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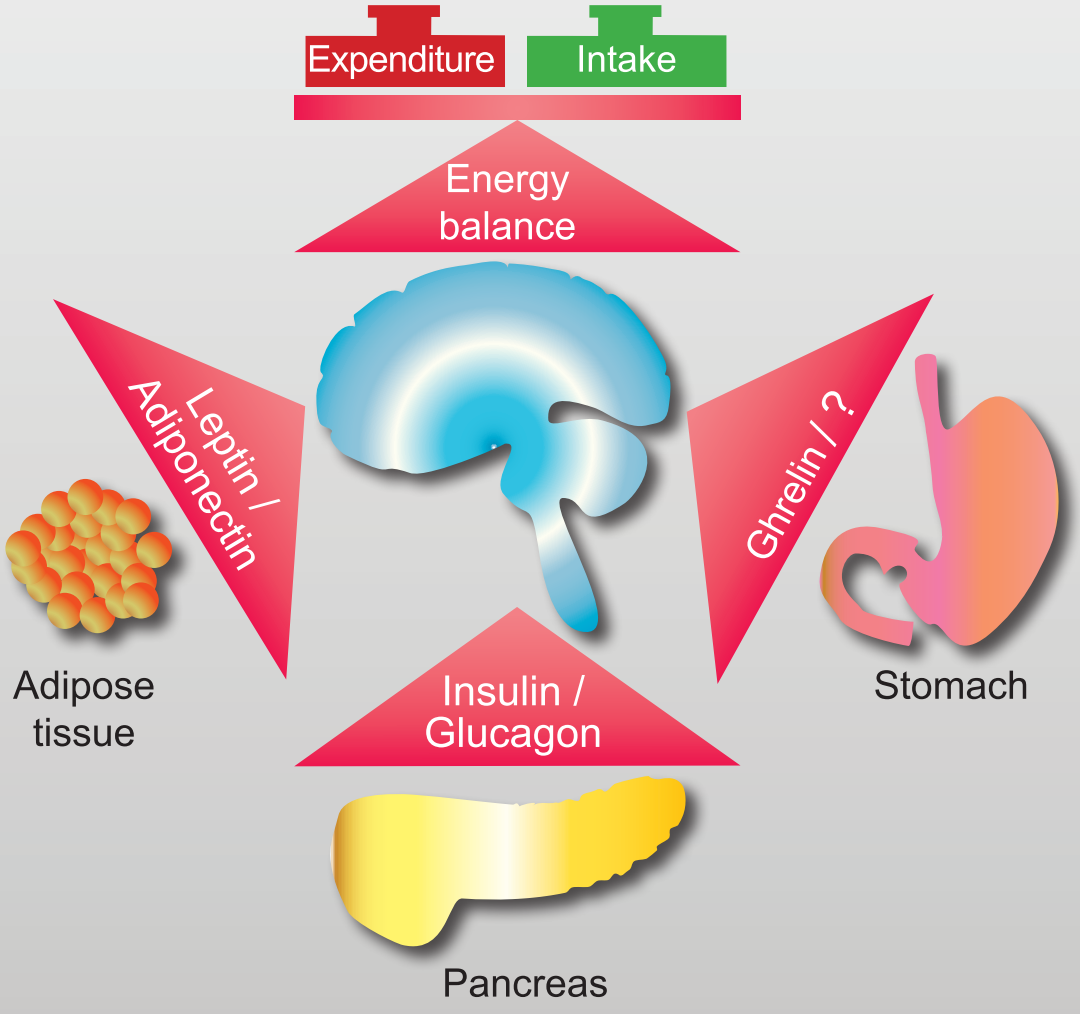

Control of energy balance.
(Taken from the paper by Ljiljana Šaranac and Zoran Gucev)

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The cover image taken from the paper from this issue by Ljiljana Šaranac, Zoran Gucev, "The Ghrelin System; beyond the Role in Energy Homeostasis".

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10. Reed ML. *Si-SiO_2 interface trap anneal kinetics*, PhD thesis. Stanford University: Stanford, 1987.

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11. Apostolides PJ, Lawton MT, David CA, Spetzler RF. Clinical images: persistent primitive trigeminal artery with and without aneurysm. Barrow Quarterly 1997; 13(4). http://www.thebarrow.org/Education_And_Resources/Barrow_Quarterly/204843
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Review Article

THE GHRELIN SYSTEM; BEYOND THE ROLE IN ENERGY HOMEOSTASIS

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Abstract. *The fascinating story of ghrelin started more than 30 years ago with the discovery of synthetic (non-natural) growth hormone (GH) releasing peptides. Scientists were searching for a novel peptide, ligand of orphan GH secretagogue receptor. The discovery of ghrelin is a typical example of reverse pharmacology. The new peptide quickly attracted a lot of attention by its pleiotropic nature, and provoked a burst of new enthusiasm among scientists and clinicians. Ghrelin is mainly produced in the stomach from a distinct group of endocrine cells located within the gastric oxyntic mucosa. It acts as hunger signal and long-term body weight regulator. But, ghrelin is much more than just a natural orexigenic factor and GH secretagogue. It exerts major peripheral endocrine and non-endocrine actions, and it has a role in learning and memory, glucose homeostasis, immunity, cardio protection, fertility and addiction. Exploring the actions of ghrelin and ghrelin agonists and receptor antagonists or reverse agonists could establish new treatment options for so far incurable diseases*.

Key words: *Ghrelin, energy homeostasis, obesity, anorexia nervosa, ghrelin application, sepsis*.

Introduction

The discovery of ghrelin allowed us to intrigue our students at the very beginning of the lecture with the fact that even stomach has endocrine properties.

The ghrelin-ghrelin receptor system is one of the most important mechanisms regulating energy balance and metabolism. The discovery of ghrelin in 1999 by Kojima group opened new era in energy homeostasis investigation and held the promise for new therapeutic options for a variety of health problems assigned as leading causes of morbidity and mortality all over the world [1,2].

Identified as a natural ligand for GHsR (Growth Hormone secretagogue Receptor), the small peptide provoked a burst of new enthusiasm among scientists and clinicians.

The Precursor of ghrelin is preproghrelin which is consisted of 117 amino acids. Ghrelin is present in two formes: acylated and non- acylated, both of them with remarkable activity. The ghrelin O-acyltransferase enzyme (GOAT enzyme) is critical in switching from one form to another. Furthermore the mystery is even more provocative by discovery of obestatin as the product of the same ghrelin gene, with the reciprocal activity [2].

Ghrelin is mainly produced in the stomach from a distinct group of endocrine cells located within the gastric oxyntic mucosa. Intestinal mucosa and pancreas are also capable of producing a certain amount of ghrelin. The expression of ghrelin within the brain, pituitary, thyroid gland, testis, kidney and placenta suggests the pleiotropic nature of ghrelin action [1–3].

Several hormones, including peptide YY, pancreatic polypeptide, oxyntomodulin, glucagon-like peptide-1 and cholecystokinin act as satiety signals. Only ghrelin, a small opioid peptide produced by the stomach, functions as putative hunger gut signal appearing to act both as meal initiator and a long-term body weight regulator [4].

Ghrelin Discovery

Kojima et al. discovered ghrelin in 1999 as 28-amino acid peptide from the rat stomach extracts. Many researches were hunting for this hormone, but discovery took a long time.

The story of ghrelin, from the initial development of an artificial GHS (growth hormone secretagogue) to the identification of the endogenous ligand, is a typical example of the general paradigm of reverse pharmacology [1]. This story also makes the investigators reevaluate the importance of purification of natural substances. By scanning genomic databases, some amino acid sequences could be identified, but natural substances sometimes escape. Ghrelin is such a case. Kojima's team had been searching for novel unknown peptides for almost 30 years. They discovered the opioid peptides (α-neoendorfin), neuromedins and the natriuretic peptide family. As Kojima said, it was very exciting to find a novel peptide and explore unknown physiological functions. From the beginning of 1998, they had been searching for the endogenous ligands

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of several orphan receptors, although none of the ligands - except for ghrelin have yet been discovered [5]. Professional luck or intuition led Kojima to switch the search from brain to stomach and to make great discovery. The new peptide was named ghrelin according "ghre", the Proto-Indo-European root of the word „grow" [5].

Secretion and Receptor Distribution

Two thirds of gastric mucosa, the epsilon cells of the gut and pancreas, pituitary, thyroid, kidney, lung, brain and even lymphocytes produce ghrelin. Secretory granules containing "X/A–like"-cells, recognized in gastric mucosa since 1960 are in fact ghrelin cells. They represent about 20% of the endocrine population in adult oxyntic glands. Ghrelin-immunoreactive cells are also found in the duodenum, jejunum, ileum and colon, gradually decreasing from duodenum to colon. Secretion of ghrelin has been also proven in rat hypothalamic and pancreatic cells [3]. The human ghrelin gene is located on chromosome 3p25-26. Mature ghrelin mRNA contains two transcripts (A and B). Translation produces ghrelin precursor consisting of ghrelin (28 amino acids) and obestatin (117 amino acids), further exposed to cleavage and acyl modification. Receptors (GHsR) 1a and 1b encoded by gene located on 3q26.31 are widespread including tumors and metastases. Effects include local and systemic responses [6]. GHsR1a is G-protein coupled receptor (GPCR) expressed in the brain as well as other areas of the body. Ghrelin receptor mRNA is prominently expressed in the arcuate (ARC) and venromedial (VMN) and in hippocampus [1]. Ghrelin activation of the ghrelin receptor, GHs-R1a, has many regulatory effects on physiology and behavior, such as enhancement of memory and learning, neuroprotection, immune function improvement, blood glucose control, potentiation of food and drug addiction, and cardiovascular and renal protection [2–8]. The existence of ghrelin and its receptor in the hippocampus, a region associated with learning and memory, suggests the role of ghrelin in memory formation [1].

Factors Influencing Ghrelin Secretion

Ghrelin secretion is increased by starvation, in anorexia nervosa, low BMI conditions, and during sleep. Leptin, GHRH, thyroid hormones, testosterone stimulate ghrelin secretion, while somatostatin, insulin, PYY and PP are inhibiting it. Ghrelin concentration is low in obese subjects, after food intake, in conditions accompanied by high glucose and lipid levels [1–5]. Stomach strech is not a stimulus for ghrelin inhibition which was proven by filling rat stomach with water after starvation. However the addition of dextrose did decrease ghrelin secretion, suggesting that hypoglycemia itself might be the stimulus for ghrelin release [6–9].

Helicobacter pylori infection of gastric mucosa could have a negative impact on ghrelin production and secretion, but opposite results are also published [10,11]. Weight gain following Helicobacter pylori eradication may be attributable to changes in plasma and gastric ghrelin; however, this hypothesis needs to be further investigated **[**12].

Ghrelin Action

Discovered as a potent GH secretagogue, ghrelin quickly demonstrated its pleiotropic nature. Opposite to leptin it stimulates food intake and rises BMI in rodents and humans (Fig 1). In fact this hormone is one of the most important factors regulating appetite and energy expenditure. Ghrelin is also known as starvation hormone; potent orexigenic signal acting via NPY/ Agouti Related Peptide (AGRP) and orexine neurons stimulation in nucleus arcuatus. Investigation of fasting and feeding on human plasma levels of ghrelin showed peak values after 12 hours of starvation, achieving sharp fall after 30 minutes of meal intake. Three hours later ghrelin is gradually reversed to the starting peak value. Furthermore ghrelin is a trigger for other neuroendocrine, metabolic and nonendocrine actions [13].

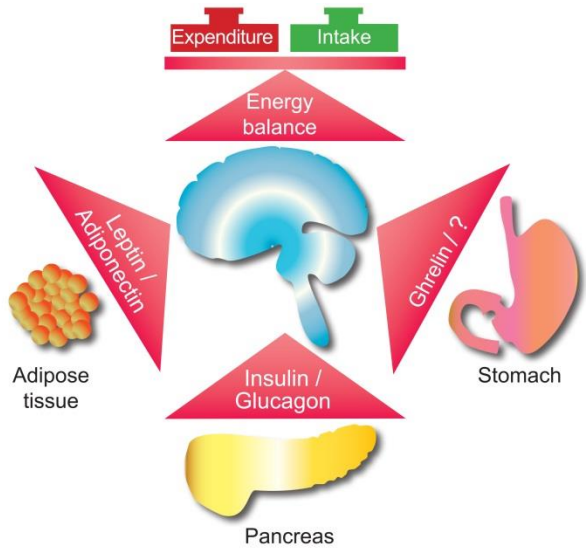

Fig. 1 Control of energy balance.

Both peripheral and central administration of ghrelin potently promotes body weight gain and adiposity through a stimulation of food intake while decreasing energy expenditure and body fat utilization [14].

Endocrine Effects

Among central effects the most prominent are appetite stimulation and stimulation of growth hormone release. Actually, ghrelin provides at the same time GH release and calories that GH needs for growth and repair. GH releasing activity results from dual action, one synergistic with GHRH and another through the inhibition of somatostatin. Ghrelin modulates lactotropic and corticotropic activity, provoking ACTH, cortisol and PRL stimulation. Additionally, it controls insulin secretion and influences thyroid function and reproduction [15,16].

Metabolic actions of GH and ghrelin are complementary. GH stimulates protein synthesis, preserves carbohydrates and stimulates energy expenditure acting by favorizing lipolysis and fatty acids oxidation in calories replete, fed state. In starvation ghrelin levels are high, acting as stimulator of energy intake and inhibitor of energy expenditure, and, at the same time, spending calories from carbohydrate intake. Additionally it stimulates glucose output by hepatocytes.

Although a potent GH releasing agent ghrelin is not necessary for longitudinal growth. Namely ghrelin knock-out mouse unexpectedly grows with normal velocity, reflecting thus the complexity of the regulation of GH system. The phenotype of ghrelin knock-out mouse includes normal size, growth rate, food intake, body composition, reproduction, and gross behavior, without any pathological changes. It could be explained by the fact that survival is more threatened by starvation than obesity [2].

However, the ghrelin-null mouse showed a significant reduction in body fat mass when the animal was fed with the high fat diet. Thus it is interesting to explore ghrelin level in idiopathic short stature [5,16,17].

Ghrelin, Endocrine Pancreas, Glucose and Lipid Metabolism

Endocrine pancreas is the main source of the ghrelin during fetal life. Acylated and non acylated forms show opposite effect on glucose metabolism. Obestatin exhibits counter effect from acylated form. Ghrelin modulates both, exocrine and endocrine pancreatic functions. Insulin level is reduced, but glucagon level remains unchanged [3, 5]. It is expressed in pancreatic cells and inhibits insulin release in mice, rats and humans. Ablation of ghrelin, GHsR or GOAT enhances insulin release. This insulin static action of ghrelin/GHsR system could optimize the amount of insulin release on systemic demand. The ghrelin blockade counteracts the obesity associated glucose intolerance in both, the life style-related and genetic obese models.

In healthy humans, total ghrelin levels are negatively associated with skeletal muscle mass , the fact that suggest a detrimental role on insulin sensitivity explored in human, animal and cellular models [15].

Effects on adipose tissue could shortly be summarized as following: adypogenic effect, lipolysis inhibition, influence on adipocyte proliferation, differentiation and apoptosis, regulating total number of adipocytes. The signalization is through MAPK and PI3K/Akt.

Ghrelin as Link between Energy Balance and Fertility

Ghrelin is more than orexigenic signal and GH secretagogue. It influences gonadal axis and the onset of puberty. It is well known that puberty onset and reproduction are metabolically gated. Conditions of starvation are associated with perturbations in the timing of puberty and subfertility. Thus ghrelin which operates as a signal of energy insufficiency and functional antagonist of leptin, may play a physiological and eventual pathophysiological role in the regulation of puberty onset and gonadal function [17]. Ghrelin receptors are identified in Leydig cells and follicular ovarian cells. It exerts inhibitory role on testosterone and LH secretion. PRL secretion is stimulated by ghrelin direct action on pituitary somatomammotroph cells. It is slight and dose-dependent PRL secretion. Ghrelin has been reported to exert an inhibitory effect on follicle-stimulating hormone, an, in particular, on luteinizing hormone, probably via an inhibitory effect exerted at the hypothalamic level on gonadotropin-releasing hormone secretion [18].

Ghrelin and Long Term Regulation of Energy Homeostasis

Ghrelin is braking boundaries between short-term and long-term regulating signals in energy balancing. Modulatory effect of both forms of ghrelin, acylated and non acylated on adipogenesis is well documented. It has been shown that both forms of ghrelin directly promote adipogenesis in rat bone marrow adipocytes [19,20). Although the orexigenic action of ghrelin itself predicts impact on weight gain, ghrelin has also been shown to be able to directly act at the level of endocrine pancreas, liver and adipose tissue, thus modulating glucose and lipid metabolism [4,5,9]. Plasma levels of ghrelin are generally negatively correlated with body weight and increase in response to fasting with a subsequent decrease upon refeeding. In humans, ghrelin concentrations progressively decrease during childhood and adolescence, as well as with advancing puberty. In adolescents, similar to adults, ghrelin concentrations are inversely related to BMI and to circulating insulin [21–24]. One notable exception is the presence of elevated ghrelin concentrations in subjects with Prader-Willi syndrome, raising the possibility that ghrelin could be part of the etiology of excess food intake in this condition [25].

Due to ghrelin's ability to promote body weight gain and adiposity via centrally mediated signaling mechanisms, modulation of the endogenous ghrelin system is considered a promising strategy to treat individuals with pathologically reduced body weight.

Ghrelin and Sepsis

Even in modern Intensive Care Units (ICU), sepsis is the leading cause of mortality. Sepsis is characterized by hemodynamic perturbation as well as excessive production of proinflammatory cytokines resulting in multiple organ dysfunction. Acute kidney injury (AKI) in septic patients increases the mortality to 50-80%. Therefore it is of survival importance to identify potential therapeutic interventions with the capability to attenuate sepsis related acute renal insufficiency. Ghrelin inhibits proinflammatory cytokine release (IL-1, IL-6 and TNF-alfa) as well as endotheline activity responsible for vascular perfusion. Ghrelin improves tissue perfusion in severe sepsis via downregulation of endothelin-1. Gram-negative bacteria endotoxin is known to cause most of hemodynamic and inflammatory responses [26–29]. In addition to any hemodynamic benefit of ghrelin in protecting against endotoxin-induced AKI, the agent's anti-inflammatory properties are no doubt critical. There are experimental data that ghrelin protects against experimental sepsis by inhibiting High-Mobility Group Box 1 (HMGB1) release and by killing bacteria. HMBG is secreted by activated monocytes and macrophages in humans and animals with sepsis and endotoxemia, acting as late proinflammatory factor. In animal models of sepsis, ghrelin improved bacterial clearance *in vivo* and showed bactericidal properties *in vitro* [28). Thus, ghrelin emerges as a natural antimicrobial and anti-inflammatory peptide, widely distributed in all body tissues and especially abundant in the proximity to physical barriers such as stomach, gut, and skin suggesting that one of its primary functions could be related to the control of innate immunity and response against infections [29–31].

Ghrelin, an Addiction Hormone

The essential goal of feeding is to provide calories and a diversity of nutrients. However, we do not eat only for energy, we also eat for pleasure. Almost all circulating gut peptides contribute to the control of food intake by signaling satiety. One important exception is ghrelin, the only orexigenic peptide hormone so far described. Ghrelin interacts with the brain reward pathways to increase food intake, alter food preference and enhance food reward, thus influencing hedonic feeding control [32,33].

Evidence is accumulating linking obesity as an environmental risk factor to psychiatric disorders such as stress, anxiety and depression. Plasma ghrelin levels are enhanced under conditions of physiological stress and ghrelin has recently been suggested to play an important role in stress-induced food reward behavior. In addition, chronic stress or atypical depression has often demonstrated to correlate with an increase in ingestion of caloric dense foods [34,35]. Recent evidence suggests ghrelin as a critical factor at the interface of homeostatic control of appetite and reward circuitries, modulating the hedonic aspects of food intake. Therefore, the reward-related feeding of ghrelin may reveal itself as an important factor in the development of addiction to certain foods, similar to its involvement in the dependence to drugs of abuse, including alcohol. Thus the ghrelinergic system could be an effective target for the development of successful anti-obesity pharmacotherapies, which not only affects appetite but also selectively modulates the rewarding properties of food and impact on psychological well-being in conditions of stress, anxiety and depression [33–37].

Ghrelin and the Cardiovascular System

The cardiovascular system has also been recognized as important target for ghrelin effects. A single injection of ghrelin provokes a significant decrease in blood pressure, without changing the heart rate, acting as antagonist of endothelin-1 [2,38]. In experimental models ghrelin showed cardioprotective effect against ischemia. Apart from vasodilatory effects, ghrelin may also have other vasoactive and anti-inflammatory properties and could be considered as therapeutic agent in conditions with atherosclerosis [39]. In hypophysectomized rats pretreated with GH secretagogues heart damage was prevented. Possible anti-apoptotic effects have also been studied on cardiomyocytes and endothelial cells in vitro. In rats with experimental myocardial infarction, ghrelin treatment improved cardiac output [40–42]. Chronic heart failure (CHF) remains one of the most challenging therapeutic problems, regarding frequency in ICU and high mortality rate, particularly so, if the CHF patient develops cardiac cachexia. Ghrelin and its analogs have been suggested to improve body weight and cardiac function in heart failure models [43].

In whom to Determine Ghrelin?

Taking into account all above it is of practical importance to suggest in whom to investigate circulating ghrelin levels. In our opinion it is crucial to determine ghrelin or ghrelin/leptin ratio in lean subjects with abundant intake of energy dense food, searching for clue in achieving healthy balance in orexitropic signaling [44]. Obese and cachectic subjects are also candidates in order to rule out possible resistance syndromes as well as in addiction syndromes.

With regard to children, SGA, IUGR neonates, septic children, patients with idiopathic short stature are certainly good candidates for ghrelin determination, being the population that deserves better understanding of their pathological conditions and opening new therapeutic approaches.

Recently, we have found an important difference in appetite targeting hormones, ghrelin and leptin, and their profiles in a study group that included 88 children and adolescents aged 6 to17.67 years, classified as obese (30 subjects), undernourished (25 subjects) and lean (33 subjects). Two orexitropic hormones showed different profiles in 3 different nutritional conditions; an inverse relationship was discovered between study groups in ghrelin/leptin and leptin/ghrelin ratios (unpublished data).

Clinical Application of Ghrelin

Due to its adipogenic, orexigenic and diabetogenic activities, ghrelin has emerged as an attractive target for the treatment of different pathological conditions (Tab. 1). Unacylated ghrelin agonists in animal and human studies exerted improvement in post-prandial glucose and insulin sensitivity, as well in prevention of fat accumulation. GOAT inhibitors have potential to decrease plasma acylated ghrelin, consequently resulting in reduction of blood glucose and weight gain. AG antagonists and blockers in animal and human models decrease AG central activity assessed by inhibition of GH secretion [45].

Table 1 Clinical application of ghrelin

UG	control of PPG	(45)
GOAT inhibitors	BM and BG reduction	(45)
Ghrelin agonists	GHD diagnosis	(2,5)
Ghrelin agonists	AN treatment	(47,48,50)
	AIDS, Cardiac cachexia	(1,43,46)
	Cancer cachexia	(43,45,46)
Ghrelin antagonists	Obesity	(45,46,49)
	Prader –Willi Sy	(25)
Ghrelin analogs	Gastroparesis	(51)
	Postoperative ileus	(51)
Ghrelin	Sepsis (animals)	(29,30,31)

UG – Unacylated ghrelin, GOAT – ghrelin O – acyltransferase enzyme, PPG – postprandial glucose, AN – Anorexia nervosa

Ghrelin offers great potential for clinical application in diagnosis of pituitary function in GH deficiency, in eating disorders like anorexia and bulimia nervosa as well as in Prader-Willi syndrome, gastrointestinal diseases, cardiovascular disease (heart failure and dilated cardiomyopathy), osteoporosis, in aging, in catabolic state or chronic wasting syndrome, in cachexia (cancer and cardiac cachexia), AIDS, postoperative patients [1,43,46]. Anamorelin, an oral ghrelin receptor agonist with appetite enhancing and anabolic activity is recently successfully applied in anorexia-cachexia syndrome [47,48].

At present, ghrelin is a unique peripheral orexigenic signal that is effective upon its intravenous injection. Thus blocking or neutralizing ghrelin's action may be a reasonable approach to reversing a chronic obesity [46,49].

In Japan, ghrelin receptor agonist is clinically used as diagnostic agent for growth hormone secretion deficiency. Recently, it has also been used as therapeutic agent in patients with severe form of anorexia nervosa. One-year intra-nasal application of such agonist in a severely emaciated AN patient improved hypoglycemia and body weight [50].

Repeated administration of ghrelin can stimulate appetite in patients with functional dyspepsia. Ghrelin also accelerates gastric emptying in diabetic gastroparesis and postoperative ileus after partial colectomy. Therefore, ghrelin analogs could represent a new class of prokinetic agents in the future [51]. However, most ghrelin compounds are peptides and need to be injected several times per day, which affects the quality of life of patients in human clinical studies.

Ghrelin, ghrelin agonists, receptor antagonists and reverse agonists are certainly potential diagnostic and therapeutic options for the future.

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Review Article

BLASTOCYSTIS HOMINIS: A MYSTERIOUS AND COMMONLY DISREGARDED PARASITE

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Abstract. *Blastocystis hominis (B. hominis) is an anaerobic, single-cell protozoan, commonly present in human and animal stool samples. It can be found in healthy people as well and it still has not been elucidated whether it is a commensal organism or a pathogen. Blastocystosis is a disease caused by the protozoan in humans. The prevalence of the parasitosis varies both between the countries, and between certain population groups within individual countries. Due to poor hygienic conditions, common exposure to animals and intake of contaminated water and food, people in the developing countries have got a higher prevalence of blastocystosis, but economically developed countries have not been spared either. The taxonomy of B. hominis is still a matter of debates. For the reasons of genetic diversity, it has been suggested that the name B. hominis should be replaced with „Blastocystis species". Seventeen subtypes of the species have been so far identified, and a definitive characterization of Blastocystis spp. is possible at the molecular level only. The parasite is transferred by the fecal-oral route. A variety of hosts have been identified, and animal-to-human and vice versa transfers have been documented. The most common manifestations of the infection with the organism are diarrhea, abdominal pain, nausea, and bloating. This infection has also been associated with the irritable bowel syndrome (IBS), non-specific colitis, chronic inflammatory bowel disease (CIBD), and urticaria. The diagnosis can be made using the methods of conventional microscopy (CVM), phase-contrast and electron microscopy, cultivation, serodiagnosis, and by using molecular methods. The infection caused by the parasite does not always require treatment. In symptomatic patients, the first line medical treatment is metronidazole. Further studies are required to resolve all dilemmas regarding the parasite.*

Key words: *Blastocystis hominis, diarrheal syndrome, diagnosis, treatment.*

Introduction

Blastocystis hominis (*B. hominis*) is an ubiquitous parasite spread widely in the tropical climate areas. It is commonly present in human and animal stool specimens (in birds, rodents, reptiles, amphibians, fish, cockroaches). Its role as the cause of an infection has not been fully elucidated. Throughout the literature, the organism has been reported as a commensal organism, but also as a pathogen. In recent years, numerous studies have been published reporting that the infection with *B. hominis* is common in immunocompromised individuals [1,2].

The organism belongs to single cell, anaerobic eukaryotes (protists). Brittain and Swayne, independently of each other, detected the microorganisms studying a cholera epidemic in London in 1849, wrongly identifying them as the cause of cholera [3]. *Blastocystis*, the name of the genus, was given by Alexeieff in 1911, and the following year the name of the species was suggested by Emile Brumpt [4]. Zierdt et al. performed a reclassification and classified the organism among protists, based on its morphological and phenotypic characteristics (one or several nuclei, cellular organelles similar to mitochondria, endoplasmatic reticulum and Golgi apparatus, inability to grow on fungal culture media, resistance to antifungal agents, and sensitivity to antiprotozoal drugs) [5].

Classification

At the end of the XX century, Silberman et al. classified the organism among eukaryotes, of the Heterokontophyta type, based on the molecular analyses of small subunit (SSU) rRNA (SSU-rRNA) and elongation factor 1 (EF-1α) [6]. Although the taxonomy such as this was controversial when related to other studies demonstrating a similarity of *Blastocystis spp*. parasite with protists, the subsequent studies confirmed the assertion presented above [7, 8]. There are over 100.000 members of the Heterokontophyta order, commonly termed heterokonts or stramenopiles, and *Blastocystis spp*. becomes a new member of the complex group of the so-called „botanical protists“ [9,10]. By way of phylogenetic analysis of

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SSUrRNA and HSP70c, a close connection between *Blastocystis spp*. parasite and stramenopiles has been confirmed, in spite of its absence of flagella and tubular elongations [9]. Despite the classification at a molecular level, there is a difference in morphology between blystocystis and other stramenopiles (flagella surrounded by lateral hairlike mastigonemes) [11,12].

The origin of the organism at the species level has not yet been resolved. There are several host-specific species: *B. hominis* in people, and *B. ratti* in rats [13]. Host diversity is well known, and human-to-animal and vice versa transfer is well documented [14]. In humans, any of the isolated species is termed *B. hominis* [4], although, due to its genetic diversity, the suggestions have been put forward that the term *B. hominis* should be replaced with *Blastocystis species* [13].

Because of all these facts, a step towards classification of different species has been made based on their ultrastructural morphological characteristics visualized by way of electron microscopy [15]. Host specificity and pathogenic potential of different isolates correlate with sequence variations in SSU-rRNA [14]. Conserved and variable regions within 18 SSU-rDNAs constitute the basis for identification of phylogenetic relations between the species [13]. Moreover, rRNAs are made use of in diagnostic PCR analyses with high sensitivity [16]. A recent genetic classification of *B. hominis* into subtypes (STs) is the equivalent to earlier species classification [13]. SSU-rDNAs correlate with subtypes and 17 have been reported so far, so that definitive characterization of species is possible at a molecular level only [13].

Host-specificity is determined by STs, where ST1 and ST8 colonize/infect humans (not only them, however, but other hosts as well) [13]. ST9 has been found only in humans, while ST10-17 are present in other hosts as well [14,15].

Biology and Morphology

B. hominis is a strict anaerob with observed intacellular structures similar to mitochondria, but lacking cytochrome enzymes. Intracellular organelles are involved in different metabolic pathways (metabolism of amino acids, biogenesis of iron and sulphur, and tricarboxylic acid cycle). The organism is able to synthesize certain essential cellular phospholipids and to accumulate them most commonly within the storage vacuoles [3]. The average generation time of *Blastocystis* species is 17–22 h, although it depends on the medium used for cultivation. Generation time differs among different STs [17]. The microorganism enters apoptosis after exposure to harsh conditions (exposure to room temperature, air, or antiparasitic agents such as metronidazole). The phenomenon serves as a mechanism aimed to increase the number of viable cells in such conditions [18].

B. hominis has got several different morphological forms. Vacuolar, granular, ameboid, and cystic forms are the ones best described so far. Other morphological forms have also been found on electron microscopy (avacuolar and multivacuolar, of small dimensions and rarely present). In fresh stool samples and culture samples, vacuolar and granular forms are the ones most commonly encountered; they can be visualized using phase-contrast microscopy, with light microscopy of native and stained sample preparations and with electron microscopy [4].

Vacuolar form. This form of *B. hominis* is spherical, containing a central body representing a large vacuole, occupying approximately 90% of the cell, and a thin layer of peripheral cytoplasm situated immediately beneath the cell membrane. The nuclei can be distributed peripherally throughout the cytoplasm. There can be seven nuclei at the most, but there are two nuclei on the average, situated at the opposite ends of the cell [19]. Mitochondrion-related organelles and Golgi apparatus are located peripherally in the cytoplasm. Mitochondria look like roses placed around the nucleus. These structures may protrude within the central body and can have a fiberlike appearance [13]. It has been discovered that the central body is a membrane-enclosed vacuole, containing carbohydrates, fats, and basic proteins. These substances are accumulated within the vacuole by way of the action of the Golgi apparatus and via clathrin-mediated endocytosis [20]. The body most probably has storage and apoptosis-related roles [3,9]. Vacuolar forms can be of different sizes (ranging from 3 μm to 120 μm), but measuring 5 μm to 15 μm on the average [13]. It is generally accepted that this form is most commonly seen in asymptomatic carriers of *B. hominis* [1].

Granular form. This form is very similar to vacuolar forms of *B. hominis*, but contains the granules within the cytoplasm which are often centrally situated. In 1989, Dunn et al. proposed that these structures were similar to myelin-like inclusions, small vesicles, crystal granules, and drops of fat. The granules can be metabolic, reproductive, and lipid ones [13]. It is possible that reproductive granules have a role in schizogony. On the average, there are two nuclei in the cytoplasm (four at the most). They have a slightly smaller diameter compared to vacuolar forms, and measure 9.0 μm to 28.3 μm [19]. They are more frequent in older cultures and the cultures treated with antibiotics, and there has also been the hypothesis that their existence is an indicant of cell death [1].

Ameboid form. This form of *B. hominis* is most rarely encountered. It is irregular in shape, with 1–2 pseudopodia (being stationary nevertheless), with considerable adhesion abilities, enabling its attachment to the bowel mucosa [1]. There is a large vacuole in its cytoplasm, and this form is in fact transformed into cystic form. Since they resemble neutrophils and macrophages, they can easily escape recognition on routine stool sample examinations. Zierdt has suggested Gram staining of unfixed smears to be undertaken for their identification, since these forms undergo lysis when exposed to air, while leukocytes remain intact [4,9]. It is more commonly present in individuals with symptoms of digestive tract infections and in cultures, indicating the pathogenic potential of this form of *B. hominis* [1,21].

Cystic form. These are round or oval, and with smaller dimensions (3–6 μm) [13]. These forms found in certain animals are larger [15]. Cystic forms have a thin, multilayered wall with/without a surface envelope [19]. Their condensed cytoplasm has got several mitochondria and storage vacuoles. The number of nuclei within the cysts varies from 1 to 4. A cyst may survive about a month exposed to air and the temperature of 25°C and enables further spread of the infection – it is a form infectious for humans [1,9].

Vegetative forms are transformed into other vegetative forms with different morphology and can thus escape identification in stool samples [3]. Avacuolar and multivacuolar forms are the most dominant forms *in vivo*, and these also most commonly remain unrecognized on microscopy [22].

Life cycle

Infectious, cystic forms of *B. hominis* are transmitted by the fecal-oral route [23]. The infection may occur after an intake of untreated water or uncooked water plants contaminated with cysts, and also via dirty hands [24, 25]. In an adequate host, the cyst develops via the process of excitation into vegetative forms within the large bowel [9].

Further continuation of the life cycle depends on the subtype compatibility with the host [13]. Other forms can also develop from vacuolar ones. After a period of time after the infection, vacuolar forms form the cysts in the bowel lumen [22]. The encystation occurs during the passage through the large bowel, and the cysts are then excreted via feces. Fecal cysts can be covered with a fiber-like layer which gradually disappears during the cyst development. A thin fibrillar surface layer detected in stool samples plays a significant role in the survival of this parasite *in vivo* [22].

It is thought that different modes of reproduction exist when this organism is concerned (binary fission, budding, plasmotomy, multiple fission, endodyogeny, schizogony). Binary fission is nevertheless the most common mode [26].

Virulence

The studies conducted to establish the pathogenicity of *B. hominis* parasite have been so far unconvincing and disputable. There are some acceptable explanations of pathogenicity related to the species STs and virulence (27, 28). Symptomatic patients are usually infected with ST1 - 4, and 6 (with ST3 being the most common, followed by ST1 and ST2). Subtype-related variations in pathogenicity have been observed as well, which probably can explain the differences between the patogenic and non-pathogenic potentials of the species.

Ameboid *B. hominis* form, which excretes proteases, is the most virulent one. It is predominant in symptomatic patients and these forms should be sought in stool specimens in patient screenings [22,28–30]. In addition to proteases, other hydrolytic enzymes have been identified as well by way of electrophoresis. Lysates lead to cytoskeletal changes and induce apoptosis in epithelial cells, which results in increased bowel permeability. Cystein proteases stimulate mucosal cells to produce interleukin-8. This mechanism is responsible for the loss of fluids and bowel inflammation in the affected. Proteases cleave secretory IgA and help in immune evasion and survival of the parasite [3,31].

Whole-genome sequencing has been done for ST7. The genes have been identified which code the proteins that alter bowel homeostasis. The genes responsible for the production of nonribosomal peptides and polyketides (antibacterial and inducing bowel dysbiosis) have also been identified. The target genes coding for hydrolases have been also described (capable of altering the bowel mucous layer and exposing the epithelium for parasite adhesion). Expression of serine proteases and glycosyltransferases disturbs the firm bonds in the bowel mucose epithelium, leading to increased bowel permeability [3,32].

The molecules responsible for extraintestinal manifestations of the infection are relatively unknown. *B. hominis* antigens stimulate T-helper II cells, leading to an IgE-mediated allergic reaction. The organism probably activates the complement cascade, which leads to the release of anaphylatoxin and mast cell activation. Iron deficiency anemia associated with *B. hominis* infection is still awaiting explanation [33,34]. In general, virulent strains are larger, with an uneven, rough surface, they grow slowly and demonstrate an increased affinity to bind to lectins.

The relationship between the severity of infection and clinical manifestations is still unclear [35–38]. One study has proposed that 32kDa proteases of ST3 could be the virulent factors responsible for protein degradation, while another study has found that a *B. hominis* 29 kDa antigen could be used as a pathogenicity marker, enabling differentiation of symptomatic from asymptomatic *B. hominis* infections [39,40]. Increased IgA levels have been described in symptomatic individuals with *B. hominis* infection, compared to healthy asymptomatic carriers of *B. hominis* [41].

A recent study about the impact of *B. hominis* parasite on the expression of gamma interferon and proinflammatory cytokines of the cecal mucosa in rats has shown a significant upregulated transcription of type 1 gene and proinflammatory cytokines IFN-gamma, IL-12, and TNF-α. This suggests that *B. hominis* infection in rats stimulates specific local host responses, involving T cells, monocytes, macrophages, or natural killer cells [42]. Studies on mice inoculated with high doses of *Blastocystis spp*. have shown a loss of weight in mice and onset of diarrhea [43–45]. Studies have also demonstrated that *Blastocystis spp*. can attack the lamina propria, submucose, and muscle layers, and to invade the epithelium of the rat colon in view of the increased levels of hyaluronidase in the urine of rats infected with *Blastocystis spp*., which stil is not a sufficient proof of similar events in people [46,47]. Laboratory rats constitute a good model of the pathogenesis of *Blastocystis spp.* infection, in con-

trast to mice which have not been naturally infected with *Blastocystis spp.* [48].

There have been several reports suggesting that *Blastocystis spp.* could be associated with urticarias in humans [9]. Ameboid forms of ST3 *Blastocystis spp.* have been identified in the cases of acute urticaria, and authors believe that cutaneous symptoms can be caused by a disruption in the immune homeostasis [49]. In another study, *Blastocystis spp.* ST2 has been demonstrated in a patient with severe gastrointestinal complaints and chronic urticaria, in absence of any other infectious agent. The complaints persisted after the initial antibiotic therapy, but were eliminated after combined metronidazole and paromomycin therapy [50].

Clinical Significance and Treatment

In view of the controversies related to the pathogenicity of *B. hominis* in humans, the results of numerous studies confirmed/excluded *B. hominis* as a disease cause. In certain studies, the individuals susceptible to an infection with the parasite have been mentioned: HIV infected individuals, patients with cancer or with other immune difficiency conditions, children from the developing countries, frequent travellers [13].

It seems that clinical manifestations of blastocystosis depend on the subtype of the parasite. ST1 subtype has been found in those with symptoms of the infection [51], while in Columbia it has been documented in asymptomatic examinees [52]. *B. hominis* of ST2 has got a controversial pathogenicity, positively demonstrated in some studies [52–54], and disputed in the others [55,56]. Regarding the most common *B. hominis* subtype ST3 isolated in humans, there have been little evidence that could indicate its pathogenicity. Around 40% of those infected with ST4 of *B. hominis* have got gastrointestinal symptoms, found as well in those with the clinical picture of severe diarrhea [57,58]; ST5 subtype has been found in those with symptoms of the infection [59], in those coming into contact with animals and in animals themselves [60,61]. *B. hominis* subtype ST6 causes diarrhea in a third of the infected, and *B. hominis* subtype ST7 is also associated with the onset of diarrhea [57].

Clinical characteristics of the disease are non-specific and consist of abdominal pain, acute/chronic diarrhea, nausea, anorexia, bloating, perianal itching (abdominal pain and diarrhea being the most common complaints). The symptoms range from mild and moderate, to severe acute and chronic events. The number of parasites found in stool specimens determines the severity of symptoms and signs of the infection [1,9,22,37,38].

B. hominis is associated with the irritable bowel syndrome (IBS), since the changes in the intestine occur (caused by this disorder) that favor the development of the parasite [13]. It is believed that low-intensity inflammation occurs as the consequence of constant immune activation caused by the parasite and persistent antigen exposure of the host [62]. Moreover, increased levels of IgG2 immunoglobuline against *B. hominis* have been found in the examinees with IBS [63]. Blastocystosis has been associated with non-specific colitis too, as well as with chronic inflammatory bowel disease (HIBD) (including Crohn's disease and ulcerous colitis) [14,64,65].

Eosinophilia and skin changes (primarily urticaria) are rarely encountered in patients [9,13]. There have been several individual cases of *B. hominis* infection in patients with chronic kidney disease [66,67] and arthritis [66, 68-71). A high prevalence of the infection (95.8%) has been described in immunocompromised patients (HIV positive individuals and those with AIDS) [72].

However, not all of the infected develop symptoms and signs of the disease. These are asymptomatic individuals – there are many more asymptomatic cases than those with symptomatic *B. hominis* infection [27,30,41,56,64].

There is also the question whether the infection with this parasite requires treatment. In symptomatic patients with confirmed infection (the finding of *B. hominis* in the stool specimen), it is necessary to examine the presence of other infective agents in the gastrointestinal tract, since there is a real possibility of coinfection with other pathogens as well [13].

Metronidazole is a first choice drug in cases of proven infection. It's effectiveness, however, has been known to vary. It is effective in some patients, but it cannot produce complete eradication of the infection (especially a severe one). There is a possibility that non-responders have been infected with resistant *B. hominis* subtypes. The studies dealing with metronidazole efficacy have not elucidated the association between *B. hominis* subtypes and treatment failure [13].

Trimethoprim – sulfamethoxazole is the second choice drug for those who failed to respond to metronidazole treatment. It has been demonstrated that paromomycin, a wide spectrum antibiotic indicated in acute and chronic intestinal amebiasis, is successful as the treatment of *Blastocystis* infections associated with skin lesions (predominantly urticaria) [73–76].

Yakoob et al. have studied the efficacy of garlic and other dietary herbs *in vitro* in comparison with metronidazole in individuals infected with *B. hominis*. The authors assessed the efficacy of garlic and metronidazole in concentrations of 0.01 and 0.1 mg/ml. They found that garlic and metronidazole were equally effective in both concentrations. *Blastocystis* isolates were not sensitive to other tested herbs such as ginger, black pepper, and white cumin [77]. It has been established that probiotics such as *Saccharomices boulardii* are equally effective as a symptomatic treatment as metronidazole [13,78].

Diagnosis

Conventional microscopy of (CVM) (with/without concentration method):

- **native preparations** (unstained/Lugol stained). If Lugol's solution is added, parasites are stained golden yellow. However, due to *Blastocystis* polymorphic structure, a wrong identification can occur and their misinterpretation as fungi, *Cyclospora spp*., and drops of fat [13]. Classical vacuolar forms do not have to be predominant in fresh stool sample, while smaller forms can be hard to identify [60]. **For the diagnosis of *Blastocystis* infection to be made, several stool samples (at least 3) are required, more than 5 cysts in the visual field without other parasites** [1].
- **stained preparations** (by Giemsa, Gram, Wright, iron hematoxylin) [80]. A common staining in the diagnosis of *Blastocystis* is **trichrome staining**. With this method, the large central body is usually stained green to gray. Inclusion bodies in the cytoplasm stain light to dark red.

Phase-contrast microscopy

Phase-contrast microscopy is more convenient than light field microscopy when greater magnifications are required and when samples are colorless or their details are so minute that color cannot be discerned well [80]. Phase-contrast microscopy enables the study of morphological features of the cells and of their reproduction via cell division.

The fundamental principle of phase change visualization in phase-contrast microscopy is the separation of background light from the specimen-scattered light, which enables better visualization (highlighting) of the required image details; the phenomenon is based on the property of cells to have a refractive index different than that of the surrounding medium [9].

Transmission electron microscopy (TEM)

In the routine diagnosis of *Blastocystis* TEM is not used; it is used however in the demonstration of atypical forms of the parasite [9].

Cultivation

When CVM specimens are positive to the vacuolar forms of *B. hominis*, cultivation on the Löwenstein-Jensen (LJ) medium is performed: a 48-hour incubation in anaerobic conditions, when white, very bright and mucous colonies grow [81]. A native preparation with physiological solution is made of the suspect colonies and is inspected microscopically, when vacuolar forms of *B. hominis* are seen [82].

Xenic and monoxenic laboratory cultures of *B. hominis* isolates, growing together with non-standardized or individual known types of microorganisms, can be kept alive in the Jones' or Boeck-Drbohlav condensed medium [83,84]. The Jones' medium is the medium of choice for the studies involving cultures for parasite identification from patient specimens [81,85–88].

Axenic cultures, i.e. the cultures without any other living organism(s), demonstrate a rich growth in different media [89,90]. *B. hominis* cells can grow on a solid medium as well, and its colonies macroscopically appear similar to bacterial colonies [91]. The colonies may survive up to 2 weeks and can be preseeded in a liquid or solid medium [92]. It is interesting that the same isolates cultured in a liquid medium reach their maximum cell density around day 4 after inoculation, and enter the dying phase on day 5, so that the growth of their subcultures is made difficult [9]. This indicates that growth characteristics of the same isolates in a solid medium are essentially different from the characteristics of *Blastocystis* isolates grown in a liquid medium.

Axenic cultures of *Blastocystis* isolates are very important for molecular and biochemical research. Axenization can be accomplished by the addition of antibiotic cocktails in order to eliminate bacteria and fungi. Several combinations of antibiotics have been so far described, used with variable success. The procedure is generally a demanding one, lasting for weeks or months, without any guarantee that the bacterial contaminants would be eliminated in the end. It has been supposed that some of the isolates require the presence of bacteria for their survival, so that the removal of all bacteria can result in the death of the parasite [9].

Lanuza et al. have improved the method for *Blastocystis spp*. axenization and succeeded to axenize 25 out of 81 isolates. The time required for axenization was about 3 weeks [93]. In addition to an antibiotic treatment, some authors have pointed out that physical methods can contribute to the success of axenization – separation of parasites from the mass of sprouting bacteria [93–96].

Serodiagnosis

The infections caused by *B. hominis* lead to IgA and IgG immune response, and antibodies can be demonstrated using indirect immunofluorescent (IFA) and enzyme-linked immunosorbent assay (ELISA) tests [97].

An IFA assay involves a highly specific and sensitive method for the confirmation of *B. hominis* parasite. Commercially available immunofluorescent antibodies are used, specific for *B. hominis*. Based on polyclonal antibodies, the subtypes of *B. hominis* ST1-ST3 and ST5 can be determined using the ELISA test [97].

Symptomatic infections are associated with elevated IgG antibody titer. In asymptomatic infections, IgA response is weak or absent. The strongest immune response is reported for chronic infections. Since the knowledge concerning host immune response to *B. hominis* infection is still limited, and since antigen diversity of the parasite is obvious, serologic tests are not routinely used, but their use is of key importance in epidemiological and other scientific research. These methods are far more sensitive and specific

than CM and the tests are nowadays commercially available [9,97].

Molecular methods

Amplification of *B. hominis* DNA obtained from fresh stool samples or from culture is convenient for the purpose of epidemiological and screening studies, and genotyping should be included in the analysis as well [9]. The development of a real time polymerase chain reaction (PCR) method for sufficiently sensitive and rapid detection of *Blastocistis spp*. and the ability to differentiate between the genotypes present in the specimen would be equally useful in screening and in epidemiological studies [9].

Epidemiology

B. hominis is a ubiquitous parasite. Its prevalence varies between the countries and from one population group to another. People in the developing countries have got a higher prevalence of blastocystosis due to poor hygienic conditions and intake of contaminated water and food [51].

However, *B. hominis* has got a wide geographical distribution and can be found in economically developed countries as well [97,98]. A study involving the whole territory of the USA, conducted by the Center for Disease Control and Prevention and using the 1987 data, reports the prevalence of *B. hominis* infection of 2.6% in the general population [99], while a study published in 2000, performed in private laboratories in 48 states of the USA, shows the prevalence of blastocystosis of 23% [100]. In Canada, *B. hominis* was the most common cause of protozoal infections in 2005 (101).

B. hominis was the parasite most commonly isolated in Indonesia, in the HIV infected and those with AIDS tested before the administration of antiretroviral therapy [102] and in Turkey in cancer patients [103]. An increased prevalence of the parasite in individuals in contact with animals suggests the possibility of a zoonosis [27].

The distribution of genotypes established using the PCR method has been reported in several countries: Bangladesh, Germany, Pakistan, Japan, Singapore, Greece, Turkey, China. The ST3 subtype established in China is the most predominant one, while ST1 has been found in a lesser degree in Singapore, Greece, and Germany [13]. There are also mixed type infections with different subtypes, most commonly with ST1 and ST3 [51,55,104,105].

A recent study in our country has shown that children with blastocystosis have colitis as the most common large bowel pathology, without any significant difference between non-specific colitis and HIBD. The infection is most commonly found in children aged 2 to 3 years, followed by those 16 to 18 years of age. Significantly higher number of the infected live in houses compared to flats, and possess domestic animals and/or pets. A positive fecal occult blood test, iron-deficiency anemia, elevated erythrocyte sedimentation and CRP are characteristic of those with *B. hominis* infection and HIBD who have a larger number of parasites in their stool samples. Mesenteric lymphadenitis and splenomegaly are the most commonly described pathologic changes on the abdominal ultrasound of children infected with *B. hominis* hospitalized for the complaints of abdominal pain and/or diarrhea. The author concludes that the establishment of a pathogenic significance of *B. hominis* contributes to the recognition of this protozoan as a pathogen and stresses the necessity of a treatment for the condition [106].

The first studies in the region of Niš concerning the prevalence of blastocystosis in patients with/without infection symptoms have been published about a decade ago; the prevalence of 4.05% was then established among the healthy, and 0.36% among those with symptoms of the infection [107]. Based on still unpublished data of the Parasitology Laboratory, Public Health Institute, the total prevalence of *B. hominis* has been reduced in the last decade (2.7%) in asymptomatic individuals.

Conclusion

Blastocystis hominis is still a mysterious and perhaps scientifically disregarded parasite in human pathology. Differentiation among *Blastocystis* species is not possible using the routine methods. The use of DNA methods enables detection of genetic variations in these parasites with still uncertain taxonomy. Epidemiological molecular studies are especially useful in the establishment of transmission patterns, host specificity, and in the surveillance of chemotherapeutic resistance.

Distribution and genotypic diversity of *Blastocistis spp*. have not been studied so far in Serbia, which necessitates a systematic research of the parasite and filling in of the epidemiological map, as well as the establishment of significance of the organism in human pathology.

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Review Article

ANTIOXIDANTS AND THEIR IMPORTANCE DURING MUSCULAR EXERCISE: A REVIEW

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Abstract. *Physiological levels of reactive oxygen species, as an essential part of the homeostatic milieu, are required for normal functioning of skeletal muscle. High levels of reactive oxygen species promote contractile dysfunction resulting in muscle weakness and fatigue, oxidative stress, apoptosis and necrosis of muscle cells. It is known that both resting and contracting skeletal muscles produce reactive oxygen species and reactive nitrogen species. The first suggestion that physical exercise results in free radical-mediated damage to tissues appeared in 1978. The newest researches investigate the mechanisms by which oxidants influence skeletal muscle contractile properties and explore how to protect muscle from oxidant-mediated dysfunction. Principal antioxidant enzymes include superoxide dismutase, glutathione peroxidase, and catalase. Numerous non-enzymatic antioxidants exist in cells within skeletal muscle fibers, the most abundant of which include glutathione, bilirubin, α-Lipoic acid , uric acid, and ubiquinones, or coenzyme Q (CoQ) andflavonoids. Dietary antioxidants are vitamins C- L ascorbic acid , vitamin A, retinol and their provitamins, carotenoids (especially β-carotene), vitamins E, tocopherol (especially α-tocoferol), folic acid or folates. The usage of endogenous enzymatic and non-enzymatic antioxidants protects muscle from strong damaging effects caused by free radicals during acute exercise or longer term physical exercise. Scientific researches now confirm that the long-term use of antioxidants is safe and effective. The actual recommendation for physically active individuals is to ingest a diet rich in antioxidants.*

Key words: *physical activity, human body, antioxidants, diet.*

Introduction

Free radicals production is a normal process in the life of aerobic organisms. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are continuously produced during normal skeletal muscle metabolism [1,2]. Some of them have beneficial effects, notably as a part of the body's natural immune system [3].

Low and physiological levels of reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, the hydroxyl radical and nitric oxide (NO) increase blood flow to skeletal muscle during physical exercise [4,5].

Exercise was associated with an increased formation of free radicals, reactive oxygen species (ROS) and reactive nitrogen species (RNS) [6,7]. In young individuals, ROS are required for normal force production in skeletal muscle, for the development of training-induced adaptations in endurance performance, as well as for the induction of the endogenous defense systems [8]. A greatly increased rate of free radical production, ROS and RNS, caused by exhaustive exercise, may exceed the capacity of the cellular defense system causing attack of free radicals on the cell membranes initiating the skeletal muscle damage and cause the oxidative stress, apoptosis and necrosis of the muscle cells [9–12].

The first suggestion that physical exercise results in free radical-mediated damage to tissues appeared in 1978 and after that there have appeared numerous data about exercise and oxidative stress [13]. Under physiological conditions, these deleterious species are mostly removed by the cellular antioxidant systems which include endogenous human body enzymic and non-enzymic antioxidants that work together to scavenge free radicals and who protect muscle function during physical exercise by maintaining an effective balance between free radical production and preventing potentially deleterious free radical effects. Antioxidant activity was determined by the ability of each compound to scavenge the long-lived free radicals [5,14–19].

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Both enzymic and non-enzymic antioxidants exist in the intracellular and extracellular environments to detoxify ROS. These scavengers are located throughout the cell and provide protection against free radicals' toxicity using different approaches. Primary strategies include conversion of free radicals into less-active molecules (i.e. scavenging), and prevention of the transformation of less-reactive free radicals into more damaging forms (i.e. prevention of the transformation of H_2O_2 into the damaging hydroxyl radical) [14]. Maintenance of oxidative –antioxidant homeostasis is critical for the normal function and survival of all aerobic organisms because an imbalance between ROS and antioxidants is referred to as oxidative stress [2,19]. Intense and prolonged exercise can result in oxidative damage to both proteins and lipids in the contracting myocytes [20].

In general, slow-twitch, mitochondria-rich (type I) fibers have an increased content of protective systems compared with fast (type II) fibers [15,21].

Enzymic and Non-enzymic Human Body Antioxidants

During intense and exhaustive exercise cells continuously produce free radicals and reactive oxygen species (ROS) as part of metabolic processes in skeletal muscle. These free radicals are neutralized by the antioxidant defense system. These endogenous human body protective systems comprise enzymatic and non-enzymatic antioxidants.

a) Primary antioxidant enzymes

The group of primary antioxidant enzymes in human muscles comprises both mitochondrial and cytosolic isoforms of superoxide dismutase (EC 1.15.1.1; SOD), manganese containing SOD and cuprum, zinc-containing MnSOD and CuZnSOD, respectively, catalase (CAT) (EC 1.11.1.6) and glutathione peroxidase (GPX), (EC 1.11.1.9; [21]. These enzymes are responsible for removing superoxide radicals, H_2O_2 and organic hydroperoxides. Additional antioxidant enzymes such as thioredoxin, glutaredoxin, and peroxiredoxin reductase also contribute to cellular protection against oxidation [13,18]. An acute exercise or longer term exercise result in increased activities of superoxide dismutase, catalase or glutathione peroxidase in animal and human muscles [6,23–26].

1. Superoxide dismutase (EC 1.15.1.1; SOD)

Superoxide dismutase (SOD) forms the first line of defense against superoxide radicals in order to form hydrogen peroxide (H_2O_2) and oxygen (O_2):

$$2O_2^{\bullet -} + 2H^+ \xrightarrow{\text{Superoxide dismutase}} O_2 + H_2O_2$$

Superoxide dismutase must work with enzymes that remove H_2O_2, that is with catalase. In mammals, three isoforms of SOD (SOD1, SOD2, SOD3) exist, and all require a redox active transition metal in the active site to accomplish the catalytic breakdown of the superoxide anion. The catalytically active metal can be copper, iron, manganese or nickel [27,28]. Exercise training significantly increased superoxide dismutase activity in the muscle soleus [29]. Mitochondrial SOD activity was increased by 37% in fast-twitch red and slow-twitch red types of muscle and 14% in white muscle [22].

Two of the SOD isoforms are located within cells, whereas the third SOD isoform is found in the extracellular space. Manganese (Mn) or copper/zinc (Cu/Zn)-dependent superoxide dismutase (SOD), are located in the matrix and intermembrane space of mitochondria, and quickly dismutate the superoxide generated by mitochondria to H_2O_2 to prevent oxidative stress [3,27,28,30].

The relative allocation of the SOD_1 and SOD_2 isoenzymes varies across tissues. In skeletal muscle, 15–35% of the total SOD activity is in the mitochondria, and theremaining 65–85% are in the cytosol. SOD activity is highest in oxidative muscles that contain a high percentage of type I and type IIa fibers compared with muscles with low mitochondrial volumes. SOD activity in skeletal muscle is not constant and can be modified by activity Endurance exercise training promotes 20–112% increases in the activities of both SOD1 and SOD2 in the exercised [13].

2. Glutathione peroxidase (EC 1.11.1.9; GPX)

All glutathione peroxidases (GPX) enzymes catalyze the reduction of H_2O_2 or organic hydroperoxide (ROOH) to water (H_2O) and alcohol (ROH), using reduced glutathione (GSH), or in some cases thioredoxin or glutaredoxin as the electron donors [13,31]. Glutathione peroxidase (GPx) removes H_2O_2 by coupling its reduction to water with oxidation of reduced glutathione (GSH):

$$H_2O_2 + 2GSH \xrightarrow{\text{Glutathion peroxidase}} GSSH + 2H_2O$$

Selenoproteome has five glutathione peroxidases in mammals (GPX1-GPX5) [20,31]. The highly oxidative fibers - type I, contain the highest GPX activity. The fact that many GPX isoenzymes will reduce a wide range of hydroperoxides ranging from H_2O_2 to complex organic hydroperoxides makes glutathione peroxidases (GPX) an important intracellular antioxidant to protect against ROS-mediated damage to membrane lipids, proteins, and nuclei acids. Glutathione peroxidases (GPX) increase in skeletal muscle fibers during regular and endurance exercise training along with increased cellular concentrations of glutathione in skeletal muscles, causing the risk reduction of cellular injury [3,13,14,31]. The reduction of GSSG back to GSH is done by glutathione reductase, a flavin containing enzyme, whereby NADPH provides the reducing power [3,31]. Skeletal muscles produce NADPH primarily via isocitrate dehydrogenase (ICD) through citric cycle but many tissues produce NADPH by glucose-6-phosphate dehydrogenase via the pentose pathway [1].

3. Catalase (EC 1.11.1.6)

Catalase (CAT) has principal biochemical functions to catalyze the break down of H_2O_2 into H_2O and O_2, i.e., 2 $H_2O_2 \rightarrow 2\ H_2O + O_2$. Catalase is widely distributed within the cell. Iron is a required cofactor attached to the active site of the enzyme. Catalase degrades H_2O_2 only when H_2O_2 reaches high concentrations [32,33]. Highly oxidative muscle fibers have the highest CAT protein levels and fibers with low oxidative capacity have the lowest enzyme activity [3,13].

The depleted activity levels of superoxide dismutase, catalase, glutathione peroxidase in the exercise animals indicated decreased antioxidative defense system in the muscle [26].

Accessory antioxidant enzymes

In addition to the primary antioxidant enzymes, cells contain the thioredoxin, glutaredoxin, and peroxiredoxin, the enzymes systems that directly or indirectly participate in the maintenance of redox balance [33].

The thioredoxin antioxidant system is composed of thioredoxin (TRX) and thioredoxin reductase [34–36]. TRX is the major ubiquitous disulfide reductase responsible for maintaining proteins in their reduced state. Oxidized TRX is then reduced by electrons from NADPH via thioredoxin reductase. Selenium (Se) is essential for the activity of thioredoxin reductase, explaining why this trace element is required for cell proliferation [34,37].

Glutaredoxin (GRX), similar to TRX, is a thiodisulfide oxidoreductase that is involved in the protection and repair of protein and non-protein thiols during periods of oxidative stress. GRX protects thiols by the transfer of electrons from NADPH to disulfide substrates, and this catalytic cycle is coupled with glutathione and glutathione reductase [34,37,38]. Human cells contain three different GRXs; GRX1 is located in cytosol, whereas both GRX2 and GRX5 are located in the mitochondria.

Peroxiredoxin (PRX) was discovered in 1988 and is a novel peroxidase capable of reducing both hydroperoxides and peroxynitrate with the use of electrons provided by a physiological thiol like TRX. Although peroxiredoxin (PRXs) may defend against cellular oxidative stress, the importance of their antioxidant role in mammalian cells remains unclear [36].

The effects of regular exercise on the TRX, GRX, and PRX systems in skeletal muscles remain unknown. Exercise-induced changes in one or all of these antioxidant systems could contribute to the redox adaptation to exercise [35,36,39].

Non-enzymic Antioxidants and Dietary Compounds

b) Vitamins (phytonutrients) as antioxidants

Vitamins are organic compounds required by humans in small amounts from the diet.

The group of dietary antioxidants, dietary phytonutrient, vitamins, include vitamins C- L ascorbic acid, vitamin A, retinol and their provitamins, carotenoids (especially β-carotene), vitamins E, tocopherol (especially α-tocopherol), folic acid or folates. In humans, vitamins function as metabolic regulators in small amounts from the diet, influencing a number of physiological processes important for exercise or sport performance. Many of the B-complex vitamins are involved in processing carbohydrate and fats for energy production, an important consideration during exercise of varying intensity. Several B vitamins, phyridoxal phosphate and vit. B2 (riboflavin, lactoflavin) are also essential for biosynthesis of hemoglobin in red blood cells, and for oxygen delivery to the muscles during aerobic endurance exercise [21].

It may be concluded that physically-active individuals might benefit from supplementation of vitamins [3].

1. Vitamin C (L-ascorbic acid)

Vitamin C has a main biological role as a reducing agent. Ascorbic acid is required for the hydroxylation of amino acids lysine and praline in protocollagen in the body. Without this hydroxylation protocollagen cannot properly cross-link into normal collagen fibrils. Thus, vitamin C is obviously important for maintenance of normal connective tissue. Vitamin C is also necessary for bone formation, since bone tissue has an organic matrix containing collagen as well as inorganic calcified portion. Collagen is a component of the ground substance surrounding capillary walls [40].

Various studies have demonstrated beneficial physiological effects of vitamin C supplementation in physically-active people. At physiological pH ascorbic acid exists as the ascorbate anion which is widely distributed in mammalian tissues. Vitamin C (L-ascorbic acid) distributes in the aqueous phase of the muscle cells. The antioxidant roles of vitamin C are numerous. Vitamin C can directly scavenge superoxide, hydroxyl and lipid hydroperoxide radicals. It modulates the intracellular redox status through maintaining sulfhydryl compounds, including glutathione in their reduced state. Increasing the concentration of vitamin C might interfere with antioxidant systems lowering the level of reduced glutathione as well as the activities of glutathione metabolic enzymes related to glutathione metabolism, such as glutathione reductase, glutathione peroxidase, and glutathione-S-transferase [41]. Furthermore, vitamin C plays a key role in recycling vitamin E. In this reaction native vitamin C, L-ascorbate is converted to a dehydroascorbate radical. This radical can be reduced back to native vitamin C by glutathione (GSH) [3].

2. Vitamin A (axeroftol, retinol)

The family of lipid-soluble antioxidants includes vitamin A (axeroftol, retinol) and provitamins- carotenoids, including β-carotene, vitamin E, α-tocopherol. Vitamin E, α-tocopherol, belongs in a family of lipid-soluble vitamin. These molecules are hydrophobic lipid soluble antioxidants located primarily in cell membranes and their primary function is to protect muscle membranes against oxidation. Because of their cellular location and their radical scavenging capacity, carotenoids are efficient biological antioxidants against lipid peroxidation.

The antioxidant activity of β-carotene, as a radical-scavenging antioxidant against lipid peroxidation, was much smaller than that of α-tocopherol [42].

3. Vitamin E-tocopherol

Vitamin E is potent lipid-soluble antioxidant in cell membranes and other lipid components of the cell and therefore it is essential for human nutrition [43,44]. In nature, compounds with vitamin E activity include α-, β-, γ-, g- and δ-tocopherols (TCP) as well as α-, β-, γ- and δ-tocotrienols (TCT) (44). The capacity of vitamin E to prevent oxidation of unsaturated fatty acids is its primary function in the body. Supplementation with tocotrienol-rich fractions (TRF) from palm oil, a potent antioxidant from the natural Vitamin E family, may help in the prevention or treatment of several diseases [45]. Studies have shown vitamin E supplementation as an efficient means of reducing exercise-induced muscle damage due to free radical formation [18].

Vitamin E is a particularly important antioxidant because of its capacity to convert superoxide and hydroxyl radicals to less-reactive forms. Vitamin E can also break lipid peroxidation chain reactions which occur during ROS-mediated damage to cell membranes. In addition to its direct antioxidant properties, evidence indicates that the beneficial effects of vitamin E in cells also comes from its ability to control gene expression of several proteins (46). Many studies in humans have demonstrated antioxidant protection by high-dose vitamin E supplementation [3,47–49]. The supplementation with both vitamins E and C only prevented increases in lipid peroxidation (43).

4. Folic acid, folacin

Folic acid, folacin, belongs in a group of water soluble vitamins. Before functioning as a C_1 carrier, folic acid must be reduced within cells to tetrahydrofolate (THF also H_4folate) through the action of dihydrofolate reductase (DHFR), an NADPH-requiring enzyme [50–52]. Through participating in biosynthesis of S-adenosyl methionine (SAMe) folic acid has an important function in glutathione synthesis. SAMe is a precursor for the synthesis of cysteine and thus glutathione (Fig. 1). SAMe effectively increase intracellular glutathione concentration in patients with liver disease [53,54]. Folate, vitamin B6, and vitamin B12 are required for homocysteine metabolism (Figure 1) by serving as cofactors for methionine synthase (B12), cystathionine synthase (B6), and cystathionase (B6) and as a substrate (5-methyltetrahydrofolate) for methionine synthase [55]. Cellular antioxidant metabolisms are linked by methylation and the transsulfuration pathway, which converts *Methionine- Homocysteine cycle* to cysteine, the important component in glutathione synthesis [54,56].

In situations where antioxidant defenses are compromised or where ROS production is grossly excessive, ROS are mediators of contraction-induced damage to skeletal muscle, (53).Through participating in biosynthesis of S-adenosyl methionine (SAMe) folic acid has an important function in glutathione synthesis. SAMe is

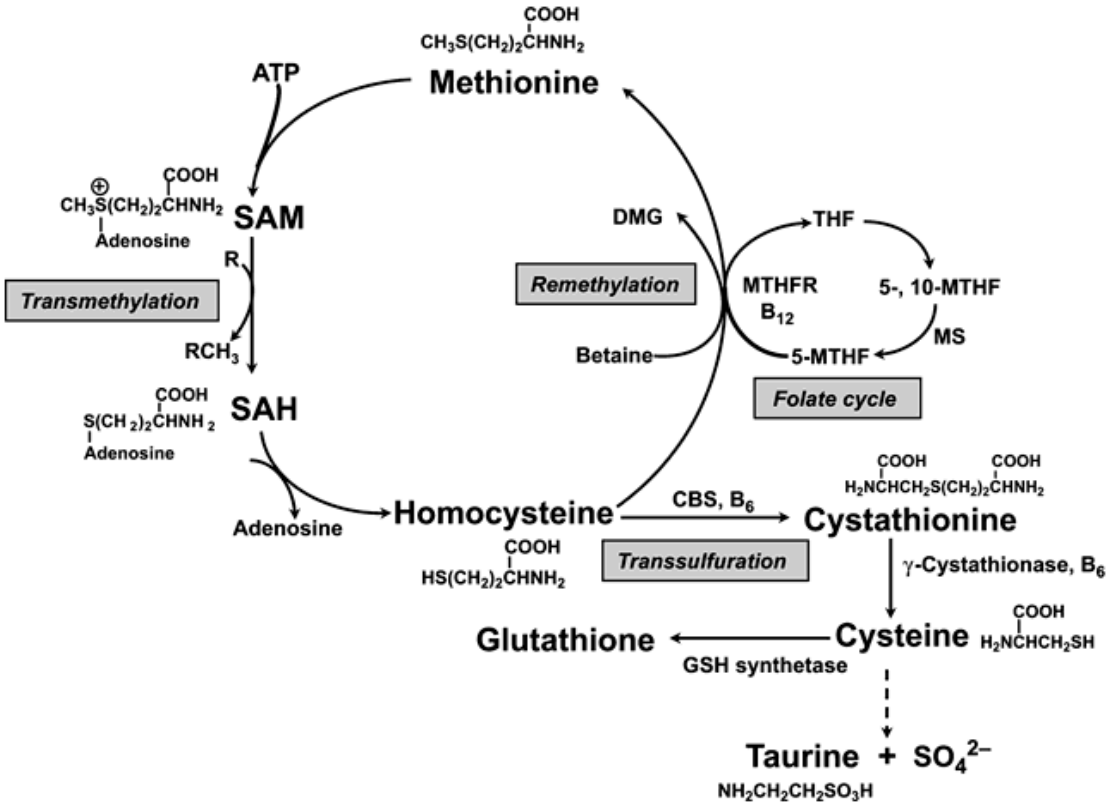

Fig 1 Folate (as THF), vitamin B6, and vitamin B12 are required for biosynthesis of Glutathione [54].

a precursor for the synthesis of cysteine and thus glutathione (Figure 1).

SAMe effectively increase intracellular glutathione concentration in patients with liver disease [53,54,57]. Literature data present conflicting results of vitamins' protective effects as antioxidant. However, despite the literature data, we could support that physically-active individuals might benefit from natural, human body constitutive, and diet rich in antioxidants [49].

Human Body Non-enzymic Antioxidants and Dietary Compounds

Beside several anti-oxidant enzyme systems and vitamins, cells contain endogenous non-enzymic anti-oxidants and naturally dietary compounds, to be used as scavenger free radicals molecules and to work against cellular damage [18].

The most important nonenzymatic antioxidants in muscle fibers include glutathione (GSH), bilirubin, α-lipoic acid (LA), uric acid, ubiquinone or Coenzyme CoQ10.

The group of naturally dietary compounds, besides phytonutrients-vitamins, includes phytochemicals, flavonoids, chlorophylls, and others, present in most edible fruits and vegetables.

c) Human body non-enzymic antioxidants

1. Glutathione

Glutathione (GSH) is a tripeptide and is the most abundant nonprotein thiol in cells [3,12,54,57,58]. This antioxidant is primarily synthesized in the liver and transported to tissues via the circulation. GSH content varies across organs depending on their function (3,58). The concentration of GSH found in skeletal muscle fibers varies across fiber types; for example, (slow) type I fibers contain 600% higher GSH content (approximately 3mM) compared with (fast) type IIb fibers (3). As an antioxidant, GSH has multiple roles in cells. GSH can directly react with a variety of radicals by donating a hydrogen atom. Endogenous glutathione and thioredoxin, thiol antioxidants, are modulated with high oxygen consumption and ROS generation during physical exercise, controlling cellular function through redox-sensitive signaling and protein-protein interactions [59]. Exercise increased GSSG content and decreased GSH/GSSG in mitochondria. These data provided direct evidence that oxidant production in skeletal muscle is increased during prolonged exercise, with both mitochondrial respiratory chain and NADPH oxidase as potential sources [60–62]. GSH serves as a substrate for GPX to eliminate H_2O_2 and organic hydroperoxides. Furthermore, GSH is also involved in reducing other antioxidants in the cell including vitamins C and E. Growing evidence indicates that exercise training results in an elevation in the activities of both superoxide dismutase and glutathione peroxidase along with increased cellular concentrations of glutathione in skeletal muscles. The exercise-induced increase in GSH within muscle fibers is due to increased activity of a key enzyme involved in GSH synthesis, glutathione synthase (γ-glutamylcysteine synthase**)**, [23,58,63].

Glutathione (GSH) and bilirubin are prominent endogenous antioxidant cytoprotectants. The water-soluble GSH primarily protects water soluble proteins, whereas the lipophilic bilirubin protects lipids from oxidation [64].

2. Bilirubin

Bilirubin is the final product of hemoprotein catabolism as heme oxygenase cleaves the heme ring to form biliverdin; biliverdin is then reduced by biliverdin reductase to form bilirubin. Exercise training significantly increased serum bilirubin levels [65]. Heme oxygenase (HO) has been shown to be important for attenuating the overall production of ROS [66,67].

Although both biliverdin and bilirubin are reducing species, bilirubin is considered to be the best physiological antioxidant cytoprotectant. Bilirubin, at micromolar concentrations in vitro, efficiently scavenges peroxyl radicals. Bilirubin is a potent antioxidant that can protect cells from a 10,000-fold excess of H_2O_2. The potent physiologic antioxidant actions of bilirubin reflect an amplification cycle whereby bilirubin, acting as an antioxidant, is itself oxidized to biliverdin and then recycled by biliverdin reductase back to bilirubin. This redox cycle may constitute the principal physiologic function of bilirubin [67–69]. Bilirubin possesses strong antioxidant potential against peroxyl radicals and has been shown to protect cells from toxic levels of hydrogen peroxide. It has been suggested that the powerful physiological antioxidant actions of bilirubin are a result of an amplification cycle whereby bilirubin acting as an antioxidant, is itself oxidized back to biliverdin and then recycled back to bilirubin via biliverdin reductase [67,68]. The data support the idea of a "beneficial" role for bilirubin as a physiological, chain-breaking antioxidant. Small amounts of plasma bilirubin are sufficient to prevent oxidation of albumin-bound fatty acids as well as of the protein itself. The data indicate a role for albumin-bound bilirubin (Alb-BR) as a physiological antioxidant in plasma and the extravascular space [70].

3. α-Lipoic acid (LA)

α-Lipoic acid is a naturally occurring compound and can be obtained from the diet. It is concluded that alpha-lipoic acid supplementation diminishes oxidative damage. Studies suggest that LA also acts as a powerful micronutrient with diverse pharmacologic and antioxidant properties [4, 44]. As an antioxidant, LA directly terminates free radicals, chelates transition metal ions (e.g. iron and copper), increases cytosolic glutathione and vitamin C levels and prevents toxicities associated with their loss, [71,72]. α-Lipoic acid (ALA), as lipoamide, is an endogenous intracellular thiol that acts as a cofactor in the multienzyme complexes that catalyze the oxidative decarboxylation of a-keto acids such as pyruvate, a- ketoglutarate, and branched chain α-keto acids [73–75]. Their binding to an enzyme complex generally limits its function as an antioxidant. Normally, α-lipoic acid is present in very small quantities in tissues.

ALA and DHLA couple has shown the ability to react with reactive nitrogen and oxygen species (RONS) such as hydroxyl radical, hypochlorous acid and singlet oxygen and reduce glutathione disulfide, tocopherol radicals and ascorbate [75].

Alfa-LA has been applied in sport as a dietary supplement. Alfa-LA supplementation attenuated exercise-induced oxidative damage in various tissues [76]. Alfa-LA can assist in the recycling of vitamin C and E [73,74,77]. On the other hand, long-term Alfa-LA administration led to enhancement of lipid peroxidation [75].

4. Uric acid

Uric acid is a by-product of purine metabolism in humans and is potentially an important antioxidant in human biological fluids. Uric acid (UA) may function either as an antioxidant (primarily in plasma) or pro-oxidant (primarily within the cell). In the plasma, urate can prevent lipid peroxidation only as long as ascorbic acid is present [78]. At physiological pH, almost all uric acid is converted to urate [79]. As an antioxidant, urate is able to protect against oxidative damage by acting as an electron donor. It appears that urate could be an antioxidant scavenger in skeletal muscle during exercise [79]. High UA concentrations are associated with increased serum antioxidant capacity and reduced oxidative stress during acute physical exercise in healthy subjects [80].

5. Ubiquinones

Ubiquinone, or coenzyme Q (CoQ), plays an important role in the production of chemical energy in the mitochondria. Coenzyme Q_{10} (CoQ_{10}) is the major form of ubiquinone in human subjects. CoQ_{10} has gained considerable attention as an agent capable of influencing cellular bioenergetics and counteracting some of the damage caused by free radicals [3,81]. Humans can synthesize ubiquinones, hence, coenzyme Q_{10} cannot be considered a vitamin [82]. Coenzyme Q_{10} is soluble in lipids and is found in virtually all cell membranes, as well as lipoproteins; a cause that is essential in mitochondrial electron transport chain [83,84]. Reduced forms of ubiquinones, ubiquinols, are lipid-soluble and are efficient antioxidants, better antioxidants compared with ubiquinones [85]. Approximately 50% of the total cellular ubiquinone is located within the mitochondria, 30% in the nucleus, with the remaining 10% located in the endoplasmic reticulum and the cytoplasm. The presence of high concentrations of quinol in all membranes provides a basis for antioxidant action either by direct reaction with radicals or by regeneration of tocopherol and ascorbate [83].

CoQ_{10} is also a micronutrient. However, its bioavailability is limited compared to that of other lipid-soluble antioxidants like vitamin E. CoQ_{10} is the only lipid-soluble antioxidant synthesized endogenously. Ubiquinol inhibits the peroxidation of cell membrane lipids and also that of lipoprotein lipids present in the circulation [86].

Ubiquinone (Co Q10) is suitable for therapeutic use in the treatment of some muscular diseases [83,87]. It might therefore be used during strenuous exercise. Zuliani U et al. (1989) have evaluated the effect of prolonged treatment with Co Q10 (100 mg/day per os for one month) on the biological changes induced by prolonged work on an ergometer bicycle in athletes; they observed any variation before or after the period of treatment with ubiquinone (Co Q10) [87].

Oxidative stress generated by physical exercise increases tissue ubiquinone levels by increasing biosynthesis in human body. Acute and chronic supplementation of CoQ_{10} may affect acute and/or chronic responses to various types of exercise [85,88]. CoQ_{10} supplementation increased total CoQ concentration in the slow-twitch muscles and was useful for reducing exhaustive exercise-induced muscular injury by enhancing stabilization of muscle cell membrane [89].

Coenzyme Q_{10} is available without prescription as a dietary supplement. Supplemental doses for adults range from 30-100 mg/day, which is considerably higher than normal dietary coenzyme Q_{10} intake. Supplementation of healthy men with 120 mg/day for three weeks did not increase skeletal muscle concentrations of coenzyme Q_{10} [90].

d) Dietary compounds, phytochemicals

The group of naturally dietary compounds includes phytochemicals such as flavonoid and chlorophylls and others present in most edible fruits and vegetables.

Phytochemicals are chemicals produced by plants that may affect health.

1. Flavonoids

Flavonoids are nearly ubiquitous in plants and are recognized as the pigments responsible for the colors of leaves, especially in last autumnal burst of hues and the many shades of yellow, orange, and red in flowers and food. The flavonoids are found in fruits, vegetables, nuts, seeds, herbs, spices, stems, flowers, as well as tea and red wine [91,92]. They are abundant in seeds, citrus fruits, olive oil, tea, and red wine. Flavonoids are polyphenols or polyhydroxylated phytochemicals. The two main classes of polyphenols include flavonoids and phenolic acids [81,91]. Flavonoids exert positive effects on human health; they are required for best activity, especially antioxidant and antiproliferative activity [81,93]. The mechanism of antioxidant activity of flavonoids can be characterized by direct scavenging or quenching of oxygen free radicals or excited oxygen species as well as inhibition of oxidative enzymes that generate these reactive oxygen species [92,94]. Some of them have been proposed to be beneficial in exercise and exercise performance. The well-known flavonoids are quercetin, catechins, and resveratrol, and they have received great scientific attention. Quercetin supplementation increases mitochondrial biogenesis of exercise skeletal muscle [81,96].

2. Chlorophyll

Chlorophyll is one of the main photosynthetic pigments, found in particularly large amounts in higher plants, dark green, leafy vegetables. Chlorophylls are important antioxidants found in foods. They give plants and algae

their green color. Green, leafy vegetables collect light and energy by photosynthesis, and transform it into a powerful compound called chlorophyll. Sunlight and chlorophyll are our best friends [1,44,96,97]. The basic structure of chlorophyll is a porphyrin ring similar to that of heme in hemoglobin, although the central atom in chlorophyll is magnesium instead of iron. The long 20-carbon phytol tail attached to the porphyrin ring makes chlorophyll hydrophobic, fat-soluble and allows chlorophyll to incorporate into biological lipid membranes. Magnesium in chlorophyll contributes to muscle toning, contraction and relaxation. Chlorophyll *a* and chlorophyll *b* are natural, fat-soluble chlorophylls found in plants. The most important and widely present form in the plants is chlorophyll [44]. In these naturally occurring chlorophylls the functional group of C7, a -CH3 or a -CHO group, define the *a* and *b* forms of chlorophyll, respectively. These naturally occurring *a* and *b* derivatives of chlorophyll are present in plants as breakdown products and as the products of chlorophyll digestion [1,44,97]. Chlorophyllin is a semi-synthetic mixture of sodium copper salts derived from chlorophyll. During the synthesis of chlorophyllin, the magnesium atom at the center of the ring is replaced with copper and the phytol tail is lost. Unlike natural chlorophyll, chlorophyllin is water-soluble [44, 97–101].

Chlorophyll and his liquid derivatives are powerful antioxidants from the diet [44,99]. This antioxidant action can provide many of the benefits of eating vegetables and fruits that contain chlorophylls. As an antioxidants and health supplements chlorophylls prevent the formation of free radicals caused by oxidation of molecules and neutralize existing free radicals, making them harmless [97,101].

Most of benefits of liquid chlorophyll and their derivatives lack extensive research.

Conclusion

The present review tries to elucidate the mechanisms by which antioxidants influence the decreasing of toxic free radical effects on the skeletal muscle function in normal, physiological condition, and under intensive physical exercise. With this article we wish to clarify the normal acting of natural antioxidants, as a normal body constituent and compound of diet and to stimulate further research in this area.

Recent scientific data confirm that the long-term use of antioxidants is safe and effective. The actual recommendation for physically active individuals is to ingest a diet rich in antioxidants.

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Review Article

EVIDENCE-BASED STROKE REHABILITATION

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Abstract. *Stroke can have different clinical characteristics and consequences, with unequal disability and outcome, thus demanding individual approach, specific skills and general knowledge. Treatment of stroke has significantly improved during the last twenty years, mainly because of clinical and experimental studies, adequate medicamentous therapy, and the use of new technologies as well. Use-dependent rehabilitation strategy includes repetitive training with proper adjustment of the program. Other rehabilitation practices should also be incorporated, such as self-care, recreation, and home-based activities. Motivation of the patients, improving quality of life, functional independence, activities of daily life are crucial. Holistic approach means that the patient as a whole should be considered and treated. Scientific evidence is sufficient to confirm the necessity of physical rehabilitation of patients after stroke in order to achieve the optimal results. Current evidence on the effect of physical therapy in stroke rehabilitation is presented.*

Key words: *stroke, physical therapy, rehabilitation, evidence.*

Introduction

Stroke represents non-traumatic injury of the brain, induced by occlusion or rupture of cerebral vascular supply causing neurologic deficit, mainly damage of motor control, sensation, cognition, disequilibrium, and sometimes leading to coma. Proposed modern term instead of stroke is cerebrovascular accident [1,2].

Stroke is the fourth leading cause of death after heart failure, cancer, and lower respiratory tract diseses. Cerebrovacular disease is the most important cause of long term disability. Stroke mainly affects older individuals, with about 70% of stroke occurring in persons over 65 years, but it can also occur in children [1,2].

Different vascular pathologic factors cause stroke. Ischemic brain damage, whether thrombotic or embolic, predominates with approximately 85%, while intracranial hemorrhage represents about 15% of all strokes. Clinical stroke syndromes and consequent impairment and disability are based on affected artery.

Predominant causes of stroke are: atherosclerosis, hypertension, smoking, hypercholesterolemia, diabetes mellitus, and other factors. Non-modifiable risk factors are age, sex, previous stroke, and race. Sedentary way of life predisposes to cardiovascular disease and stroke. When stroke affects the left hemisphere language, comprehension and communication can be seriously damaged, while stroke in the right hemisphere alters intuition, solving, reasoning, judgment and vision. Cognitive problems after stroke are often inadequately addressed, though they are the most important predictor of professional inability. Older patients with stroke often have bigger motor and cognitive affection due to reduced brain vessel density and ischemia [1–3].

Certain gender differences were noted concerning outcome of stroke. Women exhibit greater disability and have a lower rate of full recovery than men. However, age and psychosocial adjusment tend to be more influential than gender itself.

The first six hours and emergency medical management in acute stroke unit are crucial for treatment of stroke. This is followed by further diagnostic evaluation, stroke prevention, and rehabilitation [4,5].

Rehabilitation of Stroke

Neuroplasticity is defined as the ability of central nervous system to remodel after injury. Indeed, adaptive plasticity of adult brain after damage was confirmed. Both basic and clinical studies have confirmed that rehabilitation must accompany any other medical or surgical intervention in treatment of stroke.

Functional recovery after stroke can be improved using different sensorimotor techniques, such as: strengthening, balance training, postural control, and improving range of motion. ***Brunnstrom*** early movement technique, *Bobath* neurodevelopmental technique, proprioceptive neural facilitation, and task-oriented approach have traditionally been used, but without suffi-

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cient evidence and proof of better results of one technique over the other [6–8].

Upper limb rehabilitation after stroke can produce significant improvement, with adequate compensatory strategies and promotion of recovery of the limb. Constraint-induced movement therapy (CIMT) is based on the fact that patients with upper extremity loss are more dependent on unaffected arm, with negative feedback. Repetitive movements, reacquisition of skills and cortical reorganization are included in CIMT. The original and modified CIMT were investigated in clinical trials that showed improvement of motor control and functional skills. EMG triggered neuromuscular electrical stimulation (NMES) is adequate for stroke rehabilitation, since some clinical studies have confirmed improvement of motor function and better activity of affected hand [9].

Lower limb rehabilitation after stroke was primarily performed using body weight-supported treadmill training (BWSTT). The aim is to make step movement of the limb and to ameliorate bipedal walking. Many repetitions that are needed, increased weight bearing and action of two therapists pose limitations to this technique. Also, clinical studies have confirmed that BWSTT is also beneficial for cardiovascular fitness of stroke patients.

New technologies, such as robotic-assisted training, were used and studied for rehabilitation of upper and lower extremities after stroke. The advantages are: induced passive or assisted movements, tactile feedback and skill acquisition and easy repetition of movements. Further studies on robotic-assisted training are needed, particularly for rehabilitation of finger movements after stroke. Transcranial magnetic stimulation, cortical brain stimulation, and neuronal transplantation were applied in clinical studies, and autologous marrow stromal cells in preclinical trials were used, all with good results [10].

Spasticity increases motor impairment and disability after stroke through an increase of tonic stretch reflexes. Treatment of spasticity includes exercises and botulinum toxin injections. Daily exercises, application of static resting splints prevents contractures. Botulinum toxin is particularly effective and repeatable.

Cognition, language, and communication difficulties after stroke are frequent. Certain spontaneous recovery after stroke is expected, while aphasia tends to recover much slower. Rehabilitation is focused to improving speaking, understanding, reading, and writing, as well as developing compensatory techniques. Many treatment options exist for rehabilitation of aphasia [11].

Dysphagia after stroke is treated using compensatory mechanisms. Percutaneous gastrostomy has been used successfully in store patients with severe dysphagia.

Pain syndromes, and particularly shoulder pain, are frequent complains after stroke. Neuromuscular electrical stimulation (NMES) of affected muscles reduced pain, and improved activity of daily life.

Control of bladder and bowel can be altered after stroke. The treatment includes timed voiding, and adequate medication based on urodynamic tests, or even intermittent catheterization in some cases.

Psychosocial changes after stroke include: sadness, anxiety, depression, anger, frustration, confusion, and other. Coping with these problems is essential in rehabilitation program, so motivation, counseling, and support are used [12,13].

Medical comorbidities in stroke are numerous, frequent, and potentially devastating. Venous thromboembolism, pneumonia, hypertension, heart failure, diabetes mellitus, malnutrition, and psychological deconditioning are the most important and should be treated adequately [14].

Organization and Levels of Rehabilitation

Instead of classical treatment programs in inpatient and outpatient ward, recent stroke rehabilitation is organized as a continuum according to current patients demands that change over time. This resulted in significant improvement of overall results of rehabilitation. Healthcare system, community resources, and patient's medical condition and motivation are important for the optimal result [15,16].

Early stroke rehabilitation prevents prolonged bed rest and complications. Exercises in bed, gait training, activities of daily life are executed. Early rehabilitation was more influential on the outcome than the duration of rehabilitation, as found in meta-analyses.

Acute hospital rehabilitation is a team program including doctors, nurses, and equipment. It is essential that a patient can tolerate treatment for three or more hours per day.

Long-term acute rehabilitation is indicated for persons with prolonged complex disease. Adequate respiratory care, nutrition, and medical treatment are applied as long as needed. The intensity of treatment and specific demands are organized according to the tolerance of the patients.

Outpatient day rehabilitation includes the same rehabilitation program without hospital stay. Medical stability and social support are mandatory.

Outpatient rehabilitation is centered to single-modality training provided by physical, occupational and speech-language therapist as well.

Home therapy is the most familiar and appropriate environment for the patient and physical therapy. Stability of medical condition and sufficient social support enable home rehabilitation.

Specialized equipment, such as: adapted feeding devices, bathing equipment, dressing and walking devices assist patients to become more independent and have better performance. They are included depending on the level of functional activity [17,18].

Outcome of Rehabilitation

Data from medical references on the effect of physical rehabilitation after stroke are numerous, but they are difficult to interpret adequately, because of used methodology, samples, different rehabilitation programs, and outcome measures. Outcome can be measured by: morbidity, mortality, impairment, hospitalization, functional consequences, and quality of life. The insufficient number of randomized studies for rehabilitation of different diseases was mentioned in the Report of Disability published by the World Health organization [19].

Analysis of patient's condition and improvement in post-stroke rehabilitation should be presented longitudinally in order to better document and compare the results of treatment. Such longitudinal follow up was found in only one third of the studies in the literature, while others are mainly cross sectional studies, often without mentioning the dynamics of changes. All the studies emphasize that physical therapy after stroke results in improvement of physical performance, functional status, and quality of life. Independence can be obtained by most of the patients, ranging from 65% to 85%, with less favorable social and professional results, ranging from 45% to 65%. The most prominent aspects with improvement are: locomotion, mobility, self-care, and sphincter control. Communication and social cognition are improved to a smaller rate [19,20].

Predictors of outcome are numerous, and include: severity and type of impairment, cognitive factors, comorbidity, social support, and type and quality of rehabilitation. The list of influential factors is long: age, education, type, severity, and location of stroke, previous stroke, heart failure and other comorbidities, dementia, presence of coma, cognition, language, balance, bowel and bladder function, depression, motivation, support, and others. However, it is difficult to apply numerous data on prediction factors to each individual patient with stroke. Documentation of certain factors that are present is very important, but clinical implications are complex. Also, stoke can impede different aspects of physical, cognitive, emotional, and vocational functions.

Control group, without therapy, for the studies of stroke rehabilitation is mandatory for randomized controlled trials, but this is impossible and unethical. On the other side, many other factors (motivation, support, adaptability, etc.) can influence the effect of rehabilitation. Individuality and diversity of factors makes them difficult to compare and divide into clinical groups. Spontaneous recovery is also uneasy to differentiate from rehabilitation results. Variability in stroke recovery is a well-known fact. It depends on initial severity of hemiparesis, age, location and site of infarct, stroke type, nonmotor parameters, and impairment in the first 72 hours [21,22].

The first randomized controlled trial of stroke rehabilitation was published in 1980. Ever since that, the studies have been documenting the improvement achieved by physical therapy of stroke. Statistically significantly better functional independence and outcome were universally reported. Treatment in stroke rehabilitation unit produced less mortality, earlier discharge and better functional scores.

Meta-analyses confirmed that focused stroke rehabilitation is significantly better than other groups, particularly for mobility, personal care, and perception. Also, home based physical therapy was found effective to improve the outcome. The most important contribution of all the studies performed on stroke rehabilitation is that they emphasize certain clinically important aspects for proper rehabilitation programs. They have confirmed the importance of focused personal care, mobility training, team work, and education of patients and families [22,23].

More than 1000 randomized controlled trials have been published concerning stroke rehabilitation. The best period for the studies is the first days after stroke, since this is when spontaneous biological recovery happens. However, most of the studies are conducted more than 6 months after the stroke. Better standardization of terms used for recovery, the differences between the experimental and control arms, precise definition of usual and standard care are needed to improve the reliability of conducted studies of post stroke rehabilitation [24–27].

Randomised controlled trials indicate that **early supported discharge** (EDS) after stroke with continued rehabilitation program at home gives good results, primarily in reduced dependency. At home, patients are instructed to problem solving skills, supported in daily activities, and assisted in achieving the previous activities. This is realized through professional visits of physiotherapists, and other included professionals of the team. EDS demands adequate organizational changes, defining desired performance to be achieved. Increased capacity to solve problems improves patients' self-efficacy and independence. Studies have also confirmed cost reduction with implementation of EDS [28].

Management of medical demands, change of behavior of life, treatment of emotional consequences of stroke are the mainstay of support and self-management of patients. Solving problems, making adequate decisions, use of resources, proper relationship with the medical team, and feedback are dominant aspects of post-stroke support.

Multidisciplinary health care team in recovery after stroke has now significantly improved the treatment and outcome of stroke patients. Stroke unit care and thrombolysis have reduced acute morbidity and mortality, but long term consequences of stroke are frequent, and deserve individual, permanent and team approach. Interdisciplinary teamwork adds the group effort and responsibility to each individual task in - planning and treatment of stroke. Hospital stroke team consists of neurologists, physiotherapists, occupational therapists, speech and language therapists, nurses, and others. Cochrane review indicates that such team approach improves the results of treatment of stroke. Physical therapy of stroke effectively improves function and mobility, however, it is not determined which ap-

proach gives the best result. Early supported discharge reduces the length of stay, and according to systematic Cochrane review adds to activities of daily life of stroke patients. Long term rehabilitation support of patients with stroke is essential for further improvement of outcome, independence, and active life [29].

Physical exercise was applied in stroke patients aiming to document physical dysfunctions, improve motor performance, and prevent further damage. References prove the importance of physical training for physical independence, quality of life, and reduction of cardiovascular complications.

Physical training improves cerebral angiogenesis, vasomotor reactivity, reduces apoptosis and activates motor unit. Also, pre-stroke physical activity can improve recovery with decreased brain damage and better motor outcome. It also reduces the initial stroke severity, edema, infarct volume, inflammation, metabolic disorders. Preventive effects of pre-stroke physical activity (both walking 1h/day/5 day per week, or vigorous aerobic activity) are documented by better functional outcome, milder severity, and better motor improvement.

Both animal and human researches have documented positive effect of acute exercise on muscle activation regulation in cerebral ischemia. Decrease of motoneuronal recruitment from spinal and supraspinal tracts, and increase of cortical activation of the unaffected side, with the decrease on the affected one, confirm compensatory neural process.

Chronic exercise after stroke was more extensively studied. Literature clearly states that physical therapy is the first line intervention for reduction of chronic disability after stroke. Treadmill training in early phases after stroke induces neuroplasticity through influence on brain vasomotor function, angiogenesis, neurotrophic factors, apoptosis and inflammation. Adequate brain oxygenation is essential for decreasing volume of cerebral infarct, and for less functional damage. Regeneration of striatonigral and corticonigral projections in ischemic brain, increased mitochondrial biogenesis, and improved cholinergic homeostasis were found after intensive treadmill training in stroke. All these processes lead to reduced muscle fatigue, improved walking speed and endurance. Overall better sensorimotor functions are directly correlated to the increase of aerobic capacity.

Volume, intensity, frequency, and timing of training directly affect proper neuroplasticity and recovery. Early intense training in the first 24 hours is not recommended. More intense exercises after stroke led to better recovery, but the influence of intensity of training on neuroplasticity in stroke is insufficiently studied [30–34].

The combination of exercise and medicaments is essential for functional outcome after stroke. Indomethacin, D-amphetamine, S-nitorogluthatione, and others exhibited neurovascular protective effect after cerebral ischemia in preclinical trials. Clinical significance of these medicaments combined with training is to be confirmed in clinical studies.

Published studies confirm that **aerobic training** improves cardio respiratory fitness, functional recovery, endurance, and quality of life after stroke. Also, aerobic training reduced cognitive dysfunction, promoting neuroplasticity in post-stroke period. Such increased brain plasticity produces better executive functions and long term memory. New brain imaging technologies have documented the increase of both white and gray matter, better synaptic connections, and brain activation patterns after aerobic training. Functional Magnetic Resonance Imaging (fMRI) confirmed increased activation of brain regions for attention control and inhibitory functioning, while activation was reduced in behavior conflict regions. Neuroplasticity improved by aerobic training was further documented using measurement of circulating neurotrophic factors (*brain derived neurotrophic factor, insulin-like growth factor-1, and vascular endothelial growth factor*). However, the exact influence of circulating growth factors on cognition in older persons is now unclear [35,36].

Only three clinical studies concerning the influence of aerobic training on **cognitive functions** after stroke were published. Two out of three studies found amelioration of cognitive dysfunction with better functional results, motor learning, and speed of information processing. On the other side, eight weeks after training, the long-term preservation of achieved results was absent. Patients with stroke, opposite to healthy persons, did not exhibit improvement of cognitive functions.

Aerobic training combined with other exercises in rehabilitation of stroke was also studied. The addition of muscle strengthening techniques of lower extremities improved attention, executive functions and voluntary motor control. Combination of aerobic exercises with stretching, balance and task-specific practice resulted in better verbal memory and flexibility of cognition, without influence on executive functions. Aerobic and behavioral training improved cognition, but without total recovery [35,36].

Few combinations of aerobic training and pharmacological treatment (memantine, citicoline, recombinant tissue plasminogen activator, S-nitrosoglutathione) improved motor recovery after stroke, but for cognitive function the results were less efficient. Promotion of cognition was more efficient when low-intensity aerobic exercises, starting one week after stroke, were applied.

Cognitive and motor functions in stroke patients must not be considered independently. Complex cognitive functions and sensorimotor control show complex interaction in the process of learning and control of specific movements. This fact is even more important for older persons, since functional brain reactivation makes motor and cognitive functions more interrelated.

Aerobic capacity is best ameliorated using moderate continuous aerobic training, with several 30-60 minutes of exercise sessions per week, and 40-80% of the maximal heart rate reserve. Training with higher intensity could increase aerobic fitness, and oxygen uptake. Muscular and cardiovascular adaptations were improved

when high intensity interval exercise (repeated intense training with active or passive rest) was administered. Forced treadmill exercises were proven superior to other types of training. Rehabilitation program in subacute post stroke period also reduced the complications of prolonged inactivity.

So, current recommendations for application of aerobic exercises are based on the effect on cardiovascular and motor functions, and not on cognitive functions. Thus, there is no standard recommendation for exercises concerning recovery of cognitive functions after stroke [37,38].

Self-management support after stroke is important for reducing chronic disabilities. It includes all activities one has to perform in order to efficiently manage the long-term management of stroke effect on the person and his family. Meta-review of literature has confirmed the important impact of stroke on self-image, and different support needed in the whole period of post-stroke rehabilitation and recovery. The following recommendations were made according to these facts: the support for stroke patients need to be changed according to the stage of recovery; collaboration of health care professionals enable adequate decisions of patients in different stages of stroke recovery; individual psychological, emotional and behavioral support from the start to adaptation, social reintegration, and developing skills is needed; social groups of patients and supporting persons should be conducted [39].

Powered robotic exoskeletons represent a new technology aimed to improve walking with lower extremity weakness. Early conventional physical therapy of walking after stroke improves speed and endurance, but it is very demanding for the therapists. Exoskeletons reduce the need for physical strain, and also enable repetitions. Treadmill-based robotics was also developed leading to improvement of walking after stroke, but without change in speed and endurance. Current clinical studies explore safety and efficiency of exoskeletons for gait in subacute and chronic stroke, with more convincing results in the sub-acute phase [40,41].

Virtual reality (VR) is a technology generated by the computer with interaction with a user, enabling precise movements using sensorimotor feedback. Recent studies have confirmed effectiveness of VR in stroke rehabilitation, especially for gait and balance. More flexible software and individualized treatment are needed.

VR enables acquisition and retention of motor skills through repetitive, defined, motivating task specific training. Current metacentric studies of VR in stroke rehabilitation are limited, mainly because of study designs and small sample size [42–45].

Noninvasive brain stimulation (NIBS) facilitates neuroplasticity. Transcranial magnet stimulation (TMS) and transcranial direct current stimulation (tDCS) are commonly used for noninvasive brain stimulation. The use of NIBS demands meaningful rehabilitation program that is task specific. It should be applied with other procedures that promote neuroplasticity, such as body weight support treadmill training, functional electric stimulation of the lower limb and bio feedback. Adequate timing of NIBS during a gait cycle is mandatory. Important questions for adequate use of NIBS are: which hemisphere should be stimulated, location and extent of stimulation, and individualization of treatment. Because of high individual variability of patients to NIBS customized stimulation is recommended. Furthermore, genetic and imaging biomarkers to predict outcome, and definition of target sites should be clarified in the future [46].

Functional MRI has confirmed that motor recovery of paretic limb after stroke is associated with hyper activation in different brain areas, such as contralesional hemisphere and subcortical structures. Mental practice is internal stimulation created by auditory, visual, tactile, or kinesthetic stimulation without movement. Mental practice and physical therapy are used in post-stroke rehabilitation [47].

Sleep problems in stroke are frequent and multifactorial, sometimes reducing acute recovery with longer hospitalization, fatigue, demotivation, and sedentary life style. Sleep is also of primary importance for neuroplasticity and memory consolidation. **Transcranial direct current stimulation** (tDCS) for sleep disorders after stroke is a safe technology that alters brain plasticity, modifies neuronal activity, delivering a weak electric current (1-2 mA). Both anodal and cathodal tDCS are used. One randomized study confirmed that anodal tDCS produced performance gains in the paretic hand. Studies that analyzed motor rehabilitation program combined with tDCS indicate additional improved motor outcomes. Further studies are needed. Individual variations caused by mental health status, motivation, and many physical factors, such as fatigue, significantly influence the outcome of use of tDCS [48–50].

In conclusion, scientific evidence is sufficient to confirm the necessity for physical rehabilitation of patients after stroke. It represents the cornerstone during the early phase and continues throughout the whole post stroke period.

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Original Article

HEARING LOSS IN RHEUMATOID ARHRITIS

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Abstract. *The incidence of hearing loss in patients with rheumatoid arthritis (RA) and the treatment have been differently reported. The aims of this study were to establish the presence and to differentiate the type of hearing loss in patients with RA, and to investigate the results of corticosteroid and methotrexate treatment on hearing loss in RA. Longitudinal, prospective study included 87 patients aged between 18 and 70 years diagnosed with RA. Disease Activity Score (DAS 28 Se) and Health Assessment Questionnaire (HAQ) were measured. 38 Patients were treated with prednisone; intratympanic appliction was used in 11 persons, and in case of weak or absent improvement after steroids methotrexate was applied for further treatment. Predominantly sensorineural hearing loss was present in 56.3% of the patients, without correlation with the duration of disease and clinical activity of RA. Corticosteroid therapy, both peroral or intratympanic contributed to hearing improvement in 60.0%. Audiometric tests are recommended in patients who suffer from RA in order to control hearing in rheumatoid arthritis and analyze the effect of proposed therapeutic procedures.*

Key words: *Rheumatoid arthritis, hearing loss, corticosteroids, methotrexate, intratympanic application.*

Introduction

Rheumatoid arthritis (RA) is a disease of unknown origin, characterized by disseminated erosions of articulations, and different systemic inflammatory changes. Humoral immune alterations (rheumatoid factor, interferon gamma, interleukin 2), as well as cellular alterations (lymphocyte activation) are characteristic for RA. Reduced complement, leukocytosis, increased sedimentation, and higher gammaglobuline level are usually found. The diagnosis is predominantly based on clinical, radiological, and laboratory parameters [1,2].

RA causes proliferative synovitis in symmetric peripheral joints, which differentiates it from other systemic diseases of connective tissue. Frequent multiorganic changes, such as pericardial effusion, subcutaneous nodes, polyneuropathy, vasculitis, nephropathy, and keratoconjunctivitis are characteristic for RA.

Hearing and balance impairment has been documented in patients with RA. Concerning hearing loss, bilateral, symmetric, slowly progressive hypoacousia predominates in these patients [3,4].

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Previous researches resulted in controversial data on the incidence and treatment of hearing loss in RA [5–7].

The aims of this study were:

1. To document the presence and type of hearing loss in patients with RA.
2. To compare activity and clinical parameters of RA with hearing status.
3. To examine the effectiveness of corticosteroid and methotrexate treatment on hearing loss in RA.

Patients and Methods

Longitudinal, prospective study comprising of 87 patients aged between 18 and 70 years diagnosed with RA was conducted during the period 2002-2010 at the Institute for Rheumatology and Clinic for Otorhinolaryngology. The mean age was 56.2±.8.7 years, female to male ratio was 70:17, and average duration of disease was 13.4±6.1 years.

The diagnosis was made according to the American College of Rheumatology Classification Criteria [1] which included: morning rigidity of joints, arthritis of more than 3 joints, arthritis of hands, all of them lasting for at least 6 weeks, subcutaneous nodes, seropositivity for RF, and radiologic erosions and decalcifications. Exclusion criteria were: other causes for conductive and sensorineural hearing loss, other systemic and chronic diseases, professional noise exposure, malignant diseases,

use of ototoxic and immunomodulator drugs. Laboratory tests included: sedimentation rate, leukocytes, fibrinogen, immunoglobulins, CRP, and RF.

Disease Activity Score (DAS 28 Se) was measured and Health Assessment Questionnaire (HAQ) was conducted. DAS 28 Se is index with strong correlation to the functional ability of patients, as well as with the outcome of the disease. The results were divided into: 3.2-5.1 moderate activity, and >5.1 indicating high activity. HAQ scale comprises of 20 questions in 8 categories. The results range from 0.0 (the best) to 3.0 (the worst functional ability).

The patients gave written consent, and the study was approved by the Institutional Ethics Committee.

Control group included 40 healthy subjects with similar age (average 55.3±6.2 years), and sex ratio (F: M ratio 31:9).

Hearing threshold for seven frequencies was measured using audiometer Madsen ZO 2020. Improvement of hearing was defined as the increase for more than 15 dB for one frequency (250, 500, 1000, 2000, 4000, 6000, 8000 Hz), or any improvement for two or more frequencies.
No previous steroid treatment was given to any patient for at least for six months.

The patients with hearing loss were offered peroral or transtympanic steroid therapy. 38 patients were treated with prednisone (30 days, peroral dose 60 mg/day). Intratympanic appliction was used in 11 persons (methylprednisolone, weekkly 0,5 ml in 40 mg/ml). Weak or absent improvement after steroids was further treated by methotrexate (7,5 mg/wk, with gradual increase to 25 mg/ml, for 8 weeks).

Student's t test and chi^2 test were used, and statistical significance limit was defined as $p<0.05$.

Results

Among patients with RA a total of 49 patients (56.3%) had hearing loss. There were 41 females, and 8 male patients, without significant difference from the patients with normal hearing. Hearing loss was predominantly sensorineural, documented in 91.8% of them, and in other 8.2% it was conductive, or mixed. Hearing loss accompanied by tinnitus, fullness, or vertigo was verified in 19.5% of patients, while bilateral progressive sensorineural hearing loss was present in 10.3% of them. Subjective average duration of hearing loss was 3.5±2.3 years.

Control group had hearing loss in 12.5 %, and it was mainly mild sensorineural. Distribution of patients across age was similar in both groups.

Longer duration of RA, as well as higher values of DAS 28 Se, and HAQ scale produced more frequent incidence of altered hearing. However, the data did not reach statistical significance for any of these parameters (Table 1).

After peroral steroid therapy hearing improvement was documented in 60.5 % of the patients, while in others hearing remained the same. Intratympanic application of steroids resulted in better hearing in 63.6% of the patients (Table 2). Improvement of hearing was achieved only for higher frequencies, over 2000 Hz. Also, better results were achieved for mild to moderate initial hearing loss, than for pronounced sensorineural alteration of hearing. After follow-up for three to five months there were no significant changes of obtained improvement (Table 3).

In total, 38.8% of the patients did not have any change of hearing after steroid therapy. However, only 47.4% of them accepted further methotrexate therapy. The results of such treatment were not satisfactory, with improvement in only 11.1 % (Table 2).

Table 1 Clinical characteristics and hearing loss in patients with rheumatoid arthritis

Parameter		RA without HL		RA with HL		RA Total	
Sex	Female	29	(76.3 %)	41	(85.4 %)	70	(80.5 %)
	Male	9	(23.7 %)	8	(14.6 %)	17	(19.5 %)
Age (y)	41–50	7	(18.4 %)	8	(6.3 %)	15	(17.2 %)
	51–60	18	(47.4 %)	21	(42.9 %)	41	(47.1 %)
	61–70	10	(26.3 %)	13	(32.6 %)	24	(27.6 %)
	>70	3	(7.9 %)	4	(8.2 %)	7	(8.1 %)
Disease duration (y)	0–10	23	(60.5 %)	17	(34.7 %)	40	(46.0 %)
	11–20	11	(30.0 %)	25	(51.0 %)	36	(41.4 %)
	21–30	3	(7.9 %)	6	(12.3 %)	9	(10.3 %)
	>30	1	(2.6 %)	1	(2.0 %)	2	(2.3 %)
DAS 28 Se	<5.1	7	(18.4 %)	3	(6.1 %)	10	(11.5 %)
	>5.1	31	(81.6 %)	46	(93.9 %)	77	(88.5 %)
HAQ	<1.0	2	(5.3 %)	1	(2.0 %)	3	(3.4 %)
	1,125–2,0	20	(52.6 %)	22	(44.9 %)	42	(48.3 %)
	>2.125	16	(42.1 %)	26	(53.1 %)	42	(48.3 %)

RA = rheumatoid arthritis, HL = hearing loss, DAS 28Se = Disease Activity Score, HAQ = Health Assessment Questionnaire

Table 2 Results of different treatment for hearing loss in rheumatoid arthritis judged by audiometry and subjective assessment of patients

Treatment		No	Audiometry				Subjective assessment			
	Initial HL		Improved		Unchanged		Improved		Unchanged	
Cortico per os	20–30 dB	17	13	(76.5 %)	4	(23.5 %)	10	(58.8 %)	7	(41.2 %)
	31–40 dB	16	9	(56.3 %)	7	(43.7 %)	6	(37.5 %)	10	(62.5 %)
	>40 dB	5	1	(20.0 %)	4	(80.0 %)	0	(0.0 %)	5	(100 %)
	All cases	38	23	(60.5%)	14	(36.8%)	16	(42.1 %)	22	(57.9 %)
Cortico intratymp	20–30 dB	1	1	(100 %)	0	(0.0 %)	1	(100 %)	0	(0.0 %)
	31–40 dB	5	4	(80.0 %)	1	(20.0 %)	3	(60.0 %)	2	(40.0 %)
	>40 dB	5	2	(40.0 %)	3	(60.0 %)	1	(20.0 %)	4	(80.0 %)
	All cases	11	7	(63.6 %)	4	(36.4 %)	5	(45.5 %)	6	(54.5 %)
MTX	20–30 dB	2	0	(0.0 %)	2	(100 %)	0	(0.0 %)	2	(100 %)
	31–40 dB	4	1	(25.0 %)	3	(75.0 %)	0	(0.0 %)	4	(100 %)
	>40 dB	3	0	(0.0 %)	3	(100 %)	0	(0.0 %)	3	(100 %)
	All cases	9	1	(11.1 %)	8	(88.9 %)	0	(0.0 %)	9	(100 %)

MTX = methotrexate, HL = hearing loss

Table 3 Improvement of hearing in dB after different treatment modalities and after follow-up for hearing loss in rheumatoid arthritis across frequencies.

Treatment		125 Hz	250 Hz	500 Hz	1 KHz	2 Hz	4 KHz	6 KHz	8 KHz
Cortico per os	Before	21.4±3.2	21.4±3.3	21.4±3.2	30.6±5.1	36.5±7.1	42.5±4.7	48.6±7.1	51.3±8.5
	After	19.4±4.2	21.4±5.1	21.4±5.1	26.4±3.2	30.6±5.1	30.6±5.1	40.3±4.4	42.5±4.7
	Δ dB	2.0	0.0	0.0	4.2	5.9*	11.9*	8.3*	8.8*
	Follow	19.9±5.1	22.2±4.8	20.7±6.1	28.3±4.1	32.7±6.1	32.5±4.8	43.2±5.6	46.2±5.5
	Δ dB	1.5	–1.0	0.7	1.3	3.2	10.0*	5.4*	5.1*
Cortico intratymp	Before	23.8±3.4	23.8±3.4	23.8±3.4	40.6±5.9	40.6±5.9	50.1±6.6	54.2±7.9	55.8±9.9
	After	20.5±4.9	20.5±4.9	23.8±3.4	36.5±7.1	36.5±7.1	38.7±8.2	38.7±8.2	44.6±9.7
	Δ dB	3.3	3.3	0.0	4.1	4.1	11.4*	15.5*	11.2*
	Follow	21.7±6.3	24.2±6.1	22.4±5.1	37.2±8.1	39.7±6.2	40.2±9.9	35.7±7.6	46.9±9.3
	Δ dB	2.1	–0.6	–0.6	3.4	0.9	9.9*	18.5*	8.9*
MTX	Before	28.8±5.5	25.6±5.9	25.6±5.9	30.3±9.7	39.5±7.2	55.1±8.5	55.1±8.5	54.8±7.2
	After	28.2±3.4	28.8±3.4	25.6±5.1	25.6±5.1	35.6±7.3	45.7±9.8	51.1±8.0	54.8±9.2
	Δ dB	–0.6	–3.2	0.0	4.7	4.1	9.4*	4.0	0.0
	Follow	28.8±5.5	26.3±7.1	25.6±5.9	30.7±8.3	40.2±8.6	45.7±9.9	52.6±9.1	55.3±8.7
	Δ dB	0.0.	–0.7	0.0	–0.4	–0.7	5.4	2.5	-0.5

MTX = methotrexate, * = significant

Subjective assessment of patients concerning their hearing was comparable to audiometric data, but overall estimation of treatment improvement was lower than found on tonal audiometry (42.9% for subjective versus 61.2% improvement for audiometry) (Table 2).

Discussion

Hearing loss in patients with rheumatoid arthritis (RA) is differently reported, usually affecting two thirds of them, but the values are ranging from 25.2% to 72.2%. Hearing loss in RA was significantly worse for 200, 500, and 6000 Hz [2,3,8]. There are also reports that found no significant hearing loss in RA compared to control group with matched age and gender [9].

Sensorineural hearing loss in RA is attributed to vasculitis caused by immune complexes inside inner ear, antibodies against inner ear, and ototoxic effects of drugs used for treatment of RA [10].

Transient evoked otoacoustic emissions in RA were found to be lower than in controls. Distortion product of otoacoustic emissions in RA patients were similar to control [9,11]. Acoustic reflexes were with normal thresholds, but with occasional prolonged latency. Besides increased hearing threshold, patients with RA have higher air-bone gaps, and increased wave I latency on ABRs [12,13].

Conductive hearing loss was less frequently reported in patients with RA, ranging from 4% to 17.1% [1,3]. The supposed mechanisms were hypermobility of conductive middle ear mechanism, or discontinuity of ossicular chain. Tympanometric studies did not find any important changes [1,14].

The incudo-malleolar and incudo-stapedial joints are true diarthroses, and may be subjected to the same

rheumatoid lesions. Scanning electron microscopic study of auditory ossicles from patients with RA confirmed significantly higher incidence of surface and articular changes. Longer duration of disease caused more intense ossicular alterations [15].

Hearing was worse in older patients, with longer duration of disease, with active disease, and RA nodules. Decrease of hearing threshold significantly correlated to the duration of RA, with all frequencies affected after 15 years of disease [10]. The results were opposite in other studies [16]. For example, no correlation between hearing loss and age, sex, disease duration, disease characteristics, and therapy were documented [7,9,28].

There is no clear association between the activity of RA and hearing loss [1]. The increase of sedimentation rate, interleukin 6, metalloproteinase 3 correlated well, contrary to RF [3].

The recovery of hearing loss in persons with RA was not sufficiently studied. A positive result of Western Blot predicted functional recovery of hearing in patients with RA [3].

Steroid treatment of autoimmune ear diseases produced significantly better results in cases without systemic diseases (excellent results 33% versus 25%) [17,18]. Glucocorticoids can reverse hearing loss; however, their important side effects prevent long term treatment in chronic diseases. Experimental studies in C3. MRL-Fas(lpr) autoimmune mice treated with corticoids confirmed better hearing threshold, and reduced anti-nuclear antibodies and immune complexes 19].

Methotrexate is considered as a gold standard for patients with RA, mainly because of its tolerability, efficacy, and ease of use. Methotrexate treatment was used for immune mediated cochleovestibular diseases (mean duration 12.9 months) and it improved hearing in 69.6% [20,21]. Other studies concluded that methotrexate in low doses (7.5 to 20 mg/wk) improved hearing and balance in autoimmune inner ear disease (AIED). However, later randomized, double-blind, placebo controlled trial reported that methotrexate was not effective in maintaining the hearing improvement achieved with pronisone therapy in patients with AIED [7].

Some other therapeutical modalities for hearing loss in RA were proposed and tested. TNFalpha inhibitor in combination with methotrexate was used in autoimmune sensorineural hearing loss [22]. Etanercept is a powerful antagonist of TNF that binds to and inactivates the cytokines. However, substantial efficacy of etanercept has not been confirmed for improvement of hearing in patients with immune mediated cochlovestibular disorders [23]. Vasodilatators and antioxidants were also proposed for treatment of hearing loss in RA [13]. Successful use of adalimumab was verified for RA with sensorineural hearing loss [24].

The highest levels of steroids in perilymph were found after intratympanic injection. Also, limited permeability of the blood-labyrinth barrier prevents systemic absorption of applied corticosteroids [25,26].

There were no significant differences concerning age and sex in RA patients with and without hearing loss. Also, age-matched control group eliminated the effect of possible associated presbyacousis. More importantly, duration of disease, DAS and HAQ scales indicated higher incidence of hearing affection, but the differences were not significant. So, we need more specific tests to predict inner ear changes in patients with RA [27–30].

According to the results of this study steroids resulted in hearing improvement in approximately 60.0%, both for peroral and intratympanic application. Contrary to this, methotrexate had virtually no influence of hearing. The disadvantage of steroid treatment is limited time of use. Also, the prediction of success or failure of treatment is difficult. Thus, initial peroral steroid therapy is advisable in RA with hearing loss. This is also acceptable for the patients. The positive outcome of such option can be further treated with steroids intrtympanically. Though intratympanic use of steroids was reported in references, there are no references for application for hearing loss in RA [25,26].

The slight discordance found in this study between patient's assessment and audiometric findings after the therapy is very important, since the patients must use medication and tolerate its side effects. They usually report less favorable results of treatment than indicated by audiometric tests.

New studies should be undertaken to compare different options of treatment of hearing loss in RA.

Conclusion

Hearing loss was present in 56.3% of the patients with rheumatoid arthritis, and it was predominantly sensorineural. Duration of disease and clinical activity of rheumatoid arthritis did not correlate to the incidence of hearing loss. Corticosteroid therapy, either peroral or intratympanic resulted in hearing improvement in about 60.0% of these patients. Results of methotrexate treatment of hearing loss in rheumatoid arthritis were unsatisfactory. Intratympanic steroids are safe for prolonged treatment of hearing loss in RA. Audiometric tests are recommended in order to control hearing in rheumatoid arthritis, and analyze the effect of proposed therapeutic procedures.

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Original Article

STRESS, D -TYPE PERSONALITY AND CORONARY DISEASE

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Abstract. *Stress is a non-specific state of a body caused by stressors. Stressor is a stimulus which, with its activity, produces a certain response which may be: physiological, emotional, cognitive and behavioural. The purpose of the research was to determine the connection and differences between personality traits (negative affectivity, social inhibition and psychosomatic inclination) of healthy people and patients with coronary disease, as well as their role in the development of the disease. The research was conducted on the sample of 43 respondents, with and without a coronary disease. It was found that there is a link between negative affectivity and social inhibition so that these two traits can be observable with part of the sample with a coronary disease. Dimensions of A type behaviour (achievement motivation), although it cannot be claimed for sure, have a significant role in the development of a coronary disease, that is, they are more noticeable. This was confirmed by our initial assumption, where this dimension is more dominant with women who have coronary problems.*

Key words: *A type behaviour, D type personality, coronary disease.*

Introduction

This research is a small contribution to the understanding of the connection between personality and the development of a coronary disease. We gathered the literature about this problem so we could start to present it:

Stress, stressor, concept and definition

Stress is a non-specific state of body caused by stressors (an activity of physical and social surroundings). It can be constructive and pleasant, such that, as a rule, does not lead to the organic defects, in which case we speak about EUSTRESS. Stress like that is affection, a reward or success. The other type of stress causes morpho-functional disorganization and organic damages and is called DESTRESS [1].From the viewpoint of the Psychology of learning, stress (stimulus) affects body, O (organic, internal variable) would be changes inside the body caused by stressors, R (reaction) would be a reaction to stress. After a great many exposures to stress, a tendency to react stressfully is developed. The change of stressors, or the meaning of a stressor, would help the neutralization of a stimulus (stressor), the change of reaction to stress or the elimination of the stressful reaction. We react to a stimulus only when it has a meaning for us (neo, neobehaviorism) [2].

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Stressors and the division of stressors

A stressor can be a stimulus of physical, social or psychological nature, which disturbs a healthy functioning of an individual [1].

1. Physical stressors: noise, vibration, physical exhaustion, magnetic effects, ionizing radiation, high and low temperature, humidity, weightlessness, electromagnetic radiation.
2. Chemical stressors: stressors of chemical nature which lead to poisoning.
3. Psychic stressors: loss of a loved one, divorce, moving house, job loss, wedding, pregnancy and childbirth, jail, retirement, financial problems, aging, change of weight due to aging.

D type personality

D type personality, called distressed type, is characterized by two traits, negative affectivity and social inhibition [1]. Negative affectivity is manifested by increased negative distress, and social inhibition represents a tendency towards inhibition and the expression of negative emotions in a social contact. D type persons are worried, of pessimistic perspective on the world, they are tense and unhappy. They are easily irritated and rarely have a positive attitude to life. They feel uncomfortable with unknown people and have few social connections [1]. They can be depressed, chronically tense, tired, exhausted. They have an increased activity of the sympathetic nervous system, the tendency to blood clotting and show signs of inflammatory processes [1]. Depression is most often associated with the variability of heart rate, increased heart irritability to psychological stress and higher ten-

dency to blood clotting. D type personality has huge inclinations to developing cardiovascular disorders. There is no difference in systolic and diastolic blood pressure between the D type and other types, but D type heart problems are caused by psychic stress [3,4].

Coronary disease

Myocardial heart attack – caused by thrombosis of lumen of one or more coronary blood vessels, which occurs on the ground of atherosclerotic plaques destabilization. This results in a necrosis of myocardium due to the reduction of myocardial blood flow [5,6].

Coronary vasospasm – antiplatelet therapy (aspirin, blockers of platelet receptors P2Y12) and calcium antagonist. Do not administer beta blockers since they provoke vasospasm [7]. Coronary vasospasm may have a significant role in the development of **Atherosclerotic lesions** [8].

Aortic stenosis – is a narrowing of an aortic valve, with a consequential obstruction which occurs during pumping out of blood from the left ventricle. It includes real stenosis, subvalvular and supravalvular stenosis [9].

The heart as the organ of neurosis

The conversion of symptoms into organs is characteristic of those people who retain their emotions in their bodies. Blushing, palpitations and shivering are reactions to specific eustress or distress emotions if we perceive an emotion as an O (organic) variable to a stimulus, and a stimulus can be an event. Arterial hypertension is characterized by a strong, instinctive tension which results from a strong tendency towards aggression as well as from a tendency to free oneself from that aggression. One appears calm on the outside, while not giving vent to one's impulses. Physiological research should provide some additional explanations for hormonal perturbations which cause arterial hypertension. We live in an aggressive world so this happens to people who believe or who have been taught that aggression is a bad thing [10]. Cytokines and other humoral mediators of inflammation activate the neurotransmitter systems which respond to stress. The sympathetic part of the autonomous nervous system is responsible for the regulation of the blood flow. Asthma, eczema and functional vascular diseases are caused by acute stress [11].

The goal of research was to establish the connection between type A behaviour and type D personality and coronary disease.

Material and Methods

Sample

The sample included 25 examinees with coronary disease (10 women and 15 men). There were 18 examinees without coronary problems (10 women and 8 men). Total sample included 43 examinees (20 women and 23 men). The examination took place in a room without noise in collaboration with a physician in the Life impulse clinic and in the coronary department of the General Hospital in Niš.

The instruments

The adaptation of Jenkin's Activity Scale for the evaluation of the type A behaviour consists of 12 supposed items of Likert's type and it is intended for the evaluation of the two components of behavioural style: the aspiration towards achievement and impatience - irritability. The aspiration towards achievement relates to activity, ambition and the degree of how serious a person takes his/her job, whereas impatience – irritability involves intolerance, anger, hostility, as well as the preoccupation with the lack of time [12].

The scale for evaluation of the type D personality consists of 14 items through which Negative affective attachment and Social inhibition are put into operation. Negative affectivity implies a stable tendency to experience sadness, depression, anxiety whereas social inhibition pertains to the inhibition in expressing the aforementioned emotions in social contacts. The responses can be placed on a five-level Likert's scale [13]. According to the test for measuring the efficiency of the system for the regulation and control of organic functions, **HI from the bacterium KON-6,** a tendency towards pshychosomatic mental disorders, is put into operation. HI regulator is formed through the joined action of subcortical centres for the regulation of the organic which are mostly placed within the region of the hypothalamus and their subordinate cortical centres for regulation and control. The disorder of this regulator causes the disorder of the basic systems of organs: cardiovascular, respiratory, gastrointestinal and urogenital as well as the functional disorders of the sensor and motor systems and the secondary creation of the hypochondriac reaction system in reaction to these basic organic functions [14].

Results

Table 1 presents mean values, standard deviation and standard errors of D-type and A-type behaviour, dimensional values of examinees with coronary disease and healthy examinees. Table 2 shows mean values, standard deviation and standard error means of dimensional values of D-type personality for male and female examinees, separately for those with and without coronary artery disease.

H0 – There is statistically significant difference in dimensional values of D-type personality and A-type behavior between examinees with coronary disease and healthy examinees, regardless of their gender.

Statistically significant difference of aspiration for achievement, an A-type behavior characteristic, is more pronounced in diseased ($t = -2.682$, $p = 0.10$) examinees. A-type behavior characteristics are more pronounced in diseased examinees. There is no statistically significant difference of D-type characteristics for the sample group.

It can be assumed that responsibility regarding life and profession is higher for diseased examinees.

This hypothesis is partially confirmed. Further hypotheses are made to determine the extent of characteristics for healthy and diseased examinees with respect to gender.

H1 – There is statistically significant difference of D-type personality between male examinees with coronary disease and healthy male examinees. Statistically higher dimensional values are found n negative affection dimensional values of D-type personality among diseased male examinees than on the same values for healthy male examinees ($t = 2.748$, $p < 0.05$). Hypothesis is confirmed.

It is assumed that dimension values of D-type, for the whole sample group, are more pronounced in diseased male examinees, regardless that this is not confirmed by H0. Diseased males make social contacts harder than healthy males and have stronger unpleasant emotions.

H2 – There is statistically significant difference of A-type behavior between male examinees with coronary disease and healthy male examinees. No statistically significant differences were found on dimensional values of A-type behavior between diseased male examinees and healthy male examinees. Higher statistically significant values in tendency towards psychosomatics are found in diseased male examinees than in healthy male examinees ($t = 2.284$, $p < 0.05$). This hypothesis is rejected. H0 had shown statistically significant difference in a dimension of A-type. Diseased males have more difficulties with conflicts and have tendencies towards physiological reactions when under stress.

H3 – There is statistically significant difference of D-type personality between female examinees with coronary disease and healthy female examinees. No statistically significant differences were found on

Table 1 Mean values, standard deviation and standard errors of D-type and A-type behaviour dimensional values of examinees with coronary disease and healthy examinees

	Coronary problems	N	Mean	Std. Deviation	Std. Error Mean
Negative feelings	Yes	25	12.5200	6.50718	1.30144
	No	18	10.0556	8.05476	1.89852
Social inhibition	Yes	25	9.5200	4.62889	0.92578
	No	18	9.6111	4.96030	1.16915
Aspiration toward achievement	Yes	25	23.7200	5.48878	1.09776
	No	18	27.5556	3.01413	0.71044
Impatience-irritability	Yes	25	15.8800	3.50381	0.70076
	No	18	16.6667	6.24971	1.47307

Table 2 Mean values, standard deviation and standard error means of dimensional values of D -type personality for male and female examinees

	Coronary problems	N	Mean	Std. Deviation	Std. Error Mean
Males					
Negative feelings	Yes	15	13.6667	8.08585	2.08776
	No	8	5.3750	3.42000	1.20915
Social inhibition	Yes	15	10.5333	4.65781	1.20264
	No	8	7.3750	4.80885	1.70018
Aspiration toward achievement	Yes	15	17.0000	3.85450	.99523
	No	8	14.2500	6.60627	2.33567
Impatience-irritability	Yes	15	24.0667	5.56092	1.43582
	No	8	28.0000	2.92770	1.03510
Tendency towards psychosomatics	Yes	15	63.4000	24.85903	6.41857
	No	8	42.1250	11.05102	3.90712
Females					
Negative feelings	Yes	10	10.8000	2.39444	0.75719
	No	10	13.8000	8.85438	2.80000
Social inhibition	Yes	10	8.0000	4.37163	1.38243
	No	10	11.4000	4.52647	1.43139
Aspiration toward achievement	Yes	10	23.2000	5.63323	1.78139
	No	10	27.2000	3.19026	1.00885
Impatience-irritability	Yes	10	14.2000	2.09762	0.66332
	No	10	18.6000	5.52167	1.74611
Tendency towards psychosomatics	Yes	10	59.5000	12.96362	4.09946
	No	10	64.9000	16.25115	5.13907

dimensional values of D-type personality between diseased female examinees and healthy female examinees. This hypothesis is rejected.

H4 – There is statistically significant difference of A-type behavior between female examinees with coronary disease and healthy female examinees. Lower statistically significant values on impatience-irritability characteristic of A-type behavior are found in diseased female examinees than in healthy female examinees ($t = 2.356$, $p < 0.05$). This hypothesis is confirmed. Sensitivity in certain life situations is more pronounced in diseased females.

H5 – There is statistically significant difference of D-type personality dimensions between healthy male examinees and healthy female examinees. No statistically significant values have been found. This hypothesis is rejected.

H6 – There is statistically significant difference of A-type behavior dimensions between male examinees and female examinees who suffer from coronary disease. No statistically significant values have been found. H0 could point out for a statistically significant difference of A-type dimensions for diseased and healthy females, as is confirmed by H3.

H7 – There is statistically significant difference of A-type behavior dimensions between healthy male examinees and healthy female examinees. No statistically significant values have been found. This hypothesis is rejected.

H8 – There is statistically significant difference of A-type behavior dimensions between male examinees and female examinees who suffer from coronary disease. Lower statistical significance values are found on impatience-irritability of A-type behavior characteristics for female examinees suffering from coronary disease than for male examinees suffering from coronary disease ($t = -2.090$, $p < 0.05$).

This hypothesis is confirmed.

H9 – There is statistically significant correlation of A-type and D-type characteristics for a whole sample group. Both D-type personality dimensions correlate with impatience-irritability of A-type behavior dimension. There is a higher correlation with negative affectivity (rho = 0.534, $p < 0.001$) than with social inhibition (rho = 0.379, $p < 0.05$). Two dimensions of D-type personality correlate significantly between each other (rho = 0.568, $p < 0.001$).

H10 – There is statistically significant correlation of A-type and D-type characteristics for healthy examinees. There is statistically significant correlation between negative affectivity and social inhibition, both dimensions of D-type personality (rho = 0.775, $p < 0.001$). There is statistically significant correlation between impatience-irritability, characteristic of A-type behavior, and negative affectivity, characteristic of D-type personality (rho = 0.714, $p = 0.001$). Unpleasant emotions can often be caused by our attitude towards life and situations we are in.

H11 – There is statistically significant correlation of A-type and D-type characteristics for examinees with coronary disease. There is statistically significant correlation between negative affectivity and social inhibition, both dimensions of D-type personality (rho = 0.396, $p < 0.05$).

H12- There is statistically significant correlation of A-type and D-type characteristics and tendency towards psychosomatics for the whole sample. Statistically significant correlation between tendency towards psychosomatics, negative affectivity (rho = 0.745, $p < 0.001$) and social inhibition (rho = 0.424, $p = 0.005$) (characteristics of D-type personality) and impatience-irritability (rho = 0.381, $p < 0.05$) (characteristic of A-type behavior) is found. Physiological reactions to stress can often be provoked by psychological and social components.

H13 – There is statistically significant correlation between A-type and D-type characteristics and tendency towards psychosomatics for healthy examinees. Statistically significant correlation between tendency towards psychosomatics, negative affectivity (rho = 0.843, $p < 0.001$) and social inhibition (rho = 0.650, $p < 0.01$) (characteristics of D-type personality) and impatience-irritability (rho = 0.732, $p = 0.001$) (characteristic of A-type behavior) is found. Physiological state of the organism is very much conditioned by psychological and social life components. Our attitudes, optimism, pessimism, falling back, fight, communication, conflict could determinate physiological basis of psychic life.

H14 – There is statistically significant correlation between A-type and D-type characteristics and tendency towards psychosomatics for healthy examinees. Correlation between tendency towards psychosomatics and negative affectivity (rho = 0.676, $p < 0.001$) for diseased examinees is found. Physiological response to unpleasant emotions is in correlation.

Discussion

Our results showed that examinees with type A personality had more prominent tendency to coronary disease. This finding is interesting since this work was about type D personality and coronary disease. Aspiration for achievement (motives for success) was dominant in results and this motive is a part of type A personality. We cannot generalise to include the entire population with these results but we can say that some type A characteristics are correlated with coronary disease.

We found similar results with Danollet et al., that D type of personality was not the important cause for coronary disease [13,15]. It was not found in these researches that blood pressure was in correlation with D type personality. Therefore, someone who has blood pressure does not necessarily have a D type personality. According to Spence et al., A type of personality has a tendency towards a coronary disease due to more prominent motives for success and self-actualisation. People with this type of personality think about life and job very seriously. D type personality has problems in social relationships [1].

Individuals with Type A personality are hard-driving, competitive, aggressive and hurried. Type A behaviour is a risk factor for the development of coronary heart disease (CHD). However, there have been conflicting results in the literature primarily because of differences in methods and measurements of Type A behaviour. As a result, researchers have focused on subcomponents of the Type A behaviour pattern, particularly hostility and anger, which are more reliable predictors of CHD [16].

Type D personality was proposed by the psychologist Denollet in 1996 and was originally identified after observations of patients with CHD [17]. Type D personality is characterized by two personality traits: negative affectivity and social inhibition [13]. The synergistic effect of negative affectivity and social inhibition involves a higher risk of several emotional and social difficulties, such as depression, anxiety, a low level of subjective well-being, lack of social support and low quality of life [18]. Type D personality was based on the knowledge that psychosocial factors are associated with cardiovascular outcomes [19], and negative emotions and social inhibitions have been given special attention [20]. Type D personality has been associated with a variety of emotional and social difficulties, as well as increased morbidity and mortality in patients with established cardiovascular disease (CVD) [21]. Type D personality substantially affects the way in which patients with myocardial infarction (MI) perceive the availability of social support from different sources, including family, friends and others. Type D personality also plays a clinically relevant role in psychological health outcomes, as Type D patients report significantly higher levels of anxiety, depressive mood, perceived psychophysical stress, interpersonal difficulties, social anxiety and diminished psychophysical well-being and quality of life [22].

Type D personality had its origin in the cardiovascular context. In 1996, Denollet et al. proposed that Type D personality was an independent predictor of long-term mortality in patients with CHD, independently of traditional biomedical factors [17]. A subsequent study demonstrated that Type D personality patients with CHD had a fourfold increased risk of major cardiac events over 5 years, independent of disease severity [23]. Another study found that Type D personality predicted mortality and recurrent MI in patients with acute MI after controlling for both disease and depression severity [24]. Type D personality has also been studied regarding the risk of CHD in populations without clinically established CHD [25]. Findings across studies are inconsistent, as several studies have failed to find any associations between Type D personality and cardiovascular outcomes [26], and provided ambiguous evidence regarding whether Type D personality can predict CHD. Denollet et al. made the assumption that Type D personality is a significant predictor of cardiovascular events after adjustment for depressive symptoms because of the fact that the subcomponents of Type D personality, negative affectivity and social inhibition, activate different brain parts and result in different cortisol responses after exposure to social threats [27]. Research on the relationship between Type D personality and laboratory indices of cardiovascular health indicates that socially inhibited men have heightened blood pressure reactivity and that negative affectivity is related to a dampened heart-rate change during stress. Both Type D dimensions (negative affectivity and social inhibition) were associated with greater cortisol reactivity to stress [28,29]. Williams et al. compared males with and without Type D personality and found that the former group exhibited a significantly higher cardiac output during a stress condition. However, there was no relationship between Type D personality and cardiovascular reactivity in females [30]. It has been reported that 13-24% of individuals in a healthy adult population can be classified as a Type D personality. Kupper et al. found that the prevalence of Type D personality was higher among women. Moreover, females had significantly higher negative affectivity scores than males but males and females did not differ in their mean social inhibition scores. Age was not a significant covariate for either negative affectivity or social inhibition [31].

Further longitudinal studies are needed to clarify whether Type D personality and its traits remain stable over time and after life-changing events. Moreover, the influence, through disease promoting mechanisms, of Type D personality on health status in later life needs to be investigated.

Conclusion

This research has shown that personality dimensions have a significant part in a development of coronary disease. Physiological basis of psychic life is reflected upon man's constant struggle to preserve his health by changing the way of observing the position he is in, whether it is about social or motivational (inner) sphere of man's life. Our attitudes toward situations we are in contribute to better physical health. Although conclusion like this is not the goal of this paper, its goal is pure research, it can be said that some branches of psychology have offered ways of overcoming stress. Behaviorism can talk about changes of cognitive scheme towards stressors, strategies of overcoming stress, and psychotherapy affecting the motivational sphere of social life can talk about ways of resolving inner conflicts, better and more tolerant self-knowledge, self-support and choices, self-trust, hope, ability to love and understand, needs and self-actualization. Body psychotherapy can offer body relief from sustained emotions. Goal and purpose are also very significant things in human life.

At the end, it can be concluded that personality dimensions take a large part in the development of coronary disease. Higher statistically significant difference is found on A-type behavior dimension, which means that tendency towards achievement is more pronounced in examinees with coronary problems than in the healthy ones.

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