.60	11.56 ± 2.32
DTA	0.66±0.21	0.3±0.2	0.86±0.38	0.56±0.15	0.39 ± 0.19
n-6	43.08±3.04	40.4±3.9	44.27±1.80	41.51±2.47	40.84 ± 2.86
ALA	n.a.	n.a.	n.a.	0.11±0.10	n.a.
EPA	0.35±0.22	0.3±0.2	0.41±0.24	0.28±0.21	0.36 ± 0.14
DPA	0.72±0.14	0.6±0.1	0.71±0.16	0.59±0.19	0.57 ± 0.10
DHA	3.11±1.01	3.6±1.1	3.84±1.06	3.26±1.33	3.63 ± 1.12
n-3	4.18±1.18	4.5±1.3	4.96±1.28	4.58±1.88	4.56 ± 1.35
n-6/n-3	11.15±3.36	9.6±2.3	9.47±2.39	10.76±4.89	8.82 ± 1.58

Table 1. Plasma phospholipids fatty acid composition (mol %) with reference to apparently healthy male (M) and female (F) Serbian population

Sport Gender	Basketball (44) M (n=23)	Football (44) M (n=24)	Boxing (29) M (n=16)	Water polo (31) F (n=15)	Football (31) F (n=19)
Age (y)	21± 4	24±5	22.4±3.3	21.7±4.5	21.2±2.5
LA	25.67±2.50	26.00±2.85	23.03±2.63	25.60±3.02	25.06±2.04
DGLA	3.32±0.85	3.24±0.67	2.26±0.48	2.76±0.78	2.46±0.65
AA	13.98±1.92	12.64±1.66	9.29±1.72	12.44±1.89	11.50±1.25
DTA	0.93±0.28	0.61±0.13	0.64±0.33	0.71±0.25	0.61±0.24
n-6	43.90±2.16	42.49±2.33	35.20±3.47	41.51±2.47	39.63±1.90
ALA	n.a.	n.a.	n.a.	0.15±0.12	0.14±0.10
EPA	0.47±0.20	0.39±0.11	0.29±0.19	0.36±0.26	0.30±0.20
DPA	0.79±0.18	0.73±0.14	0.47±0.23	0.77±0.30	0.66±0.19
DHA	3.05±0.85	3.10±0.73	1.60±0.85	3.30±1.34	2.68±0.73
n-3	4.31±0.99	4.22±0.81	2.36±1.07	4.58±1.88	3.78±0.98
n-6/n-3	10.66±2.26	10.17±1.98	17.77±8.04	10.76±4.89	11.13±2.78

Table 2. Plasma phospholipids fatty acid composition (mol %) in elite male (M) and female (F) Serbian athletes

production of these cytokines may contribute to the amelioration of inflammatory symptoms in patients taking n-3 supplementation. Dietary supplementation with n-3 PUFAs in healthy subjects resulted in reduced levels of proinflammatory cytokines (IL-1, IL-2, PGE_2 and thromboxane 2) and decreased mononuclear cell proliferation (37). Preclinical studies indicated that decreased generation of proinflammatory cytokines is a consequence of altered gene expression, including transcriptional down-regulation and suppressed cyclooxygenase-2 activity (4). One of the possible mechanisms of n-3 PUFAs impact on inflammatory gene expression is modification of the activities of transcription factors. Two transcription factors that are likely involved in inflammation are nuclear factor κ B (NFκB) and peroxisome proliferator activated receptor (PPAR). There are 4 isoforms of PPAR: α, β, δ and γ, and EPA has been found to bind and activate at least PPAR-α and -γ isoforms. The PPAR-α is expressed in many types of human cells, including atherosclerotic plaques macrophages, while PPAR-γ is expressed in the immune cells (lymphocytes, monocytes, and macrophages), dendritic and many other cells (38). NFκB has been shown to up-regulate inflammatory cytokines (TNF-α, IL-6, IL-1), adhesion molecules and COX-2 genes. On the contrary, PPAR reduces inflammatory gene expression, and also interferes with the activation of NFκB (39). Namely, the NFκB is activated by protein kinase C catalized phosphorylation, and subsequent dissociation of its inhibitory subunit. However, n-3 PUFAs have been shown to directly inhibit protein kinase C, and thereby decrease NFκB activation (38). Furthermore, NFκB can be inhibited by directly binding to PPAR-γ, which is activated by n-3 PUFAs. *In vitro* studies have also documented decreased activation of NFκB in response to different inflammatory stimuli after treatment with EPA (40). These data are clinically significant, because reduced NFκB and consequently reduced IL-1 and IL-6 in circulation will result in decreased production of CRP, which is related to the cardiovascular disease events and their severity (41).

PUFAs status is therefore essential in development of chronic non-communicable diseases. In addition to dietary intake, PUFAs status depends on metabolic processes in the body, which are often altered in inflammation. The results in Serbian patients with chronic diseases showed that levels of n-3 and n-6 PUFAs in plasma phospholipids, as well as their ratio were usually altered: the n-6/n-3 ratio ranged from 11.2 in diabetic patients (42) to 15.4 in patients with non-Hodgkin's lymphoma (NHL) (30). Moreover, we have recently found that the n-6/n-3 ratio in NHL patients varied in response to chemotherapy from 8.5 in patients in remission, to even 19.3 in patients with progression of NHL (43). These results are in accordance with a study by Xia et al. who showed that elevated n-6/n-3 PUFAs ratio stimulated carcinogenesis, tumor growth and metastasis (44).

Besides chronic non-communicable diseases, low grade systemic inflammation is found in elite athletes engaged in prolonged, intensive trainings. Although regular moderate exercise has beneficial impact on health, chronic severe physical activity induces increased levels of cytokine and eicosanoids in circulation. For this reason, altered PUFA profiles are expected in elite athletes, as presented in Table 2. The n-6/n-3 ratio in all tested Serbian athletes is relatively high, especially in boxers. Interestingly, these sportsmen do not have high levels of n-6 PUFAs (which are even lower than in the other athletes), but very low proportion of n-3 PUFAs. Athletes engaged in heavy training programs appear to be more susceptible to infections than sedentary population due to a depression of immune system function, which could be explained by unfavorable PUFAs ratios (45). Based on these results, nutritional intervention and n-3 supplementation in all sport groups is recommended, and in boxers both n-6 and n-3 PUFAs intake should be markedly increased.

DIETARY RECOMMENDATION

In spite of convincing evidence of a relationship between PUFAs intake and development of chronic diseases, only a sparse amount of human data is available for establishing a precise quantitative estimate of the n-3 and n-6 PUFAs requirement to prevent deficiency and provide optimal intake. According to the FAO/WHO report from 2008, the adequate intake to prevent deficiency is 2.5–3.5% of total energy intake (%E), while the minimum intake levels for EFA should be 2.5 % LA plus 0.5 % ALA (46). However, an effective intake for the prevention of chronic diseases is considered to be 6–11 % E (47). In addition, 0.250-2.0g of EPA+DHA daily should be included in the diet (46). In order to improve the n-6/n-3 ratio, it will be necessary to decrease the intake of n-6 PUFAs from vegetable oils and to increase the intake of n-3 PUFAs by using oils rich in ALA, increase the intake of oily fish to 2-3 times per week or take n-3 supplements.

CONCLUDING REMARKS

Unhealthy diet with an imbalanced proportion of daily intake of n-3 and n-6 PUFAs is the main cause of low-grade systemic inflammation, which leads to the development of chronic diseases. The balanced intake of all fatty acids, particularly with regard to n-6/n-3 ratio, may help to prevent and reduce the chronic inflammation and diseases.

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ONION PEEL EXTRACTS AMELIORATE OXIDATIVE STRESS IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

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EKSTRAKTI KORE CRNOG LUKA SNIŽAVAJU OKSIDATIVNI STRES KOD PACOVA SA STREPTOZOTOCINOM INDUKOVANIM DIJABETESOM

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ABSTRACT

Background: *Onion peel extracts contain quercetin derivatives, flavonoids that have been shown to significantly improve diabetic status and to exhibit antioxidant properties in animal models. Vitamin E is an important antioxidant that is present within the cell membrane and acts as a lipid-soluble scavenger. This study aimed to compare the efficacy of an onion peel extract and vitamin E to alleviate the altered redox balance of diabetic rats.*

Methods: *Streptozotocin-induced diabetic male Wistar rats (n = 34) were randomly divided into three treatment groups. For 45 days, the first group was fed a normal diet (diabetic control group), the second group was fed a normal diet plus 20 mg/kg body weight vitamin E and the third group was fed a normal diet plus 1% OPE .*

Results: *The formation of malondialdehyde and protein carbonyls was significantly suppressed and the activity of superoxide dismutase was increased in different areas of the brain upon onion peel extract administration (P < 0.001) compared to the diabetic control group. Furthermore, vitamin E did not significantly decrease the level of oxidative stress or the blood glucose concentration in these rats.*

Conclusion: *OPE is better able to ameliorate oxidative stress and hyperglycaemia than vitamin E in a diabetic rat model.*

Keywords: *Diabetes mellitus, Streptozotocin, Onion peel extract, Vitamin E, Oxidative stress.*

SAŽETAK

Uvod: *Ekstrakt kore crnog luka sadrži derivate kvercetina, najznačajnijeg flavonoida, koji poboljšavaju dijabetički status i imaju antioksidativna svojstva na animalnim modelima. Vitamin E je važan liposolubilni antioksidant koji je prisutan u ćelijskim membranama, koji može da bude liposolubilni skevendžer. Cilj ove studije je da uporedi efikasnost regulisanja poremećene redoks ravnoteže ekstraktom kore crnog luka i vitamonom E kod pacova sa dijabetesom.*

Metode*: Mužjaci Wister pacova sa streptozotocin-inukovanim dijabetesom (n = 34) su randomizacijom podeljeni u tri grupe. Jedna grupa je bila na normalnoj ishrani (dijabetična kontrolna grupa), druga grupa je dobijala vitamin E u dozi od 20 mg/kg telesne mase tokom 45 dana, i treća grupa je dobijala 1% ekstrakt kore crnog luka tokom istog vremenskog perioda.*

Rezultati: *Malondialdehid i proizvodi stvaranja karbonilnih proteina su bili značajno smanjeni, a aktivnost superoksid dizmutaze je bila značajno povišena u različitim delovima mozga usled primene ektrakta kore crnog luka (P < 0.001) u odnosu na dijabitičnu kontrolnu grupu, dok primena vitamina E nije dovela do značajnog smanjenja oksidativnog stresa ni koncentracije glukoze u krvi.*

Zaključak: *Regulacija oksidativnog stresa, kao i hipoglikemijski uticaj ekstrakta kore crnog luka, je bila mnogo više izražena u odnosu na vitamin E u dijabetičnom eksperimentalnom modelu pacova.*

Ključne reči: *diabetes mellitus, streptozotocin, ekstrakt crnog luka, vitamin E, oxidativni stres*

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INTRODUCTION

Diabetes is characterised by hyperglycaemia and metabolic abnormalities due to decreased insulin levels or activity, which cause metabolic and physiological changes in various organs, including the brain [1]. In diabetes mellitus, 60-70% of deaths are due to diabetic neuropathy. Diabetic neuropathy is a complication of long-term diabetes that is mainly caused by hyperglycaemia. This complication produces oxidative stress in the central nervous system, which causes an imbalance in the oxidative status of the nervous tissue and leads to microvascular cerebral diseases [2,3] .

The brain was previously considered to be an insulin-insensitive tissue. However, recent molecular studies have indicated that insulin is present in several regions of the central nervous system, where it acts as a neuromodulator, inhibiting food intake and stimulating fat oxidation [4]. Cerebral glucose is increased after the onset of diabetes in rats [5]. This increase in the intracellular glucose load leads to the autoxidation of glucose, the generation of free radicals, enhanced lipid peroxidation and non-enzymatic protein glycation, and increased activation of the polyol pathway. The central nervous system is highly susceptible to oxidative stress. The vulnerability of the brain to oxidative stress induced by oxygen free radicals seems to be because the brain utilises about one-fifth of the total oxygen demand of the body for oxidative phosphorylation to acquire energy and that as it has a relatively small antioxidant capacity [6], the brain cannot neutralise the toxic effects of free radicals. Furthermore, the brain contains a high concentration of easily peroxidisable fatty acids [7], and it is known that certain regions of the brain are highly enriched in iron, a metal that is catalytically involved in the production of damaging oxygen free radical species when it is in free form [3,8]. ROS overload damages many cellular components, including proteins, DNA and membrane phospholipids [9-15]. Lipid peroxidation is the consequence of ROS, the role of which is well established in the pathogenesis of a wide range of diseases, such as Alzheimer's disease and Parkinson's disease [16-18], acute brain injuries, such as ischemia and head trauma [19-21], and some major metabolic diseases, such as diabetes mellitus (DM) [22]. Lipid peroxidation and the secondary and end products of non-enzymatic (autoxidative) fatty peroxide formation and decomposition can produce a large variety of aldehydes, including hexanal, malondialdehyde (MDA) and 5-hydroxynonenal [15]. Conceptually, these three facts indicate that MDA is an excellent index of lipid peroxidation. Protein carbonyls (PCs) are generated from oxidatively modified cellular proteins through a variety of mechanisms, including the direct oxidation of amino acid side chains and oxidation-induced peptide cleavage.

There are several proposed methods to increase insulin sensitivity and to combat against oxidative stress. Early diagnosis and prompt initiation of therapy are the main factors in reducing the population burden of diabetes. Although changes in lifestyle (weight loss, exercise, restricted diet, etc.) is always recommended to fight diabetes, the compliance rate is not at all satisfactory. In addition, several medicines are available to treat this disorder, but the aggressive use of medicine is restricted due to unwanted side effects. Therefore, the recent research trend involves the identification of a treatment with minimal side effects and maximum disease prevention. Currently, the main focus of research is on herbal remedies [23]. Many studies have indicated that diabetes can be delayed or prevented with dietary flavonoids. Flavonoids are naturally found in plant foods, and the flavonoid quercetin is one of the most common flavonoids present in foods. Some recent studies have suggested that quercetin improves diabetic status by either decreasing oxidative stress [24-26] or correcting altered hepatic gene expression [27].

Onion bulbs are the richest source of dietary flavonoids. At least 25 different flavonoids have been identified in onion bulbs, and quercetin and its glycosides are the most important ones [28]. Quercetin is present at a high concentration in the outer dry layers of the onion bulb [29]. These layers show strong antioxidant activity, and it has been proposed that quercetin is the main factor for this activity [30].

Vitamin E is a lipid-soluble vitamin with chain-breaking antioxidant activity. The major function of vitamin E is its role as a physiological membrane-bound antioxidant, protecting all cell membrane lipids from oxidative damage induced by reactive oxygen species [31].

Thus, the present study was conducted to investigate the potency of onion peel extract (OPE) and vitamin E to ameliorate oxidative stress in streptozotocin (STZ)-induced diabetes in a rat model. Accordingly, we conducted this study using an experimental rat model, assuming that the results would have similar implications in humans.

MATERIAL AND METHODS

Study location

The present study was an animal model-based case-control study that was undertaken in the department of Biochemistry with the collaboration of the department of Pharmacology at Burdwan Medical College (Burdwan, West Bengal, India).

Animals

Male Wistar albino rats (*Rattus norvegicus albinus*), between 1 to 2 months of age and weighing 150 ± 12 g (n = 34), were obtained from the appropriately maintained institutional animal house. The rats had free access to drinking water and rat food pellets. The light source in the animal room was regulated with a 12:12 hr light-dark cycle, a temperature of 22 ± 2°C and 45-50% relative humidity. All rats were acclimatised for at least 7 days before the induction of diabetes. All procedures involving animals were performed in accordance with the 'Guide for the Care and

Use of Laboratory Animals (1985)' by the NIH (Bethesda, MD, USA) and the 'Guidelines for Care and Use of Animals in Scientific Research' by the Indian National Science Academy (INSA; New Delhi, India). The study was approved by the institutional ethics committee for the care and use of laboratory animals and started after obtaining written consent [Memo No. BMC/2179/1 (3)].

Preparation of OPE

The outer dry layers of onion bulbs (*Allium cepa* L.) were extracted with 60% ethanol adjusted to pH 5.5 at 50°C for 3 hours. The extract was concentrated and then freeze dried. The amount of total polyphenol and quercetin were 616.08 ± 13.82 mg/g and 104.52 ± 7.81 mg/g as determined by the methods of Folin-Ciocalteu [32] and Hertog *et al.* [33], respectively.

Study design

STZ was dissolved in saline sodium citrate buffer (50 mM sodium citrate, 0.9% NaCl, pH 4.5) . Diabetes was induced in male neonatal Wistar rats at birth by a single intravenous injection of freshly prepared STZ at a dose of 100 mg/kg body weight. Forty-two days after STZ administration, the plasma glucose level of each rat was determined to confirm the induction of diabetes[171]. Rats with fasting plasma glucose levels that were greater than 16.65 mmol/L were considered to be diabetic and used for further studies.

Then, the rats with fasting blood glucose levels above 300 mg/dl were randomly divided into 3 groups and treated for 45 days. The first group was fed a normal diet only (diabetic control group), the second group was fed a normal diet containing 20 mg/kg vitamin E and the third group was fed a normal diet containing 1% OPE. At the end of the treatment period, all the rats were sacrificed by cervical dislocation.

Tissue sample preparation

Preparation of brain extracts

To determine the ability of OPE and vitamin E to reduce oxidative stress in different areas of the brain, rat brains were dissected and segregated in the following order: cortex, cerebellum, midbrain and basal ganglia. The brain tissues were washed gently in saline to remove any blood and then immediately frozen, first at -20 °C and then at -70 °C, and kept under these conditions (-70 °C) until the chemical analysis was performed. All assays were completed on the same day of sample collection. For homogenisation, the samples were washed and then minced with a sharp surgical blade in small volumes of ice-cold (not frozen) homogenisation buffer [0.1 M Tris-HCl (pH 7.35) and 100 µM ethylenediaminetetraacetic acid (EDTA)]. Immediately, the samples were homogenised in 10 volumes of the ice-cold buffer solution in a motor-driven glass tissue homogeniser in presence of a few properly washed particles of sand. During the whole homogenisation procedure, the homogeniser was kept on ice to dissipate any heat. Thereafter, the samples were centrifuged at 10,000 × g for 10 min at 4°C. The supernatants from the homogenates were collected and were immediately analysed for MDA content, PC product content, cytosolic superoxide dismutase (Cu^{2+}-Zn^{2+}-SOD) activity and tissue protein concentration.

Collection and processing of blood

Blood was withdrawn from tail of each rat to determine the blood glucose level. Some of the blood was separated into a heparinised vial to obtain plasma.

Biochemical assays

Blood was separated into a heparinised vial to obtain plasma and a plain vial to obtain serum . Plasma glucose was assayed photometrically using the glucose oxidase peroxidase (GOD-POD) method [35]. MDA, a marker of lipid peroxidation due to oxidative stress, was measured via its reaction with thiobarbituric acid at 532 nm [36]. The brain tissue levels of MDA were calculated using a calibration curve that was derived using 1,1,3,3- tetraethoxypropane (Fluka, Germany) as an external calibration standard. The calibration curve was linear from 1.25-2.5 nmol/ml (r^2=0.997). Oxidation-induced changes in the tissue proteins were estimated by measuring the protein carbonyl product content. The method used is based on the reaction of carbonyl groups with 2,4-dinitrophenylhydrazine to form a 2,4-dinitrophenylhydrazone-reactive carbonyl derivative that can be measured at 370 nm. [37]. The cytosolic superoxide dismutase (SOD) activity was estimated using the method of Kakkar et al. [38], where one unit of SOD was defined as the amount of enzyme that inhibits the rate of electron transfer from NADH to nitroblue tetrazolium (NBT) by 50 % under specified conditions. The protein concentration was measured using the method of Lowry et al. [39], in which the proteins in the tissue homogenates react with alkaline copper sulphate, followed by Folin's phenol reagent (SRL, India). The absorbance values of the samples were then compared to a standard curve that was prepared using known concentrations of bovine serum albumin (Merck, Germany). All photometric measurements were performed with a dual-beam spectrophotometer (UV 5704SS). The blood glucose levels are expressed in units of mmol/L, while the other parameters are expressed in their corresponding units per mg of tissue protein.

Statistical analysis

The mean values were analysed for significant differences between the diabetic control group (I) and the treatment groups (II and III) using independent t-tests. For all tests, the p-value was considered to be significant if it was less than 0.05 at a confidence level of 95 %. All statistical analyses were performed with the SPSS statistical software package (version 11.5 for Windows).

Parameters	Parameters	Group I (STZ) n = 12	Group II (STZ + vit E) n = 10	Group III (STZ + OPE) n = 12	Group I vs. Group II	Group I vs. Group III	Group II vs. Group III
Blood glucose (mmol/L)	Plasma	22.5 ± 3.49	22.89 ± 0.48	8.98 ± 1.68	p> 0.05	p<0.001	p<0.001
Tissue MDA (nmol/mg protein)	Cortex	0.95 ± 0.06	0.89 ± 0.08	0.53 ± 0.04	p> 0.05	p<0.001	p<0.001
	Cerebellum	0.67 ± 0.06	0.64 ± 0.06	0.35 ± 0.06	p> 0.05	p<0.001	p<0.001
	Midbrain	0.79 ± 0.01	0.76 ± 0.04	0.33 ± 0.05	p> 0.05	p<0.001	p<0.001
	Basal ganglia	1.39 ± 0.28	1.27 ± 0.25	0.47 ± 0.04	p> 0.05	p<0.001	p<0.001
Tissue PC (mM/mg protein)	Cortex	0.31 ± 0.02	0.29 ± 0.02	0.18 ± 0.02	p> 0.05	p<0.001	p<0.001
	Cerebellum	0.26 ± 0.04	0.23 ± 0.03	0.11± 0.02	p> 0.05	p<0.001	p<0.001
	Midbrain	0.28 ± 0.02	0.26 ± 0.02	0.14 ± 0.03	p> 0.05	p<0.001	p<0.001
	Basal ganglia	0.56 ± 0.10	0.49± 0.11	0.25 ± 0.04	p> 0.05	p<0.001	p<0.001
Cytosolic SOD (IU/mg protein)	Cortex	0.66 ± 0.05	0.75± 0.05	1.31 ± 0.04	p = 0.032	p<0.001	p> 0.05
	Cerebellum	0.59 ± 0.05	0.71 ± 0.11	0.97 ± 0.11	p = 0.026	p<0.001	p> 0.05
	Midbrain	0.44 ± 0.02	0.52 ± 0.23	0.88 ± 0.15	p = 0.017	p<0.001	p> 0.05
	Basal ganglia	0.79 ± 0.12	0.98 ± 0.17	1.29 ± 0.20	p>0.05	p<0.001	p> 0.05

Table 1. Differences between the mean values of the studied parameters in the rats of the three treatment groups.

Values are means ± SD; p < 0.05 was considered to be statistically significant.

RESULTS

To compare the efficiencies of OPE and vitamin E in reducing oxidative stress, an independent sample t-test was performed between the Group I rats and the Group II rats as well as between the Group I rats and the Group III rats (Table 1). The MDA and PC product contents were found to be significantly suppressed and SOD activity was improved in different areas of the brain upon OPE administration ($P < 0.001$, Figure 2). In addition, the Group III rats showed a lower mean plasma glucose level (8.98 (range 7.3 to 10.66) mmol/L) than the diabetic control group (22.5 ± 3.49 (range 18.97 to 26.02) mmol/L). Diet supplementation with vitamin E failed to significantly decrease both the level of oxidative stress and the blood glucose concentration (Figure 2). In fact, there was significant decrease in the oxidative parameters of the OPE-treated group (III) compared to the vitamin E-treated group (II).

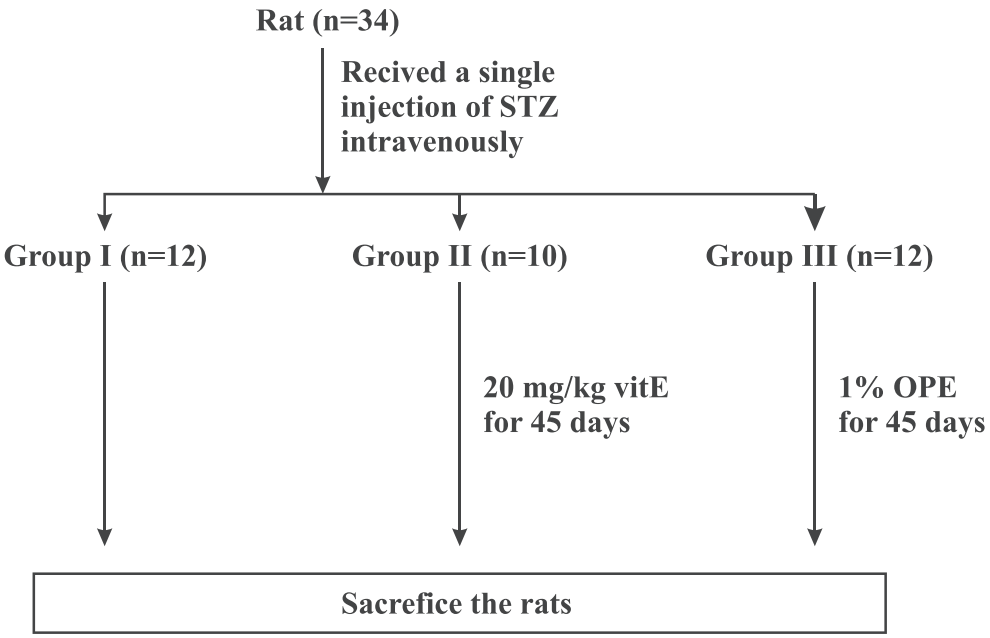

Figure 1. Study design.

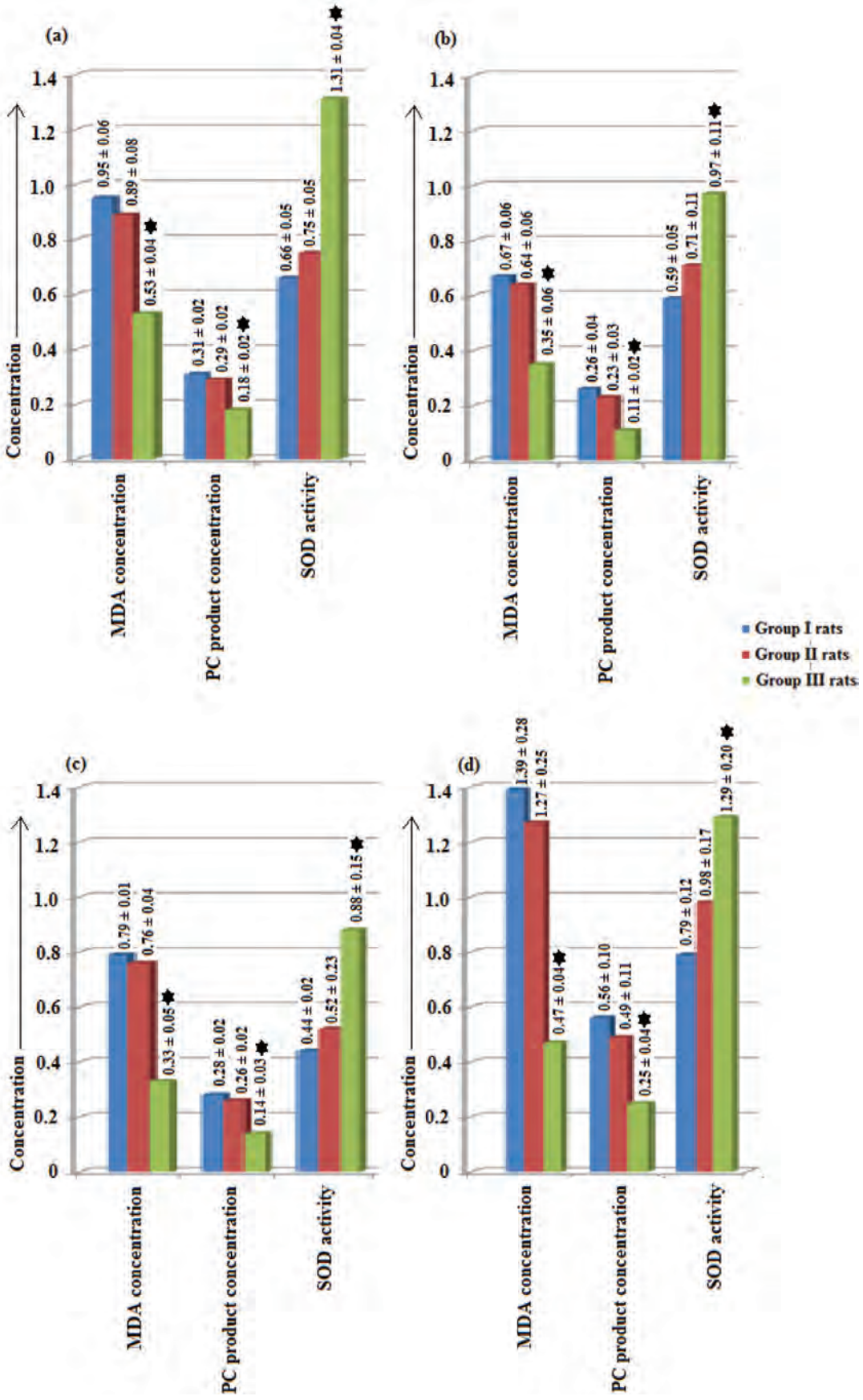

Figure 2. Histogram showing the distribution of MDA content, PC product content and SOD activity in the (a) cortex, (b) cerebellum, (c) mid-brain, and (d) basal ganglia of Group I (STZ), Group II (STZ + vit E) and Group III (STZ + OPE) rats. Asterisks indicate p<0.001.

DISCUSSION

Diabetes mellitus is a common but serious metabolic disorder that is associated with many functional and structural complications [40,41]. This disorder is associated with an increased production of reactive oxygen species (ROS) in both humans and animals. Experimental evidence has supported that ROS play roles in both pathogenesis and numerous pathophysiological mechanisms that trigger diabetic complications, which are primarily categorised as macroangiopathies or microangiopathies, the latter of which includes retinopathy, nephropathy, neuropathy, and microvascular damage to the cerebral artery [42,43]. Results of several previous studies have clearly indicated that STZ-induced hyperglycaemia causes oxidative stress, leading to compromised antioxidant activity in different areas of the brain [2,3,44-47]. Furthermore, hyperglycaemia-induced oxidative stress has been implicated in the development of diabetic neuropathy in the peripheral (PNS) and central nervous system (CNS) and nephropathy [48,49]. Free radical scavengers have been shown to protect neurons against a variety of experimental neurodegenerative conditions [50] and have been suggested to attenuate the oxidative stress and diabetic state induced by STZ [51,52]. The current study was designed to investigate the modulating effects of OPE and vitamin E on the altered redox balance observed in STZ-induced diabetic rats. We found that due to its own antioxidant activity as well as its insulin-sensitising effect, the administration 1% OPE alleviates oxidative stress more efficiently than vitamin E in all areas of the rat brain. Moreover, OPE improves hyperglycaemia, whereas vitamin E is ineffective in this area. This finding was well corroborated with a very recent work that demonstrated that OPE improves insulin action by up-regulating the expression of the insulin receptor and glucose transporters as well as by promoting glucose metabolism in peripheral tissues in diabetic rats.[53] In addition, several studies have suggested that impaired blood lipids are characteristic of subjects with insulin resistance, especially circulating FFAs. [54-56] FFAs directly activate macrophages to secrete pro-inflammatory cytokines that render muscle cells resistant to insulin [57,58]. FFAs also contribute to the increased production of reactive oxygen species and lead to the activation of stress-sensitive signalling pathways under hyperglycaemic status [59]. Upon the administration of OPE, the quercetin component of this extract acts as a strong antioxidant due to its ability to scavenge free radicals and bind to transition metal ions. By virtue of these properties, quercetin inhibits lipid peroxidation [60,61]. In the interaction of quercetin with free radicals, it can donate a proton and be converted to a radical itself, but the resulting unpaired electron is delocalised by resonance. Thus, the quercetin radical exists in a low energy state and, thus, is less reactive than its non-radical form [62]. Therefore, although the detailed mechanism awaits further investigation, we have shown that OPE improves insulin sensitivity and oxidative stress better than α-tocopherol in a rat brain model.

CONCLUSION

The present study demonstrated that OPE ameliorates oxidative stress and hyperglycaemia in diabetic rats better than vitamin E. Further studies should be performed to evaluate the use of onion peel extracts in the prevention and early treatment of type 2 diabetes.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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EVALUATION OF THE ABO BLOOD GROUP DISTRIBUTION IN SERBIAN PATIENTS WITH DIABETES MELLITUS TYPE 2

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ANALIZA DISTRIBUCIJE KRVNIH GRUPA PREMA ABO SISTEMU KOD PACIJENATA OBOLELIH OD DIJABETES MELITUSA TIP 2 U SRBIJI

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ABSTRACT

The aim of our study was to evaluate the distribution of the ABO blood groups in sample of Serbian patients diagnosed with diabetes mellitus type 2. We evaluated 114 patients from Serbia that were diagnosed with diabetes mellitus type 2. All ABO blood groups were recorded as well as the HbA/1-C values in each patient. We also analysed the presence of the following risk factors: positive family history, elevated triglycerides, increased blood pressure and overweight. Male and female patients were evaluated separately. The most frequent blood type in this group of patients was the A blood group, whereas the least frequent was the AB blood group. There was a significant increase in the frequency of the AB blood group in Serbian patients with DM type 2 compared to the frequency in the overall Serbian population ($p<0.05$), whereas no significant differences were observed for the other blood groups within the ABO system. An increase in the distribution of the AB blood group was observed in patients with increased levels of HbA1C, although this increase was not significant. The highest proportion of evaluated risk factors was observed in patients belonging to the O blood group, whereas the lowest proportion was in patients belonging to the A blood group (n.s.). The results of this study suggest a possible trend between the ABO blood groups and the risk of diabetes mellitus type 2 as well as a correlation between the ABO blood groups and risk factors in patients diagnosed with diabetes mellitus type 2. Therefore, a larger cohort study is needed to better define the initial observations based on our results.

Key words: *ABO blood groups, diabetes mellitus type 2, gender, risk factors*

SAŽETAK

Cilj rada je bio da se ispita distribucija ABO krvnih grupa u uzorku pacijenata sa dijagnostikovanim dijabetes melitusom tip 2 iz Srbije. Analizirana su 114 pacijenata iz Srbije kod kojih je dijagnostikovan dijabetes melitus tip 2. Ispitivane su sve krvne grupe iz ABO sistema krvnih grupa, kao i vrednosti HbA/1-C. Prisustvo sledećih riziko faktora je praćeno: pozitivna porodična istorija, povišeni triglicеridi, povišen krvni pritisak i prekomerna telesna težina. Zasebno su analizirani muški i ženski pol. Najčešće zastupljeni tip krvne grupe kod ispitivanih pacijenata je A, dok je AB najređe bila zastupljena. Postoji statistički značajno povećanje u zastupljenosti AB krvne grupe kod ispitivanih pacijenata u odnosu na učestalost u Srpskoj populaciji ($p<0.05$), dok za ostale krvne grupe nije uočena statistički značajana razlika. Povećana zastupljenost AB krvne grupe (mada ne značajno) je primećena kod pacijenata sa povišenim vrednostima HbA1C. Najviše riziko faktora je bilo kod ispitivanih pacijenata sa O krvnom grupom, dok je najmanje riziko faktora bilo kod pacijenata sa A krvnom grupom ali bez statističke značajnosti. Rezultati ove studije mogu biti pokazatelji inicijalnog trenda potencijalne korelacije između ABO sistema krvnih grupa i dijabetes melitus tipa 2 kao i korelacije ABO sistema krvnih grupa i riziko faktora kod ispitiavnih pacijenata sa dijabetes meliusom tip 2. Stoga su neophodna dalja istraživanja na većem uzorku pacijenata.

Ključne reči: *ABO krvne grupe, dijabetes mellitus tip 2, pol, faktori rizika*

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INTRODUCTION

Diabetes mellitus (DM) type 2 is an emerging problem worldwide and is ultimately leads to an increase in cardiovascular morbidity and mortality (1). It is estimated that 50 million adults in the United States will be diagnosed with DM type 2 by 2050 (1,2). The exact etiology of DM type 2 is unknown, but multifactorial inheritance has been suggested in numerous studies (3,4). In the work of Bastard et al., it was postulated that the risk factor obesity could be associated with inflammation to a certain degree, which might lead to the impaired glucose tolerance and eventually develop into DM (5). Previous reports have demonstrated correlation between infections and various tumors with certain types of ABO blood groups (6-8). Therefore, we hypothesised that Serbian patients with DM type 2 along with a presence of several risk factors could have a unique distribution of the ABO blood groups compared to the Serbian population. Thus, the aim of our study was to evaluate the distribution of the ABO blood groups in sample of Serbian patients with diagnosed DM type 2.

MATERIAL AND METHODS

Study group

We evaluated 114 patients from Serbia who were diagnosed with diabetes mellitus type 2. Prior to inclusion, eligible participants were informed about study protocol and provided consent. The study followed the principles of good clinical practice. The patients were age between 50-65 years of age, with 35 (56.5%) males and 27 (43.5%) females .

Blood samples were drawn from the patients to establish the ABO blood type into one of the following groups:: A, B, AB and O. The established distributions were compared with the distributions of the ABO blood groups within the Serbian population (9).

The risk factors of positive family history and elevated triglycerides were gathered from the patients' records and anamnesis, whereas increased blood pressure and obesity were assessed during the study. Additionally, the frequencies of the ABO blood groups were analysed in patients with increased HbA1C. Increased blood pressure values were defined above 90th percentile based on the age, gender and height of the patient (10), whereas excessive weight and obesity was determined based on the body mass index (BMI) and adjusted to age and gender.

Statistical analysis

The ABO blood group distribution values are presented as whole numbers and percentages. The frequencies of the risk factors for each blood group from the ABO blood group system are presented as the mean values with standard deviation (SD). For evaluation of the statistical significance between the distributions of ABO blood groups in different samples, we used the Chi squared test. Unifactorial ANOVA was used for statistical interpretation of the evaluated risk factors frequencies between the different ABO blood groups. The results were considered to be statistically significant when $p<0.05$.

RESULTS

The most frequent blood group in the evaluated patients was the A blood group (38.7%), whereas the least frequent was the AB blood group (12.9%) (Table 1). Regarding gender, the most frequent blood group in males was the A blood group (42.9%), and the least frequent was the AB blood group (14.3%). However, females showed a similar distribution of the A and O blood groups (33.3%) (Table 1). For individuals with increased HbA1C levels, the most frequent blood group was the A blood group (40.5%), and the least frequent was the AB blood group (11.9%) (Table 1). The highest proportion of evaluated risk factors was observed in patients with type O blood (2.61±0.85), whereas the lowest proportion was observed in patients with type A blood (2.33±1.01) (Table 1).

Blood groups	Serbian population (ref.9)	Patients N (%)	Patients (gender)		Increased HbA1C N (%)	Risk factors
			Males N (%)	Females N (%)		MV±SD
A	71953 (41.5)	44 (38.7)	28 (42.9)	17 (33.3)	17 (40.5)	2.33±1.01
B	30341 (17.5)	22 (19.4)	11 (17.1)	11 (22.2)	6 (14.3)	2.50±0.90
AB	12310 (7.1)	14 (12.9)	9 (14.3)	7 (11.1)	5 (11.9)	2.50±0.93
O	58776 (33.9)	34 (29.0)	17 (25.7)	17 (33.3)	14 (33.3)	2.61±0.85
Total	173380	114	65	52	42	-

Table 1. Distribution of ABO blood groups in patients with DM type 2

Blood groups	Chi squared test (p values)			ANOVA (p values)
	Serbian population vs. patients	Serbian population vs. individuals with increased HbA1C	Males vs. females	Risk factors
A	>0.05	>0.05	>0.05	>0.05
B	>0.05	>0.05	>0.05	
AB	<0.05	>0.05	>0.05	
O	>0.05	>0.05	>0.05	

Table 2. Statistical interpretation of the ABO blood group distribution between study individuals

We observed a significant increase in the frequency of the AB blood group in Serbian patients with DM type 2 compared with the frequency in the Serbian population ($p<0.05$) whereas the increase in the distribution of the AB blood group in patients with increased HbA1C was not significant. For the frequencies of the other blood groups within the ABO system, we observed no significant differences (Table 2). There was an insignificant difference in the mean values of the risk factor frequencies between the patients with different ABO blood groups (Table 2).

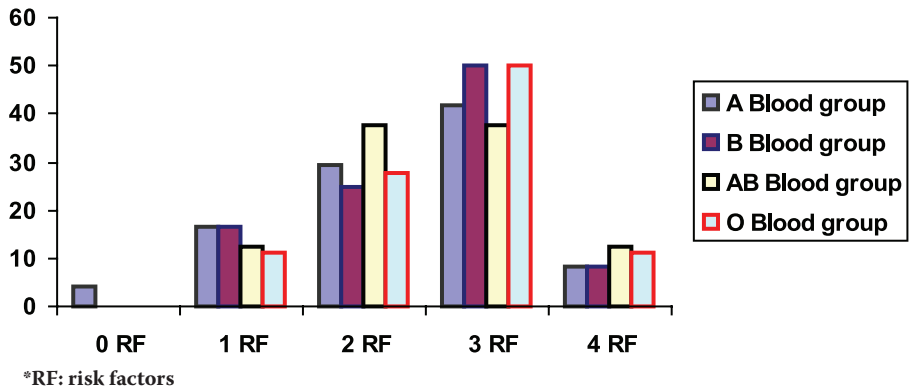
In comparing the frequency of the 4 risk factors selected within each ABO blood group, we observed that the existence of 3 risk factors was most common for all of the blood groups (A blood group: 41.7%; B blood group: 50.0%; O blood group: 50.0%), except for patients in the AB blood group, in which having either 2 or 3 risk factors occurred at similar frequencies (37.5%) (Graph 1).

DISCUSSION

Previous reports have shown that different ABO blood groups are more frequent in patients with various pathological conditions (e.g., different types of cancers, infections, etc.) (11-13). In a couple of studies performed by Serbian researchers, it was reported that the frequency of the A blood group is significantly increased in individuals with congenital hip dislocation, whereas patients suffering from tuberculosis infection were more likely to belong to the O blood group (14, 15).

The results of our study showed that Serbian individuals with type AB blood are more prone to possibly developing DM type 2 during one's lifetime compared to the overall Serbian population (9). This finding was accompanied with an increased (albeit insignificant) frequency of the AB blood group in patients with increased HbA1C levels, which can be useful for more complete analysis of obtained result. Kamil et al. (15) reported that there was no significant correlation for any of the ABO blood groups between patients with DM type 2 and controls. Similar results were observed by Cvjeticanin et al. in Serbian individuals (white population) with DM (14). However, the difference in the frequencies for different ABO blood types between various races and ethnic groups might explain to a certain degree the distinction of the AB blood group frequency between Serbian patients with DM type 2 versus the AB blood group frequency in the Serbian population (16). In support of the possible role of blood groups in the susceptibility for developing DM type 2, Nemesure et al. showed that Rhesus C+ antigen decreases the likelihood for developing DM type 2 (17).

Regarding the possible role of gender and ABO blood type in the development of DM type 2, we observed that despite the lack of any significant difference in the dis-



Graph 1. Frequency of multiple risk factors within the ABO blood groups

tribution of the ABO blood groups between genders, the A blood group was more frequent in males, whereas the O blood group was more frequent in females. These initial findings suggest to a certain degree a possible correlation between gender and ABO blood groups in the development of DM type 2. However, it should be noted out that additional studies are needed to investigate this correlation.

We have demonstrated (Table 1) that, for the patients with DM type 2 from this study, individuals in the O blood group have the highest number of evaluated risk factors, whereas those in the A blood group have the lowest number of evaluated risk factors, despite a lack of significance. These could imply to the assumption that individuals with DM type 2 and having type A blood are less sensitive to the possible influence of the evaluated risk factors, whereas those in the O blood group might be more sensitive. As shown in Graph 1, the patients with DM type 2 with a different number of risk factors have different frequencies within the ABO blood groups. These differences suggest the presence of possible predispositions for development of DM type 2 in individuals with varying numbers of risk factors and belonging to different ABO blood groups.

Although this was one of the first reports that focused on the influence of ABO blood groups, risk factors and gender on the probability of developing DM type 2 further studies with a larger cohort patient population are needed to confirm these results. However, our results could lead to more complex and broader investigations concerning the possible association between ABO blood groups and DM type 2, as well as correlation between ABO blood groups and risk factors in patients diagnosed with DM type 2.

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PHONOCARDIOGRAPHY-BASED MITRAL VALVE PROLAPSE DETECTION USING AN ARTIFICIAL NEURAL NETWORK

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FONOKARDIOGRAFSKA DETEKCIJA PROLAPSA MITRALNE VALVULE UPOTREBOM ARTEFICIJALNE NEURONSKE MREŽE

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ABSTRACT

*Mitral valve prolapse (MVP) is the most common valve anomaly and the most frequent cause of isolated mitral insufficiency. MVP has a mostly benign course and prognosis in childhood; however, complications, such as severe mitral regurgitation, infectious endocarditis, pulmonary embolism, arrhythmia and sudden death, occur more often in elderly people, demonstrating the need for prompt diagnostics and prevention. Due to its frequent occurrence, failures in diagnosing MVP and the clinical importance of early MVP detection, the aim of this study was to develop an original, non-invasive and easily applicable diagnostic method for MVP detection in children and adolescents by using an artificial neural network (ANN). Cardiac sounds were recorded by auscultation using electronic stethoscope in 48 children with MVP, 49 healthy children and 38 children with a pathological heart murmur from atrial septal defect (ASD), ventricular septal defect (VSD), ductus arteriosus persistence (DAP), aortic stenosis (AS), pulmonic stenosis (PS), aortic coarctation (ACo), mitral regurgitation (MR), mitral insufficiency (MI) and tricuspid insufficiency (TI). In electronic stethoscopes, the sound is archived in the internal memory of the stethoscope and then transmitted to a computer by a transmitter. Basic software for the check-up and sound analysis is provided along with the electronic stethoscopes and provides a phonocardiograph and spectral presentation of auscultative findings. For further qualitative analysis, the digital form (format *. e4k) of the phonocardiogram is transformed into standard *.wav format, which is the first step in the processing of the digital signal for studying and testing with an ANN. The obtained precision of MVP classification category was 71.2%. These results may be interesting for the phonocardiograph diagnosis of MVP in children and adolescents.*

Keywords: *phonocardiography – mitral valve prolapse –neural network*

SAŽETAK

*Prolaps mitralne valvule (MVP) je najčešća anomalija zalistaka i najčešći uzrok izolovane mitralne insuficijencije. MVP ima najpovoljniji tok i prognozu u detinjstvu, dok komplikacije kao što su teška mitralna regurgitacija, infektivni miokarditis, embolija pluća, aritmija i iznenadna smrt se češće javljaju kod starijih ljudi, što zahteva brzu dijagnostiku i prevenciju. Usled učestalog javljanja, otežane dijagnostike i kliničkog značaja ranog otkrivanja MVP, cilj ove studije je bio da razvije originalni, neinvazivni i lako primenljiv dijagnostički metod za otkrivanje MVP kod dece i adolescenata upotrebom arteficijalne neuronske mreže (ANN). Srčani tonovi kod 48 dece sa MVP, 49 zdrave dece i 38 dece sa patološkim srčanim šumovima nastalim usled atrijalnog septalnog defekta (ASD), ventrikularnog septalnog defekta (VSD), duktus arteriosus persistensaotvorenog arterijskog kanala (DAP), aortne stenoze (AS), stenoze plućne arterije (PS), koarktacije aorte (ACo), mitralne regurgitacije (MR), mitralne insuficijencije (MI) i trikuspidalne insuficijencije (TI) su zabeleženi auskultacijom upotrebom elektronskimog stetoskopopa. U elekctronskom stetoskopu zvuk se snima na unutrašnjoj memoriji stetoskopa a potom pomoću transmitera prenosi na memoriju kompjutera. Osnovni softver za proveru i analizu zvuka se nalazi u sklopu elektronskog stetoskopa i omogućava fonokardiografsku i spektralnu prezentaciju auskultatornog nalaza. U daljoj kvalitativnoj analizi, fonokardiogram u digitalnom obliku (format *. e4k) se prevodi u standard*. wav format, koji je prvi korak u obradi digitalnog signala, proučavanju i testiranju ANN. Dobijena preciznost svrstavanja MVP u odgovarajuću kategoriju je bila 71.2%. Ovi rezultati mogu biti interesantni u fonokardiografskoj dijagnostici MVP kod dece i adolescenata.*

Ključne reči: *fonokardiografija, prolaps mitralne valvule, neuronska mreža*

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ABBREVIATIONS:

ANN – artificial neural network
AS - aortic stenosis
ASD - atrial septal defect
ACo - aortic coarctation
DAP - ductus arteriosus persistence
FN – false negatives
FP – false positives
LSSVM - Least Square Support Vector Machine
MI - mitral insufficiency
MLP - multilayer perceptron
MR - mitral regurgitation
PS - pulmonic stenosis
MVP - mitral valve prolapse
TI - tricuspid insufficiency
TN - true negatives
TP – true positives
VSD - ventricular septal defect

INTRODUCTION

Mitral valve prolapse (MVP) is a pathological dislocation of the front or back segment or both mitral valves during systole through the plane of the mitral annulus into the area of the left atrium (1). MVP is the most frequent valve anomaly and is the most frequent cause of isolated mitral insufficiency (2). A characteristic auscultator finding is a meso-systolic click and late systolic murmur. MVP mostly has a benign course and a good prognosis in childhood; however, complications, such as severe mitral regurgitation, infectious endocarditis, pulmonary embolism, arrhythmia and sudden death, more often occur with elderly people, demonstrating the need for prompt diagnostics and prevention to preserve the structural and functional integrity of the myocardium. The true prevalence of mitral valve prolapse is not known due to the variety of research techniques and diagnostic criteria. The prevalence varies from a very low 0.4% to a very high 35%, depending on sex and age. The characterisation of heart acoustic signals by auscultation and the establishment of a diagnosis depend on the skill and individual experience of a doctor rather than just his/her theoretical knowledge (3-7). The utilisation of an electronic stethoscope and special computer program can provide a reliable diagnosis and thereby significantly reduce the number of patients who are sent for echocardiography. Considering the frequency of occurrence, failures in diagnostics and clinical importance of early MVP detection, the aim of this study was to develop an original, non-evasive and easily applicable diagnostic method for the early identification of MVP in children and adolescents by using an artificial neural network (ANN)

An artificial neural network (ANN) is a biologically inspired computational model that processes artificial nodes (neurons) and the connections between these processing elements and their parameters (8, 9). The strength of these connections is characterised by weights. Generally, ANNs are meant to be useful models for humanlike problem solving and knowledge engineering. ANNs are widely used for pattern classification and nonlinear adaptive filtering. In this paper, specific medical patterns can be treated using an ANN-based approach for a classification task.

PATIENTS AND METHODS

Subjects

In the period from 2010-2012, 2019 7- to 19-year-old school children received a general check-up at Health Centre "Zvezdara". Auscultation was performed using an electronic stethoscope (3M Littmann 4100WS). Children with characteristic findings of a meso-systolic click or meso-systolic click and late systolic murmur; healthy children who were engaged in sports and in whom auscultation revealed no murmurs or only a harmless, Still's murmur; and children with a pathological murmur from an atrial septal defect (ASD), ventricular septal defect (VSD), ductus arteriosus persistence (DAP), aortic stenosis (AS), pulmonic stenosis (PS), coarctation of aorta (ACo), mitral regurgitation (MR), mitral insufficiency (MI) or and tricuspid insufficiency (TI) were sent to University Children's Clinic Belgrade for echocardiograph confirmation of the diagnosis using a sonography apparatus, the Diasonics imager 100 and Aloka–Echo Camera–SDD-680 probes of 355 i 5 MHz. Children with a previously identified diagnosis were also included in the research. For this study, the examinees were divided into three groups:

1. MVP – 48 examinees with mitral valve prolapse
2. HEALTHY – 49 examinees without a murmur or with a harmless Still's murmur
3. OTHERS - 38 examinees with ASD, VSD, DAP, AS, PS, CA, MR, MI, or and TI

Recordings

The use of an electronic stethoscope, the data collection and processing and the corresponding phonocardiograph recordings were approved by the Ethical Committee of Health Centre "Zvezdara" in Belgrade.

Auscultation using an electronic stethoscope and recordings of heart action is performed in a sitting position, i.e., in standard conditions, when the murmur intensity is greatest. To obtain a good quality recording, the stethoscope is adjusted for the sex, age, physical strength and mass and osteomuscular structure. For each examinee, there were at least three sound recordings lasting 8-10 seconds and the best recording was selected for digital signal

analysis. The sound was converted into a digital signal in the electronic stethoscope, archived in the internal memory of the stethoscope and then transmitted and archived in a computer (Latitude E6400, 2.40 GHz, 4.0 GB, IC for the transmission of data from the stethoscope). Basic software (3M Littmann software for sound analysis V2.0) for the examination and analysis of sounds is provided along with the electronic stethoscope and provides phonocardiography and spectral analysis of auscultative recordings. All results and data are archived in one database, which contains phonocardiograph recordings in digital form (format *. e4k), which is combined with medical information in an e-card and an echocardiograph examination of the patient. For further quality analysis, the recording should be transformed into standard *.wav format, which is the first step in the digital processing of the signal.

Artificial Neural Network (ANN)

A main advantage of an ANN is the opportunity to perform nonlinear analysis. Second, due to its architecture, ANN will not malfunction when an element of the ANN fails. In addition, ANNs do not require reprogramming when new elements are encountered; instead, their behaviour will adapt to a new environment. This is known as learning or ANN training.

Figure 1 shows a basic structural element of an ANN. Each neuron consists of two parts: the net function and the activation function. The net function determines how the network inputs are combined inside the neuron. In contrast, the activation function represents a linear or nonlinear transformation, which determines the output of each neuron.

Back-propagation is a common method of training artificial neural networks. From a target value, network is trained for a set of inputs. This method is a supervised learning method and represents a generalisation of the delta rule. Back-propagation requires a dataset of the desired outputs for many inputs to form the training set and it is the most useful feed-forward network (networks that have no feedback) algorithm. Back-propagation requires that the activation function is differentiable.

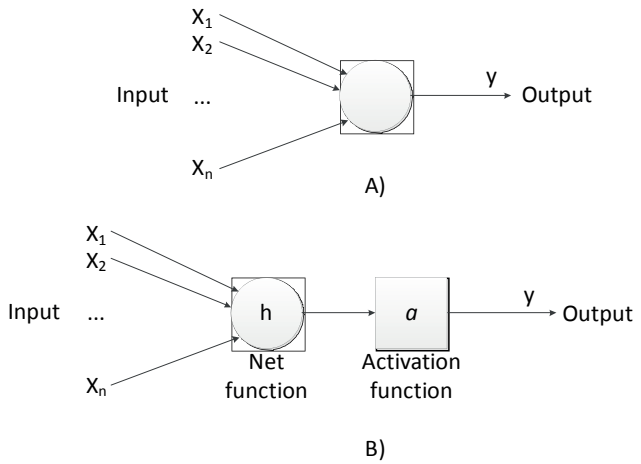


Figure 1. Basic structure of an ANN: a) neuron; b) neuron modelled via the net, and activation functions

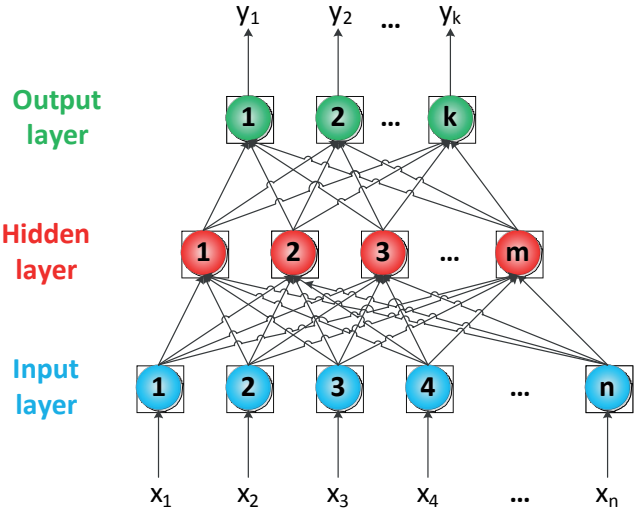


Figure 2. Multilayer Perceptron (MLP) model

A multilayer perceptron (MLP) neural network model consists of a feed-forward, layered network of neurons. Each neuron in an MLP has a nonlinear activation function that is continuously differentiable. Some of the most frequently used activation functions for MLP include the sigmoid function and the hyperbolic tangent function. A typical MLP configuration is depicted in Figure 2. Neurons are organised in three layers: input layer, hidden layer and output layer. The term "hidden" indicates that the output of these neurons will be fed into upper layer neurons and therefore hidden from the user, who only observes the output of output layer neurons. The MLP model is not permitted to create loops between neurons. An MLP provides a nonlinear mapping between its input and output.

An input matrix is created from all PCG recordings. Each PCG record is represented as a column vector in the input matrix; thus, the number of columns in the input matrix is equal to number of PCG recordings. The elements of these column vectors represent the samples of a particular PCG signal.

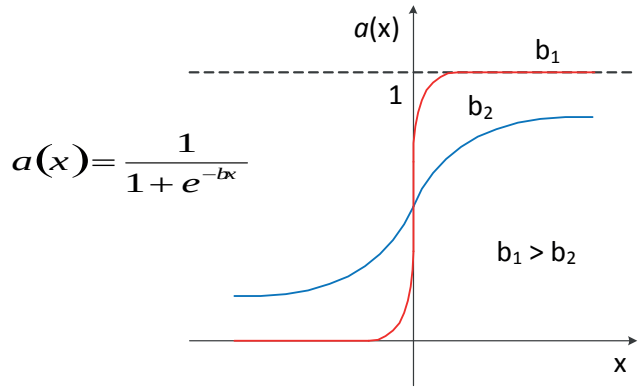
Input phonocardiograms are automatically classified into one of three possible classes (healthy, MVP, others – neither healthy nor MVP). The MLP network and back-propagation method are used for supervised learning. The net function is a weighted linear combination:

$$h_w = w_0 + \sum_{j=1}^{N} w_j x_j$$

where w_0 is the bias level (threshold), w_j is the synaptic weight for j-th neuron input, x_j is the j-th neuron input and N is the number of inputs for each neuron. A sigmoid function is used as the activation function:

$$a(h) = \frac{1}{1+e^{-h(w)}}$$

This function is shown in Figure 3 .



$$a(x)=\frac{1}{1+e^{-bx}}$$

Figure 3. Sigmoid logistical activation function

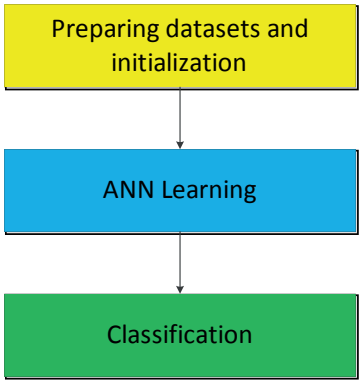


Figure 4. Block diagram of the proposed method

For learning, we chose a logistic regression cost function because our classification problem results in a discrete set of possible values. Regularisation is used to prevent overfitting and underfitting situations. The regularisation parameter lambda is useful for controlling the regularisation process. Lambda must be chosen smartly because an incorrect lambda could decrease the accuracy of the prediction result . The cost function is represented as follows:

$$J(w)=\frac{1}{N}\sum_{i=1}^{N}\left[-y^{(i)}\log\left(h_w\left(x^{(i)}\right)\right)-\left(1-y^{(i)}\right)\log\left(1-h_w\left(x^{(i)}\right)\right)\right]+\frac{\lambda}{2N}\sum_{j=1}^{n}w_j^2$$

where is *y(i)* is the input set label, *x(i)* are the input signals, *N* is the number of training examplesand and *n* is the number of neurons in the input layer. The back-propagation method uses online, batch, or stochastic learning. The batch gradient descent algorithm is implemented in the proposed solution. Gradient descent is a first-order optimisation algorithm. Negative gradient values are favoured for finding a local minimum of a function using gradient descent. The batch algorithm keeps the system weights constant while computing the error that is associated with each sample in the input. This method consumes more memory but requires fewer weight updates than the other two methods. In addition, batch learning yields a much more stable descent to local minima. The gradients of the cost function are as follows:

$$\frac{\partial J(w)}{\partial w_0}=\frac{1}{N}\sum_{i=1}^{N}\left(h_w\left(x^{(i)}\right)-y^{(i)}\right)x_j^{(i)} \qquad \text{for } j=0$$

$$\frac{\partial J(w)}{\partial w_j}=\left(\frac{1}{N}\sum_{i=1}^{N}\left(h_w\left(x^{(i)}\right)-y^{(i)}\right)x_j^{(i)}\right)+\frac{\lambda}{N}w_j \qquad \text{for } j\geq 1$$

Optimisation can minimise a continuous differentiable multivariate function . The Polack-Ribiere flavour of conjugate gradients is used to compute search direction. A line search using quadratic and cubic polynomial approximations and the Wolfe-Powell stopping criteria together with the slope ratio method are used to guess the initial step sizes. Additionally, several checks are performed to verify that the exploration is taking place and that the extrapolation will not be unboundedly large.

An input matrix is created from all PCG recordings. Each PCG record is represented as a column vector in the input matrix; thus, the number of columns in the input matrix is equal to the number of PCG recordings. The elements of these column vectors represent samples of a particular PCG signal.

For classification, a *one-versus-all* algorithm is used in which a single classifier per class is trained. For each class, prediction is performed by using a binary classifier and the result with the highest confidence score is chosen.

PROPOSED METHOD

A block diagram of proposed method is shown in Figure 4. The first step is to create training and test sets for each echo of the cross-validation method and to initialise all the ANN parameters. The second step is the back-propagation algorithm; it includes a calculation of the cost function and descent gradients and adjustment of the ANN weights. To adjust the weights, we try to minimise the cost function with the goal of minimising classification errors. The final step is classification using the *one-versus-all* algorithm.

RESULTS

While training the neural network, the database of phonocardiograms was divided into three classes : MVP, healthy and others (ill Figure 5). The analysis and selection of artificial neural network parameters suggested that 200 echoes was an optimal number for the proposed architec-

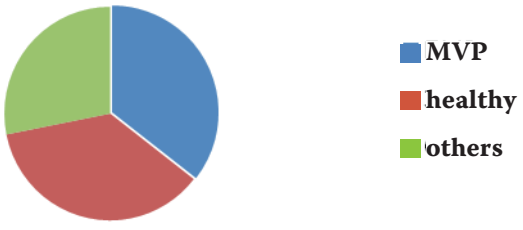


Figure 5. Dataset

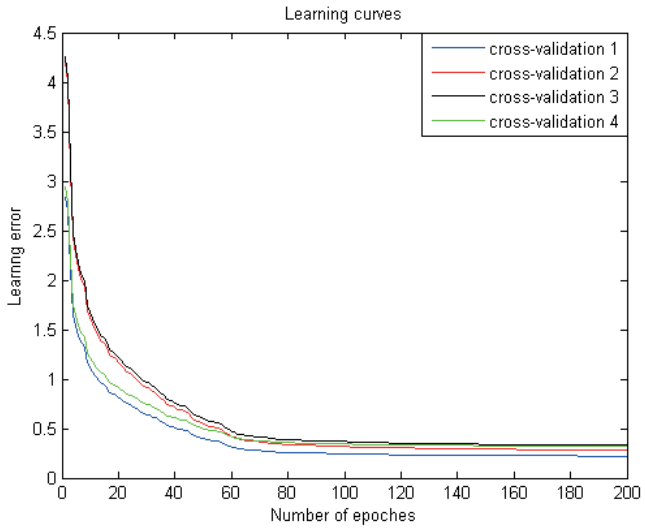


Figure 6. Learning curves for each step of cross-validation

Cross-validation 1		Real	
		P	N
Prediction	P	9	4
	N	3	17

(a)

Cross-validation 2		Real	
		P	N
Prediction	P	7	6
	N	5	15

(b)

Cross-validation 3		Real	
		P	N
Prediction	P	9	6
	N	3	15

(v)

Cross-validation 4		Real	
		P	N
Prediction	P	8	7
	N	4	14

(g)

Table 2. Confusion matrix for each cross-validation: a) Cross-validation during using the last quarter of the set for the test; b) Cross-validation using the third quarter of the set for the test; v) Cross-validation using the second quarter of the set for the test; g) Cross-validation using the first quarter of the set for the test.

ture. The enhanced number of iterations will not lead to a further reduction in training error. The presentation of the learning curve for each step in cross-validation is presented in Figure 6.

In each step of cross-validation, the accuracy of the algorithm was calculated by determining the percentage of accurately classified elements for all classes (Table 1). This factor was calculated by comparing the class elements obtained by the neural network with the classifications by a specialist doctor. A total accuracy of 71.2% in the class recognition was obtained by averaging all four steps of cross-validation.

Cross-validation 1	Recognise as 2	1
	Recognise as 3	2
Cross-validation 2	Recognise as 2	0
	Recognise as 3	3
Cross-validation 3	Recognise as 2	1
	Recognise as 3	4
Cross-validation 4	Recognise as 2	2
	Recognise as 3	2

Table 1. False recognition of PMV in each cross-validation. The second column represents the class to which the PMV signal is classified. The third column shows the number of incorrectly classified PMV signals.

The analysis of the MVP class was especially interesting compared with that of the two other classes. For this research, the following parameters were analysed and these parameters were easily obtained using a confusion matrix. In each step of cross-validation, each of these parameters was calculated by using a confusion matrix (table 2). A confusion matrix was generated with the following values: TP (true positives) – number of input sets classified as MVP; FP (false positives) the number of input sets incorrectly classified as MVP; FN (false negatives) – number of MVP input sets that were not classified and TN (true negatives) – the number of input sets that were not classified as MVP because they were not MVP.

For each class described above, the mean value obtained from four applications of cross-validation was used. Furthermore, the mean value of each parameter was calculated from each of these cross-validations. The accuracy of the MVP classification is given as follows :

$$A = \frac{\sum_1^4 \frac{TП + TH}{TП + TH + ФП + ФH}}{4} = 71.2\%$$

1. Level of classification error:

$$E = \frac{\sum_1^4 \frac{ФП + ФH}{TП + TH + ФП + ФH}}{4} = 28.8\%$$

2. Accuracy:

$$П = \frac{\sum_1^4 \frac{TП}{TП + ФП}}{4} = 59.1\%$$

3. Recall:

$$P = \frac{\sum_1^4 \frac{TП}{TП + ФH}}{4} = 68.75\%$$

4. Specificity:

$$C = \frac{\sum_1^4 \frac{TH}{TH + ФП}}{4} = 72.6\%$$

5. F-measure:

The same measures could be used for the other two classes; however, in this study, the classification of the MVP class was of primary importance. Hence, it is possible to determine which class of each classification was incorrectly identified as an MVP by the neural network.

DISCUSSION

A PCG is a display of the heart sound signals and thus shows heart sounds and murmurs that can provide useful information to the physician by complementing cardiac auscultation. The basic methodology for distinguishing cardiac murmurs using the PCG is the same as that for interpreting murmurs from auscultation. However, the PCG provides additional information about the timing of cardiac phases and events. PCG provides a digital record that can be utilised to characterise the dynamic changes that are associated with therapy and the course of the disease. PCG complements auscultation.

The major clinical drawback of PCG is that it does not present information about the frequency (pitch) of heart sounds and their components. Frequency is one of the major characteristics that is considered when interpreting murmursin clinics . A PCG does not have the ability to differentiate separate multiple (folded) frequencies of various sounds and presents no information concerning dynamic changes in the energy (power) stored in the sound. Other deficiencies arguably include signal filtration effects (change in the visual representation due to filtration) and the presence of artefacts and noise that can visually mask weak sounds. Challenges in pinpointing the start and end points of certain sounds have been reported. The end point positions will also depend on the applied filter, which introduces additional uncertainty. Manual segmentation (separation of heart sound components) may be another problem as well.

PCG never achieved acceptance as a routine clinical investigative method but did find a valuable place in clinical investigations and research. However, the current newly developed "systems science" and signal processing computational technologies in combination with digital sound recording technologies, electronic recording stethoscopes, advanced new vibration sensors and finally, extraordinary computing power, now make it possible to completely revitalise old PCG-based approaches. The addition of the frequency (pitch) dimension to the PCG signal display provides further spectral information about the heart sounds.

Don Michael (10) illustrated the intrinsic properties of various heart lesions in his monograph "Auscultation of the Heart". Similar works have recently been reported by Balster et al. (11) and Nopponen and Lukkarinen (12, 13). Tovar-Corona et al. (14, 15), DeGroff et al. (16), Tuchinda and Thompson (17, 18) utilised a wavelet-based transform to obtain time-varying scalogram maps. The spectrogram offers additional insight into time-dependent changes in murmur frequency. Donnerstein (19) correlated the frequency characteristics in a spectrogram with the Doppler echo velocity. Tavel & Katz (20, 21) reported a method for clinical differentiation of aortic stenosis from an innocent murmur using spectrogram measurements. Finally, Tavel (22) indicated great promise for this approach in clinical diagnosis.

Unfortunately, the methods described in other papers (17, 20) use various forms of the short-term fast Fourier transform (STFT) to obtain the instantaneous frequency characteristics of signals and all these methods are subject to the "quantum uncertainty" theorem , which states that a signal and its Fourier transform cannot both be concentrated (23) and that frequency and time cannot both be determined to arbitrary precision (24, 25). The resulting outcome of this drawback is a non-unique, low-fidelity image, which changes depending on the frequency resolution (26, 27). Additionally, heart sounds are nonlinear, non-sinusoidal and exponential signals and signal processing research (28) has demonstrated that the Fourier transform is not a mathematically appropriate method to study such signals.

Tuchinda and Thompson (29), Tovar-Corona et al. (14, 15) and DeGroff et al. (16) utilised a continuous wavelet based transformation (CWT) to develop maps that resemble spectrograms and present the wavelet scale variation in time (scalograms) . The CWT approach is not as well established as the traditional spectrogram approach in clinical studies (17), but the use of CWT and is presently increasing . Unlike STFT spectrograms, for CWT, the time and frequency resolution is non-uniform in the entire time-frequency domain (30). At high frequencies, there is good time resolution and bad frequency resolution. At low frequencies, the frequency resolution is better and the time resolution is worse. Thus, this results in smearing of the time-frequency representation of the signal in time at low frequencies. The speed of wavelet transform computations and the improved resolution over the STFT are the primary reasons that the wavelet transforms have become a popular analysis tool (22). The graphical results presented by Tuchinda and Thompson (29) also fail to provide sufficient qualitative resolution and have a strong visual "skewness" compared with traditional spectrograms.

There are numerous recent publications on the subject of the digital recording and analysis of heart sounds. Green et al. (31) discuss optimal methods of recording heart murmur findings using SNOMED templates, DeGroff et al. (16) suggest a potential for computerised frequency analysis to improve further the accuracy of murmur assessment and Nigam et al. (32) introduced new methods of segmenting heart sound signals. Finley et al. (33) demonstrated the diagnostic quality of email digital recordings of children's heart sounds and that these recordings allow accurate distinction between normal and pathological murmurs in >90% of cases. Kudriavtsev et al. (34) demonstrated that Still's murmurs have a narrow spectral bandwidth, a significant feature that can differentiate them from abnormal murmurs.

Clinical interest in spectrographic representations of heart sounds is clearly increasing. However, existing signal processing methods lack accuracy and resolution. Unlike other short-term Fourier transform-based approaches (19) and Gabor's transformation (22) which provides an approximation of the instantaneous energy distribution of a signal, the Wigner-Ville distribution (35, 36) has been derived to

compute the signal energy at each time instant, precisely utilising knowledge of the entire signal to compute the time-frequency properties for each moment in time.

The biomedical research community has shown more interest in the detection and classification of cardiac sounds via phonocardiography and auscultation in cardiac diagnosis. Different support-decision systems are developed using phonocardiography and automatic classifiers (37). An ANN-based classifier can be used for heart sound analysis, where different approaches may precede the classification process, such as the previously described wavelet representation (37). The phonocardiograms (38) were subjected to a fast Fourier transform to extract the energy spectrum in the frequency domain to detect heart murmurs in children. The processed signals were used to develop statistical classifiers and a classifier based on ANN. The ability to distinguish pathological and normal heart murmurs may provide valuable information about a potential diagnosis. Another example of diagnosis-oriented classification is the method proposed by Reed et al. for the classification of heart acoustics signals based on a least square support vector machine (LSSVM) using a wavelet-based feature set (38). In this paper, an ANN-based method for determining the diagnosis for a specific condition, mitral valve prolapse, is proposed .

This paper proposed an ANN-based method for automatic detection of mitral valve prolapse. The selected method uses MLP with a back-propagation batch gradient descent learning algorithm. The proposed ANN-based architecture showed 71.2% accuracy. Here, we use 200 epochs for learning and we conclude from experiments that the error does not change when more epochs are used for learning.

The adopted model of artificial neural networks includes the results using two successive layers of a perception neural networkand and has three layers: input, hidden and output. The input layer has 64033 neurons; the hidden layer has 95 neurons; and the output layer has 2 neurons . The network was trained using a learning algorithm using the backward and batch gradient algorithms. The cross-validation one against all (one- versus- all) algorithms were used for validation and classification, respectively . The training and validation of the neural network used two different data sets. Methods such as the confusion matrix mall together with the various statistical values that can be derived from it were used to evaluate the network.

Compared with other works, our method is different in that it does not use data preprocessing. The data were obtained directly from the electronic stethoscope, without selection of the most suitable cardiac cycle by an expert. The data include the full eight-second recordings Electrocardiograms that were recorded in parallel were not available but are necessary for successful segmentation, as mentioned in the presented work; therefore, it was not possible to perform segmentation prior to the analysis . Another approach to segmentation that has been described involves the use of Shannon's energies, but this method utilises a carefully selected cycle or just one cardiac cycle . Although the accuracy and specificity of our approach is less than that of the described work, we consider our method to be more objective because it does not require an expert to select quality cycles. For the same reason, that method only considers changes in the appearance of the cycle and more robust murmurs and PMV is not required to occur in every cardiac cycle. This technique differs from our approach to the classification of a specific disorder that can be detected by PCG (PMV) and the classification of other pathological and healthy signals, as in most of the described work. Again, we would note that the results are validated in a completely new set of data that the network had not previously used.

The present analysis of phonocardiographic signals usually includes electrocardiography as a reference method. Our method has shown that it is possible to create a system that would be based only on PCG.

Further research should be able to increase the accuracy and specificity of the method described. The proposed method is signal based. It does not involve calculating features or preprocessing of signals. To improve the classification ANN should be supported by specific features. Several solutions have been proposed so far and the wavelet transform is considered to be a promising tool, which can result in localisation of the click murmur syndrome in phonocardiograms.

CONCLUSION

The obtained result can be considered a useful tool for clinical support in the initial examination of phonocardiograms and may reveal mitral valve prolapse in children by phonocardiography.

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CONFLICT OF INTERESTS

All the authors of the present paper disclose that they have no actual or potential conflict of interests, including any financial, personal or other relationships with other people or organisations.

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SHARPNESS OF VISION OF PILOTS IN AIR FORCE OF SERBIA AFTER +Gz ACCELERATION IN HUMAN CENTRIFUGE

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OŠTRINA VIDA KOD PILOTA ViPVO NAKON IZLAGANJA +Gz UBRZANJU U HUMANOJ CENTRIFUGI

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ABSTRACT

The high speeds achieved during the take-off, flight, and landing of modern aircraft present limitations for the visual system. The importance of maintaining visual function during these intervals has been recognizised since the earliest stages of aviation development. Because of the great practical importance of air combat, research on visual stress during flight is of great importance receives much attention. Vision is the most important sensory function in terms of both flight safety and the quality performance of flight duties.

Visual acuity of 12 Air Force pilots was investigated before and after exposure to + Gz acceleration in a human centrifuge. This centrifuge is a combination gravity and altitude apparatus, capable of reaching accelerations of up to 20 G and simulating altitudes of up to 30,000 m. Each pilot had individual centrifuge training, and individual skills were first statedmeasured at the first time of exposure to G acceleration , and again in a one week . The training level that correspondscorresponding to the improvement of individual skills during submission to Gz acceleration was applied.

Exposure to +Gz acceleration provokes significant reactions and fluctuations in the eye. Immediately after exposure to + Gz acceleration, there was a transient decrease in visual acuity at a distance of 0.02 ± 0.04 degrees of visual angle. Pupil diameter increased from 3.5 ± 0.6 to 5.6 ± 0.5 mm. This dilation continued for 15 min following exposure to acceleration. Changes on the eye bottom were not noted.

Previous work has shown that exposure to + Gz acceleration results in an increase in the depth of the eye chamber. Energy reserves in retinal tissue and the central nervous system allow continued operation of brain and visual systems to continue for a few seconds following interruption of blood supply to the head. This enables rapid tolerance to high G loads for a short period of time, usually approximately 5 seconds.

At high initial rates of acceleration, significant changes in visual function can occur. However, the importance of maintaining visual acuity is increasing due to the applica-

SAŽETAK

Velike brzine prilikom poletanja, tokom letenja i prilikom sletanja modernih letilica predstavljaju dodatni napor za vizuelni sistem. Od samog pocetka razvoja vazduhoplovstva, funkciji vida se pridaje izuzetan znacaj. Zbog velikog praktičnog značaja u vazdušnoj borbi, uticaj +Gz ubrzanja na organ vida je veoma značajno za istraživanje. Od svih čulnih funkcija kojima čovek raspolaže vid je najvažniji kako u pogledu bezbednosti letenja tako i za kvalitet izvršavanja letačkih zadataka.

Ispitivana je oštrina vida kod 12 pilota ViPVO pre i nakon izlaganja +Gz ubrzanju u humanoj centrifugi. Centrifuga koja je korišćena predstavlja kombinaciju gravitacione i visinske laboratorije, jer pored ubrzanja od 20 G, može simulirati i visinu do 30.000 m. Svaki od pilota ima individualni trening na centrifugi, pri čemu se konstatuju individualne sposobnosti u trenutku izlaganja G ubrzanju, a zatim se u toku jedne nedelje primenjuje nivo treninga koji odgovara poboljšanju individualnih sposobnosti podnošenja +Gz ubrzanja.

Izlaganje +Gz ubrzanju izaziva značajne reakcije i fluktuacije oka. Prvo što je uočeno odmah nakon izlaganju +Gz ubrzanju bilo je prolazno smanjenje oštrine vida na daljinu za 0,02 ± 0,04 stepena vidnog ugla. Promer pupile je povećan od 3,5 ± 0,6 na 5,6 ± 0,5 mm i dilatacija pupile trajala je 15 min nakon izlaganja ubrzanju. Promene na očnom dnu nisu uočene.

U radovima drugih autora je pokazano da nakon izlaganja +Gz ubrzanju dolazi do povećanja dubine očne komore. Energetske rezerve u retini i centralnom nervnom sistemu, omogucavaju funkcionisanje mozga i vidnog aparata nekoliko sekundi od prekida dotoka krvi u glavu. Time je omogućena tolerancija naglo nastalih visokih G opterećenja u kratkom vremenskom periodu, uobičajeno oko 5 s.

Kod visokog početnog stepena ubrzanja mogu se javiti značajne promene u vidnim funkcijama. Međutim, značajno je održati oštrinu vida usled primene novih funkcionalnih displeja za brzu orjentaciju pilota u prostoru, vizuelne kon-

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tion of novel functional displays for rapid orientation of the pilot in space, the configuration of the area field, aircraft opponents, and weapon systems . Therefore, it is necessary to continue studies that will provide accurate indicators of visual acuity in the context of real Gz acceleration and spatial disorientation.

Key words: *Visual acuity, + Gz acceleration, pilot, human centrifuge, G loads*

figuracije reljefa terena, prikaza oružanih sistema, protivničkih aviona, i dodatno usložnjene orjentacije u prostoru. Neophodno je nastaviti ispitivanja koja će obezbediti precizne pokazatelje oštrine vida u stanju realnog +Gz ubrzanja i prostorne dezorijentacije.

Ključne reči: *oštrina vida, +Gz ubrzanje, pilot, humana centrifuga, G stres*

INTRODUCTION

The high speeds achieved during the take-off, flight, and landing of modern aircraft can compromise the visual system. Since the beginning of the development of aviation, great importance has been attributed to visual function. In the age of aircraft automatisation pilots have significant control over all manual and automatic devices, primarily through the sense of vision. The human body has adapted to the force of gravity, and many activities can be performed in this environment. However, the development of modern aviation has imposed loading at ten or more times the force of gravity. Such loading can cause changes in appearance caused by the inertia force, in turn as a result of the applied acceleration. Applied acceleration when flying , commonly called G loading, represents the ratio of acceleration due to gravity. Acceleration due to gravity is a physical constant represented by the symbol g with a value of 9.81 m/s2. The G value of the applied acceleration is given by the formula:

$$G = \frac{applied\ \ acceleration}{g}$$

If the body is exposed to an acceleration of 5 G, its acceleration will be 5 times that of gravity (9.81 m/s^2) or 49.05 m/s^2. The rate of change of acceleration, or acceleration gain (G), is important because of the physiological response to the forces generated during flight. In aviation, inertial forces acting on the human body are substantial during acceleration (1).

In Gz acceleration, inertia acts parallel to the longitudinal axis of the body to drive the pilot into the pilot seat. Because of the great practical importance of air combat, research on this stress remains an critical area of study. Tolerance to this stress can vary in individuals depending on food intake, environment, vascular tone, mental and physical condition, and other factors. Vision is the most important sensory function in terms of flight safety and quality performance of flight duties. It is the primary sense for the pilot and provides information on the status of his plane in space. During flight, the pilot is almost entirely dependent on their sense of sight, which allows him to read information from the instruments. Maintaining a high level of visual acuity is considered a paramount necessity for pilots, especially with the current use of extremely high-speed aircraft at all altitudes. The need for safe and successful flights has led to the generation of very strict criteria for the medical selection of candidate pilots. It is important that the pilots are knowledgeable in how to best utilizise their visual capacity, in addition to possessing excellent visual acuity.

For pilots, the concentration of visual acuity toin a very small area of the retina is a major disadvantage. To ensure that the retinal areas that provide the clearest vision are utilizised, it is imperative that pilots continually move their eyes while observing objects in the visual field. The maneuverability of modern aircraft can cause significant acceleration, with strong effects on the eye. When acceleration exceeds + 3.5 Gz and a duration of 6-12 seconds, there is a disturbance in visual function. This vision loss is caused by a redistribution of the blood to body parts below the heart, causing a decrease in the blood pressure in the head. This decreased blood pressure directly affects the sharpness of vision (4,5). This could further leads to the loss of peripheral and central vision and loss of consciousness, as; the initial dimming of vision proceeds to loss of consciousness due to insufficient blood supply to the brain. In this situation, visual disturbance is a useful reminder against exceeding our physiological capabilities, as this can advance result in to loss of consciousness. Thus, our motivation for testing the visual acuity of pilots in the human centrifuge before and after exposure to + Gz acceleration is to increase the individual tolerance capacity to +Gz acceleration of Air Force pilots.

The purpose of this study is to assess visual function after exposure to + Gz acceleration in pilots.

MATERIALS AND METHODS

The visual acuity of 12 Air Force pilots was investigated before and after exposure to + Gz acceleration in a human centrifuge, a device necessary for training pilots flying high-performance aircraft at high G loads. This centrifuge is a combination gravity and altitude laboratory apparatus, capable of reaching acceleration of up to 20 G and simulating altitudes of up to 30,000 m. Each pilot had individual centrifuge training, and individual skills were first measured at

the first time of exposure to G acceleration and then again in one week. The training level that corresponds to the improvement of individual skills during submission to Gz acceleration was applied. Before the start of testing, pilots underwent an emergency eye examination that included testing of distance and near vision, intraocular pressure, and colour vision in addition to as well as biomicroscopic examination of the fundus and the transparent media of the eye. Distance and near visual acuity was tested for each pilot using Landolt`s optotype and amounted to 1.0 G before exposure to stress. Intraocular pressure intraocular pressure of the subjects was within normal limits with normal colour vision, and all subjects were in good general health with no previous eye diseases. After exposure to + Gz acceleration, visual acuity was tested immediately, then and 15 min and 30 min following acceleration. To test visual acuity at distance, we used Landolt`s rings at a distance of 6 m. The fundus was examined 2 hours after exposure to acceleration.

For the statistical analysis of the data, a t-test was applied (Student distribution).

RESULTS

Exposure to +Gz acceleration provokes significant reactions and fluctuations of the eye. Observation immediately after exposure to + Gz acceleration, there was a transient decrease in visual acuity at a distance of 0.02 ± 0.04 degrees of visual angle. The pupil diameter increased from 3.5 ± 0.6 to 5.6 ± 0.5 mm, and pupil dilation lasted for 15 min after exposure to acceleration. Changes to the eye bottom were not noted.

DISCUSSION

Previous work (2,3) has shown that exposure to + Gz acceleration will result in an,increase in eye chamber depth. Energy reserves in retinal tissue and the central nervous system will allow continued operation of the brain and visual systems to continue for a few seconds after interruption of the blood supply to the head. This enables rapid tolerance to high G loads over a short period of time, usually approximately 5 seconds. The baroreceptor reflex significantly increases G tolerance, starting 10 s following after exposure, and may improve tolerance to more than 1G until for about 1approximately 15 seconds if there is enough time for development. Intraocular pressure results in the cessation of blood flow to the retina at a higher pressure than that at which blood flow to the brain stops. This phenomenon is responsible for visual symptoms at approximately 1 G below the level at which G-induced loss of consciousness occurs. When the initial rate of acceleration exceeds the capacity of the baroreceptor reflex, relaxed tolerance averages + 3.5 Gz for the grey veil and +4.5 Gz for loss of consciousness. At this initial stage of acceleration, the pilot has a few seconds between the beginning of visual symptoms and loss of consciousness. When the initial rate of acceleration is very high (10 G/s), the lack of blood flow to the head causes a depletion of the energy reserves of the eyes and brain. This leads to loss of consciousness without noticeable visual symptoms. The energy reserves of the eyes and the brain are depleted after approximately 5 seconds, and the baroreceptor reflex is not activated in this time frame. This feature of high Gz acceleration that causes a complete loss of consciousness with little or no visual symptoms and is potentially dangerous for pilots because of its insidious nature.

We can clearly observe the alterations in visual acuity in response to + Gz stress as well as changes in blood pressure at the level of the head and body blood flow. High + Gz stress has two primary effects; the immediate effect is a drop in blood pressure at head level that is proportional to the +Gz load. This drop in blood pressure is responsible for a 22-25 mmHg difference in blood pressure between the heart and brain for every +1 Gz (6). Additionally, high + Gz stress causes blood to accumulate in other parts of the body, such as the legs and abdomen. Acute redistribution of blood results in the reduced venous return of blood, reducing cardiac output and blood pressure at the level of the heart. This further contributes to the loss of blood pressure in the brain. The loss of retinal blood pressure, which mirrors the blood pressure drop in the brain, is the mechanism responsible for the tunnel vision, grey veil, and black veil that occur during high-G stress.

CONCLUSION

At high initial rates of acceleration, significant changes in visual function can occur. However, the importance of maintaining visual acuity is increasing due

before	1.00	1.10	1.00	1.00	1.10	1.10	1.00	1.00	1.00	1.10	1.10	1.00
after	0.90	1.00	0.90	0.90	1.00	0.90	0.90	1.00	1.00	1.00	0.90	1.00

Table 1: Visual acuity at distance before and after exposure to + Gz acceleration

before	3.30	3.50	3.60	3.30	3.50	3.60	3.30	3.20	3.60	3.40	3.50	3.40
after	5.40	5.50	5.60	5.40	5.50	5.60	5.40	5.50	5.60	5.40	5.50	5.60

Table 2: Pupil diameter before and after exposure to + Gz acceleration

to novel functional displays for rapid orientation of the pilot in space, the configuration of the area field, weapon systems, and aircraft opponents. These displays require visual verification of color images and emphasise the need for precise assessments of position in space at high speeds and dynamic loads. The protection and safety systems that are used must monitor the performance and capabilities of modern aircraft and they must conform to the maximum physiological tolerance . New generation aircraft will be more demanding and must include new applicable solutions.

Therefore, it is necessary to continue studies that will provide accurate indicators of visual acuity during Gz acceleration and spatial disorientation.

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HEART RATE MODULATIONS IN OVERTRAINING SYNDROME

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MODULACIJE SRČANE FREKVENCE U SINDROMU PRETRENIRANOSTI

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ABSTRACT

Every sports training program includes a component of repetitive overloading, but with an inadequate recovery time, such overloading may produce undesired effects, such as chronic fatigue and a lack of performance improvement. The state of underperformance known as the overtraining syndrome (OTS) is characterised by a number of physiological and psychological symptoms of maladaptation. It may take weeks, months or years to restore proper sports form after the development of OTS. Although overtraining has been studied for decades, the mechanism of overtraining and the tools for the early detection of overtraining are still not defined. In addition to other physiological, biochemical, immunological, psychological and performance markers, heart rate (HR) and its modulations are intensively investigated as a practical and reliable sign of overtraining. In general, resting bradycardia, a decrease in HR during submaximal exercise, an increase in speed of heart rate recovery and increased vagal-related heart rate variability indices are all well accepted markers of improved aerobic fitness. In contrast, changes of these HR measures in the opposite direction are commonly interpreted as indicators of detraining, chronic fatigue, non-functional overreaching or overtraining. However, based on the limited and diverse literature available, these parameters may be used for monitoring training status, optimising training programs and following the accumulation of fatigue, but their role in overtraining detection and assessment has yet to be elucidated. There is a need for well-controlled prospective studies where a longitudinal follow-up of athletes is performed. Because it is unethical to excessively train an athlete to produce OTS, an animal experimental model of OTS may advance our understanding of the "unexplained underperformance syndrome."

Keywords: *overtraining, heart, heart rate, heart rate variability, heart rate recovery*

SAŽETAK

Svaki program sportskog treninga se sastoji od ponavljanog preopterećenja, ali u nedostatku oporavka on može izazvati neželjene efekte kao što su odsustvo poboljšanja sportskog izvođenja i hronični umor. Ovo stanje pogoršane sportske forme, poznato kao sindrom pretreniranosti, je u vezi sa nizom fizioloških i psiholoških simptoma maladaptacije, a za obnovu sportske forme mogu biti potrebne nedelje, meseci, pa i godine. Iako se decenijama istražuje, mehanizam nastanka pretreniranosti, i što je još važnije sredstva za rano otkrivanje pretreniranosti, još uvek nisu definisana. Pored ostalih fizioloških, biohemijskih, imunoloških, psiholoških i markera sportskog izvođenja, srčana frekvencija i njeni modaliteti se intenzivno istražuju kao praktičan i validan znak pretreniranosti. Generalno, bradikardija u miru, niža srčana frekvencija tokom vežbanja submaksimalnog intenziteta, brži oporavak srčane frekvence nakon vežbanja i povećanje indeksa varijabilnosti srčane frekvence povezanih sa povećanjem vagalnog tonusa, su znakovi poboljšanog aerobnog fitnesa. S druge strane, promene ovih srčanih parametara u suprotnom smeru se često smatraju indikatorima netreniranosti, hroničnog umora, nefunkcionalnog preopterećenja i pretreniranosti. Na osnovu limitirane i raznolike dostupne literature, ovi parametri mogu biti predloženi za praćenje trenažnog statusa, poboljšanje trenažnih programa i praćenje akumuliranja umora, ali njihova uloga u detekciji i proceni pretreniranosti tek treba da bude objašnjena. Postoji velika potreba za kontrolisanim prospektivnim studijama u kojima bi se vršilo longitudinalno praćenje sportista, ali obzirom da je treniranje sportista na takav način da dodju u stanje pretreniranosti neetičko, dobar eksperimentalni životinjski model pretreniranosti bi mogao da pomogne u razumevanju ovog neobjašnjenog sindroma pada sportske forme.

Ključne reči: *pretreniranost, srce, srčana frekvencija, varijabilnost srčane frekvencije, oporavak srčane frekvencije*

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ABBREVIATIONS:

ANS – autonomic nervous system
FOR – functional overreaching
NFOR – nonfunctional overreaching
HR – heart rate
HRR – heart rate recovery
HRV – heart rate variability
OR – overreaching
OT – overtraining
OTS – overtraining syndrome
RHR – resting heart rate

BACKGROUND

The modern training of athletes is based on the overload principle and the negative feedback theory: the training stimulus must be strong enough to induce a disturbance of homeostasis so that the body has to initiate reactions to adapt to the training stimulus (1). Continuous exposure of an athlete to training stress initiates structural and functional changes that enable the improvement of an athlete's sports performance (2). To achieve desired adaptations and reach a sports performance conditioning level, the training load on a single exercise session and on every micro-, meso- and macro-cycle of training must be adequately dosed. According to the contemporary understanding, in a well-planned and programmed training process, the intensity of training increases stepwise, the periods of rest between the bouts are sufficiently long to ensure regeneration of the muscle functions and the periods of rest are short enough to not allow the regression of supercompensation (3). Every advantageous training program included a component of repetitive overloading, but with an inadequate recovery time, such overloading may produce undesired effects, such as chronic fatigue and the absence of performance improvement (4). The lack of improvement in sports performance or a decrease in sports performance is characterised by a number of physiological and psychological signs and symptoms of maladaptation, and it may take weeks, months or years to restore proper sports form (5). This state of underperformance was previously described by terms such as burnout, staleness, failure adaptation, underrecovery, training stress syndrome, and chronic fatigue (6). Recent literature has used the terms overreaching (OR) and overtraining (OT). Many consider overreaching and overtraining as lying on a continuum (Table 1), but this may be an oversimplification (7). The difference between OR and OT is based on the time to recovery and not necessarily the degree or type of symptoms exhibited (8). Many recent articles have referred to the work of Kreider et al. (9) for the definition of OR and OT: OR/OT represents an accumulation of training and nontraining stress resulting in a short-term/long-term decrement in performance capacity with or without related physiological and psychological signs and symptoms of maladaptation in which the restoration of performance capacity may take several days to weeks or weeks to months . It should also be noted that the terms overreaching and overtraining refer to the stimulus, and the term overtraining syndrome (OTS) refers to the resulting condition (10). By using the expression "syndrome," the possibility of a multifactorial aetiology is acknowledged, in which exercise (training) is not the sole causal factor of the syndrome (5).

DIAGNOSIS AND ASSESSMENT OF OVERTRAINING

Some researchers have referred to overtraining as unexplained underperformance syndrome (11,12). Currently, it appears that OTS represents a systemic inflammatory process with diffuse effects on the neurohormonal axis affecting host immunology and mood (8). Although in recent years the knowledge of central pathological mechanisms of overtraining has significantly increased, there is still a need to further study overtraining. The lack of improved sports performance or the decrease of sports performance is related to a number of physiological and psychological signs and symptoms of maladaptation. It may take weeks, months or years to restore proper sports form (5). The diagnosis of OTS is complicated by the fact that the clinical features are varied from one individual to another and are

Training load	intensification of training			
Outcome	Acute fatigue	Functional overreaching (FOR)	Nonfunctional overreaching (NFOR)	Overtraining Syndrome (OTS)
Performance	Increase	Temporary decrement	Stagnation	Decrease
Recovery	Day(s)	Days - weeks	Weeks - months	Months - ...

Table 1. Terminology from a joint consensus statement on overtraining by the European College of Sport Science and the American College of Sports Medicine (5).

nonspecific, anecdotal, and numerous (5). The only certain sign is a decrease in performance during competition or training (5). An excellent review of the topic published more than 20 years ago suggested more than 80 possible major symptoms in addition to performance decrements are involved (40 physiological, 12 psychological and information processing, 14 immunological and 18 biochemical symptoms) (13). Subsequent years of study have added to the list. Currently, several markers (hormones, performance test scores, psychological test scores, and biochemical and immune markers) are used, but none of these markers meet all the criteria for their use to be generally accepted (5). Many review articles have offered explanations for the mechanism behind the OTS (5,6,12,14-24). Numerous hypotheses have been proposed for OTS (glycogen hypothesis, central fatigue hypothesis, glutamine hypothesis, oxidative stress hypothesis, autonomic nervous system hypothesis, hypothalamic hypothesis, cytokine hypothesis), each with strengths and weaknesses (6). Although these hypotheses have potential, they remain speculative until more prospective studies with longitudinal follow-ups are carried out. The definitive diagnosis of OTS requires the exclusion of organic diseases, infections, disorders, dietary caloric restriction, insufficient carbohydrate and/or protein intake, iron deficiency, magnesium deficiency, allergies, and so on (5). The identification of initiating events or triggers that lead to OTS is also important. One of the most certain triggers is a training error resulting in an imbalance between load and recovery. Other possible triggers are the monotony of training, too much competition, personal and emotional problems, sleep disturbance, altitude exposure, exercise heat stress, and so on. (5, 6). Because there is no diagnostic tool to identify an athlete experiencing OTS, the solution to the differential diagnosis can only be made by excluding all other possible causal factors for changes in performance and mood state. Therefore, if no explanation for the observed changes can be found, OTS is diagnosed (5).

HEART RATE MODULATIONS IN OVERTRAINING SYNDROME

The assessment of physiological parameters has been the primary method used to identify overtrained athletes (25). As previously mentioned, exercise is a biological stimulus to which the physiological systems, in particular the autonomic nervous system (ANS) and adrenal glands, respond during and after an exercise bout to maintain homeostasis (2). Because the ANS is connected to many other physiological systems, the responsiveness of the ANS may provide useful information about the functional adaptations of the body. It is well known that the ANS has a major effect on heart rate (HR), and the development of heart rate monitors has allowed further research into the mechanisms behind HR responses to exercise and adaptations to training (2). Technological advancements in portable and wearable systems for the real-time collection of physiological data provide new opportunities for computerised diagnostics and quantitative modelling in medicine and related sports applications. For example, modern sport watches with ECG-type sensors can be used not only for programming personalised training sessions but also for the simultaneous collection of beat-to-beat (RR) time series, with an accuracy comparable to clinical ECG equipment (26). Such personal RR data can be used for systematic heart rate variability (HRV) analysis to provide early indication of developing cardiac abnormalities, detection of overtraining and other purposes. Measurement of heart rate recovery (HRR) also has the potential to be a useful tool for monitoring fatigue and prescribing training load in well-trained and elite athletes (27).

HR and its modulation are primarily determined by the inotropic and chronotropic effects of both branches of the ANS on the myocardium and the sinus node (28, 29). Sympathetic stimulation increases heart rate, contractility and conduction velocity, whereas parasympathetic stimulation has the opposite effect (30). The adaptive responses of the cardiovascular system to regular physical activity appear to include a reduction in sympathetic activity and an increase in parasympathetic activity during rest and at different intensities of exercise (31-33). These training-induced autonomic changes are coupled with a possible reduction in intrinsic heart rate, a decrease resting heart rate and an increase heart rate variability at rest (34-38). Athletes also show reduced sympathetic activity for any given submaximal work-rate compared with sedentary controls exercising at the same rate (2, 31, 34) and have a more rapid heart rate recovery following exercise (36, 39). In contrast, changes to these HR measures in the opposite direction (increases in submaximal HR, decreases in HRR and/or decreases in vagal-related HRV indices) are commonly interpreted as indicators of detraining (40, 41), chronic fatigue, non-functional overreaching or overtraining (2, 28, 42). Specifically, decreased sympathetic activation and parasympathetic dominance can lead to performance inhibition, fatigue, depression, and bradycardia (7, 43). However, it is worth noting that the interpretation of the changes in these HR measures is based on theoretical principles related to the expected ANS response to fatigue rather than on scientific evidence and that unexpected and/or unclear results have also been reported (44).

RESTING HEART RATE

The ANS plays a pivotal role in stress tolerance (17). The scientific and clinical literature suggests that overreaching and overtraining are concomitant with dysfunction of the hypothalamic–pituitary–adrenal (HPA) axis (45). Consequently, a negative adaptation to training stress potentially involves the ANS and may result in a concomitant alteration in HR (28). Increased resting HR is one of the first signs of OTS reported in the literature (46). The

detection of elevated morning resting heart rate is taken by many sport practitioners as a practical method to detect early signs of NFOR and central nervous system fatigue (47-49). This assumption should be taken with care because the morning heart rate is influenced by many factors other than training (50). A number of studies investigated the effects of overtraining on resting heart rate, but changes to RHR do not appear to be consistently present when attempting to identify either overreaching or overtraining (51, 52). After applying physiological stress to induce OR or OTS, some studies found an elevation in RHR (47, 53-56), some found a decrease in RHR (57-59), and some reported no significant changes in RHR (60, 61). Sleep provides a period of rest and restoration, and it may provide a more suitable setting for monitoring heart rate (53, 62). However, studies examining the effects of OR/OTS on the sleeping heart rate also yielded inconsistent results (53, 61-63). A meta-analysis by Bosquet et al. (28) revealed a trivial increase in RHR, suggesting that it cannot be considered as a reliable sign of OTS. Interestingly, HR was moderately increased after short term interventions (<2 weeks), and no significant alteration was found when the increase in training load was longer than 2 weeks (28). This time effect suggests that an increase in RHR may be used as a reliable sign of short-term fatigue (possibly FOR) but not long-term fatigue (possibly NFOR or OTS) (28). The analysis of these results should also account for the existence of two types of overtraining, sympathetic and parasympathetic, which result in different effects on heart rate. The sympathetic (Basedow type) overtraining syndrome is more common in anaerobic sports (6, 50) and is characterised by increased HR and blood pressure (BP); parasympathetic (Addison type) overtraining syndrome is more common in aerobic sports and is characterised by reduced HR and BP (50). It is suggested that sympathetic overtraining occurs during the early stages of OTS and the parasympathetic type occurs in the later, more severe stages of OTS (64).

HEART RATE DURING EXERCISE

During exercise, HR increases linearly with increasing workloads (65). A lower submaximal HR for the same exercise intensity is one of the most commonly observed adaptations to endurance training and is based on a number of adaptations: greater stroke volume, decreased sympathetic and increased parasympathetic activity, lower metabo-reflex activation because the muscles are more efficient at generating energy and reduced metabolite production (66). In addition, the lower sympathetic activity decreases the amount of catecholamines in circulation at a given workload, which contributes to a lower HR response (67). A comprehensive review of studies that investigated the effects of endurance training on HRmax involving a total of 314 subjects concluded that HRmax can decrease by 5-13 beats/min with aerobic training and increase by 4-10 beats/min with tapering or detraining in some individuals (65). The proposed mechanisms for explaining decreases in HRmax with training include plasma volume expansion, enhanced baroreflex function and decreased ß-adrenergic receptor number and density (2). Borresen and Lambert (2) indicated that an increase in at-rest HR and HR during submaximal exercise may serve as a diagnostic indication of overtraining. However, both submaximal and maximal HR have been shown to decrease when an athlete is overreached (68) or in the state of parasympathetic overtraining (48, 68, 69). Under normal circumstances, a lower submaximal response is viewed as an increase in performance capacity (70), but these studies reveal that performance is compromised in such cases due to the negative effects of overtraining. Among the possible explanations for the decreased submaximal heart rates observed in these athletes are a decrease in sympathetic activation (70) and a decrease in p-adrenoreceptor density and/or sensitivity (48) as well as hypervolemia leading to increased stroke volume (68, 69). The decreased maximal heart rate values should be approached with some caution, as peripheral factors may be responsible for the termination of a maximal test before the true HR maximum is reached (68). Reduced maximal heart rates and other maximal physiological measures, such as oxygen uptake and blood lactate, after increased training may be the result of reduced sympathetic nervous system activity, decreased tissue responsiveness to catecholamines, and altered adrenergic receptor activity; such reductions may also simply be a consequence of a reduction in exercise time and a reduced power output achieved with maximal effort and not related to abnormalities per se (5).

HEART RATE VARIABILITY

Advancements in technology made it easy to assess the beat-to-beat variation in resting pulse rates, i.e., heart rate variability (HRV). Heart rate fluctuation may be considered an output variable of a feedback network that is continuously monitored and regulated by the ANS (33). HRV analysis is used as a measure of cardiac autonomic balance, with an increase in HRV indicating an increase in vagal (parasympathetic) tone relative to sympathetic activity (70). Power spectral analysis has shown that parasympathetic and sympathetic activity can be expressed at specific frequencies (71), with a very low-frequency, a low-frequency and a high-frequency component. The occurrence of the very low-frequency component is unclear (33). However, the HF component is mediated by the parasympathetic system, and the LF component has been shown to be mediated by both sympathetic and parasympathetic modulation (71). For more than a decade, HRV has been suggested as a practical non-invasive method of assessing cardiac ANS status and possibly NFOR/OTS (72). This has lead researchers to suggest that HRV may be used to guide the training of elite athletes on a day-to-day basis (73-75). However, to date, studies that have investigated HRV and NFOR/OTS have revealed equivocal findings

with increases (57), decreases (70, 76) and no change (52, 59, 68, 61) shown in cardiac ANS activity. However, most spectral analysis studies support the theory that endurance training increases HRV, increases parasympathetic activity and, thus, contributes to a training bradycardia (31, 34, 77-79). The diversity of these results may be explained by a number of factors. First, the findings from the studies that have purposely attempted to induce NFOR/OTS are limited as it is difficult to differentiate between the three stages of the overtraining continuum (OR, NFOR and OTS). In contrast, studies that have investigated athletes that are already overtrained (57, 76) have not been able to provide baseline HRV values prior to the syndrome's onset, and this is important considering the intraindividuality of HRV recordings (80). Furthermore, day-to-day variability in HRV values is high due to the effects of environmental factors such as noise, temperature, light, and exercise (81), and most of these studies (55, 57, 59, 68, 76) measured HRV on single, isolated and non-consecutive days. Finally, the methods by which HRV have been recorded vary in the literature (82); timing and body positioning also vary during recordings, with some studies investigating HRV during sleep and upon waking (76), using the supine to head-tilt method (55, 57, 59, 68), supine to-standing method (83), or postexercise (44, 84).

HEART RATE RECOVERY

Heart rate recovery is the rate at which HR decreases (or the time taken for heart rate to recover) after the cessation of physical exercise (85-88). Passive post-exercise HRR is currently used in the assessment of endurance athletes to determine changes in performance or in the clinical setting as a predictor of all-cause mortality (89). HRR is dependent on the interaction between parasympathetic and sympathetic nervous activity, i.e., parasympathetic reactivation and sympathetic withdrawal. In fact, the rapid fall in heart rate after exercise appears to be a consequence of the prompt restoration of parasympathetic tone at the heart level, and the further decrease is attributed to the progressive decrease of sympathetic tone and hormonal factors (71, 90). A faster HRR reflects a positive adaptation to exercise training and possibly performance capacity (91-93). Changes in HRR and/or vagal-related HRV indices following training correlated largely with improvements in cardiorespiratory fitness-related performance variables, such as maximal aerobic speed and running/cycling performance (27, 83, 94), as well as with improvement in the more neuromuscular-related performance parameter of repeated-sprint ability (95). Altered HRR kinetics have also been occasionally considered a marker of overreaching or overtraining (13, 96), but there are not enough data available in the literature to support this contention. For example, a sudden increase in the HRR of a trained cyclist was reported with a state of acute fatigue and/or a state of functional overreaching (96). Borresen and Lambert (44) showed that HRR decreases with an increase in training load, and there was a tendency for a faster HRR with a decrease in training load. The authors speculated that the case in which the HRR decreased with an increase in training load can be explained by a sharp increase in the training load that produced symptoms of overreaching in the subjects. Interestingly, Buchheit et al. (95, 97) recently concluded that indices of HRR seem to be more sensitive markers of recently applied training loads than do indices of HRV, which reflect a long-term modulation of the autonomic nervous system with changes in training status. However, more recent work (74, 75, 98) shows that HRV can also track fast changes in training status. Although HRR seems to be a promising tool for monitoring training status, this finding should be taken with care because there is no standard for measuring HRR. The methods used to determine heart rate recovery kinetics can differ dramatically from one study to another. Some studies have calculated time constants by fitting HR decay data to mathematical models (27, 98), and others have looked at the difference between peak heart rate and post-exercise heart rate at a certain time after exercise (87, 99-104). Exercise mode (cycling, running), intensity (maximal, submaximal), and the duration of the recovery period (one to five or more minutes) also greatly differ between studies. Buchheit et al. (105) found that HRR was faster after endurance exercise trials than after repeated sprint or high intensity exercise trials performed by the same subjects. It seems that there may be more than only parasympathetic factors involved in lowering heart rate after exercise, such as blood metabolites, type of previous exercise training and type of exercise during the assessment (i.e., anaerobic vs. aerobic), and so on.

CONCLUSION

Despite years of research, no single factor has been identified that can quantify how an individual responds to training or predict overtraining with accuracy. The usefulness of a physiological marker depends on the ease and frequency with which it can be measured and on the speed with which the results can be interpreted so that frequent monitoring is possible with little inconvenience to the athlete. The functional state of the autonomic nervous system may provide useful information about the overall functional adaptation of the body in response to a training stimulus because it is interlinked with many other physiological systems. In general, resting bradycardia, a decrease in HR during submaximal exercise, an increase in the speed of heart rate recovery and an increase in the vagal-related heart rate variability indices are well accepted markers of improved aerobic fitness. In contrast, changes in these HR measures in the opposite direction are commonly interpreted as indicators of detraining, chronic fatigue, non-functional overreaching or overtraining. However, all of these measurements are affected by the individuality of athletes and extraneous factors that limit their use as sen-

sitive markers of training status. Based on the limited and diverse literature available, these parameters are proposed for monitoring training status, optimising training programs and following the accumulation of fatigue, but their role in overtraining detection and assessment has yet to be elucidated. There is a need for well-controlled prospective studies where a longitudinal follow-up of athletes is performed. Because it is unethical to excessively train an athlete to produce OTS, an animal experimental model of OTS may advance our understanding of the "unexplained underperformance syndrome."

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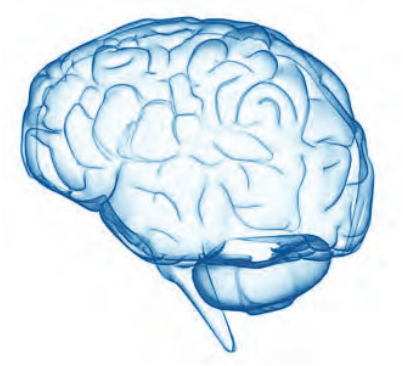

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