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VITAMIN D DEFICIENCY AND ITS IMPORTANCE - A GLOBAL PROBLEM OF TODAY, REALISTIC OR NOT?

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DEFICIJENCIJA VITAMINA D I NJEN ZNAČAJ - GLOBALNI PROBLEM DANAŠNJICE, REALNO ILI NE?

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ABSTRACT

Vitamin D, also known as the "sun vitamin" in the literature, has been examined for many years and still arouses researchers' interest due to the pleiotropic effects achieved in the human body. Because of the influence on mineral homeostasis, the initially observed effects of vitamin D on the prevention and treatment of rickets, have now been extended to a large number of diseases with different aetiologies such as cardiovascular, autoimmune, endocrine, infectious, neurological, malignant and other diseases. Due to the large number of experimental studies in animals and humans, we have exact information about the role of vitamin D in many of these conditions. Reaching an adequate level of 25(OH)D in the human body is a basic requirement for the realization of these effects; 25(OH)D is a metabolic product that reflects the vitamin D status but that does not have any biological activity. The biological activities of vitamin D can occur only after the formation of a second metabolic product, 1,25(OH)$_2$D, in the kidneys. The three main sources of acquiring vitamin D are through food, skin and supplementation. Food is not a rich source of vitamin D; it is clear that the most important influences to achieve an optimal vitamin D status in the human body are vitamin D synthesis at the skin and adequate supplementation intake. An alarming fact is that vitamin D deficiency is detected in an increasing number of people from one day to another in the general world population and that this condition has pandemic dimensions. Introducing the beneficial effects and sources of vitamin D to the general population and to medical experts with adequate supplementation regime can decrease the number of people who are vitamin D deficient.

Keywords: *vitamin D, physiological effects, pharmacokinetics characteristics, vitamin D deficiency*

SAŽETAK

Vitamin D, u literaturi poznat kao "vitamin sunca", iako je već dugo godina ispitivan i dalje pobuđuje interesovanje kod naučnika usled plejade efekata koje ostvaruje u humanom organizmu. Prvobitno dokazani efekti vitamina D u prevenciji i terapiji rahitisa usled uticaja na postizanje mineralne homeostaze danas su prošireni na veliki broj bolesti različite etiologije kao što su kardiovaskularna, autoimunska, endokrinološka, infektivna, neurološka, maligna i druga oboljenja. Zahvaljujući velikom broju ekperimentalnih studija kako na animalnom tako i na humanom modelu danas se za veliki broj navedenih oboljenja zna tačan mehanizam dejstva vitamina D. Za ispoljavanje navedenih efekata osnovni preduslov je dostizanje optimalnog nivoa 25(OH)D, prvog metaboličkog produkta vitamina D, koji iako služi kao osnovni parameter za određivanje statusa vitamina D kod ljudi ne predstalja biološki aktivnu formu vitamina D. Da bi se ispoljili navedeni biološki efekti vitamina D neophodno je formiranje sekundarnog metaboličkog produkta 1,25(OH)$_2$D. Tri osnovna izvora vitamina D su koža, hrana i suplementi. Kako hrana sadrži vitamin D u mali količinama, jasno je da na adekvatan status vitamina D najviše utiče sinteza vitamina D u koži i uzimanje određene doze suplemenata. Alarmirajući podatak je da iz dana u dan raste broj svetske populacije kod koje je utvrđeno postojanje hipovitaminoze D i da ova pojava trenutno ima pandemijske razmere. Upoznavanjem opšte javnosti sa korisnim efektima vitamina D i njegovim izvorima a stručne javnosti sa adekvatnim suplementacionim dozama može se uticati na smanjenje broja deficijentnih osoba kako u našoj zemlji tako i svetu.

Ključne reči: *vitamin D, fiziološki efekti, farkakokinetke karakteristike, deficijencija vitamina D*

ABBREVIATIONS

cAMP- cyclic adenosine monophosphate
CRE- cyclic adenosine monophosphate response element
FGF23- fibroblast-like growth factor-23
PTH- parathyroid hormone
TGFβ1- transforming growth factor β1
VDBP- vitamin D binding protein

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INTRODUCTION

Vitamin D, a lipophilic vitamin, has been examined since the 20th century and continues to be a focus of scientific research due to it pleiotropic effects in the human body. The roles of vitamin D have been observed in a large number of epidemiological and experimental studies that confirmed correlations between vitamin D status and both acute and chronic diseases. The primary recognized effects of vitamin D on mineral homeostasis and consequently on bone health have now expanded to include diseases such as autoimmune, anti-inflammatory, cardiovascular, cancer, diabetes, infectious, psychiatric and others (1-5).

The most important vitamin D discovery from a historical perspective was in 1922, when McCollum, an American biochemist, was performing experimental work on rats with rickets with his team and noted a substance in fish oil that could prevent and treat this bone disease, he called this substance vitamin D. Simultaneously, Huldschinsky observed the benefits of UV radiation in children with rickets. Both events intrigued the scientific community to specify why these events happened and triggered further testing that finally resulted in the precise definition of the chemical structure of vitamin D after several years of work (6, 7).

There are several different structural forms of vitamin D, but two forms are separated from that group according to their physiological importance for the human body; those two forms are vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol). In addition to the differences in chemical structure, they have various origins: ergocalciferol has a plant origin while cholecalciferol has an animal and human origin. According to the results of clinical trials, cholecalciferol exhibits more potent clinical efficiency than ergocalciferol due to its structural and metabolic differences, which can be important for providing recommendations about vitamin D supplementation for some indications (8).

The two cardinal causes of deficiency include insufficient exposure to sunlight and inadequate nutritional intake of vitamin D, but there are also many factors with different mechanisms that lead to this deficiency.

In this review article, we summarize the available scientific accomplishments related to vitamin D and its deficiency and the government guidelines in Europe and other countries.

VITAMIN D SYNTHESIS AND SOURCES

A characteristic of vitamin D that makes it unique among vitamins is that it is derived from exogenous and endogenous sources. A large number of observational and clinical trials have revealed that there are three main sources of vitamin D: the skin, certain foods and supplements. The most important of these three sources for achieving the optimal range of 25(OH)D in the human body is the skin, both in humans and animals (9).

The process of vitamin D synthesis in the skin is multi-step and multifactorial, which makes it significantly difficult to reach an optimal range of 25(OH)D. Two-thirds of the concentration of serum 25(OH)D has an endogenous origin, while the remaining one third is exogenous. Although 25(OH)D, as a first metabolic product, is biologically inactive in contrast to $1,25(OH)_2D$, it serves as a key marker to assess the status of vitamin D in the human body due to pharmacokinetic characteristics such as a half-life from several days to several weeks for this metabolic form, in comparison, the half-life of calcitriol is only a few hours (10).

First information about vitamin D production in the skin dates back to the beginning of the 20th century with evidence on mammalian skin synthesis, and the complete explanation of this process was revealed to the general public in 1980 by Holick and co-workers (11). As the sun is an unavoidable factor in the synthesis of vitamin D, a common synonym for this vitamin in the medical literature is "sunshine vitamin".

Vitamin D skin production begins with skin exposure to UVB radiation in the range of 290 to 315 nm when provitamin D, 7-dehydrocholesterol, from the skin is converted to previtamin D, which undergoes thermal isomerization to vitamin D3. It is interesting that the scientific population, after the chemical identification of vitamin D3 at the beginning of the 20th century, considered that this compound was a direct product of provitamin D. This claim was refuted after a solution of 7-dehydrocholesterol was exposed to UV radiation and yielded an unknown compound that was later classified as previtamin D3 (12). Unlike previtamin D3, vitamin D3 is thermostable and does not undergo further isomerisation in the body. Experimental resources demonstrate that the optimal body temperature for the thermal isomerisation of previtamin D is 37 °C. The only distinction between vitamin D2 and D3 synthesis is in the starting substance (13). An advantage of this multistep reaction is that previtamin D and vitamin D, after prolonged exposure to the sun, absorb UVB radiation and create biologically inactive products such as lumisterol, tachysterol, suprasterol, etc. (1, 4). Due to the aforementioned characteristics, prolonged exposure to sunlight is not related to toxic levels of vitamin D3, this finding is substantiated by the results of many clinical trials (14).

Synthesized vitamin D is transported from the skin via the blood to the liver where the hydroxylation reaction occurs by means of the 25-hydroxylase enzyme and is thereafter transported to the kidneys where the second hydroxylation reaction occurs by the 1-alpha-hydroxylase enzyme. The final product of the second hydroxylation is 1,25-dihydroxyvitamin D, calcitriol, which acts as a hormone. This reaction is firmly controlled by the actions of two hormones, parathyroid hormone and fibroblast-like growth factor-23 (FGF23), which have opposing actions.

Parathyroid hormone up-regulates calcitriol synthesis, and FGF23 down-regulates calcitriol synthesis. In addition to the parathyroid hormone, up regulation is stimulated by low levels of calcium and phosphorous, while down regulation is stimulated by calcitriol itself (15). A precondition for these hydroxylation reactions is the binding of vitamin D to the vitamin D binding protein (VDBP) in the bloodstream, vitamin D cannot otherwise be transported to the liver and kidney due to its liposolubility. Vitamin D production occurs in extra renal tissue such as the parathyroid glands and the prostate, colon, lung, brain and other tissues in which the aforementioned enzymes were confirmed but present in smaller quantities (4).

Some fish, such as herring, sardines, mackerel, and mushrooms, egg yolk, and offal are some types of food that contain vitamin D at specified amounts. Food is a source of vitamin D and can influence the serum vitamin D level in humans, but the list of food that contains this vitamin in certain amounts is scarce. Nutrition cannot offset inadequate endogenous vitamin D production during a specific period of the year, such as during the winter months (16, 17).

As dietary intake of vitamin D may be not adequate, one of the potential ways to achieve a desirable level of vitamin D is supplementation. It is notable that recommendations for supplementation vary from country to country and for different populations. According to the report of the European Food Safety Authority for the adult population, the upper limit for vitamin D supplementation is 100 μg/day instead of the former recommendation of 50 μg/day, while the recommended dosage is in the range of 800- 2000 IU/day (20-50 μg/day) (18). Healthcare practitioners should always keep in mind that dosing should be harmonized with the patient's pathophysiological condition in order to avoid potential intoxication due to overdose. The most common sign of vitamin D intoxication is hypercalcemia. The manifestation of hypercalcemia includes symptoms such as lethargy, nausea, vomiting, dehydration, etc. There are sparse clinical data on toxicity. In addition to hypercalcaemia, other conditions that accompany vitamin D toxicity can include hypercalciuria or nephrocalcinosis (18).

PHYSIOLOGICAL AND PHARMACOKINETIC EFFECTS OF VITAMIN D

In order to understand the role of vitamin D in the human body, it is necessary to understand its physiological and pharmacokinetic effects. Vitamin D exerts autocrine, paracrine and endocrine effects in the body by binding the vitamin D receptors that are located in most tissues and organs. Observations of many experimental studies have shown that after the synthesis of 1,25(OH)D, its biological effect is expressed by binding to nuclear vitamin D receptors, leading to complex effects on specific DNA sequences, which consequently influences the transcription of several hundred genes (4).

PHYSIOLOGICAL EFFECTS OF VITAMIN D

There are a growing number of studies that have emphasised the effects of vitamin D in humans and animals, and this wide range of effects can be divided to skeletal and non-skeletal effects. Skeletal effects of vitamin D have been examined for many years, while non-skeletal effects are being investigated.

Vitamin D plays an essential role in the maintenance of calcium and phosphorus homeostasis, consequently affecting bone formation. Calcium and phosphorous absorption from nutritional sources is poor, only 10-15% of calcium and 60% of phosphorous are absorbed, while at optimal vitamin D concentrations, the absorption of calcium and phosphorous is 30-40% and 80%, respectively (19). During certain periods when there is an increased need for Ca^{2+}, such as during development, pregnancy and lactation, 60-80% of Ca^{2+} in food can be absorbed if the vitamin D status is sufficient; the status is sufficient when there is an the increased concentration of circulating 1,25-dihydroxyvitamin D, which reflects the extent of mineral absorption (1). The role of vitamin D in the prevention of bone diseases was established for different ages. A meta-analysis of the effects and dose dependence of vitamin D with or without calcium supplementation on non-vertebral and hip fractures in older subjects showed a risk reduction of 29% for non-vertebral fractures and 15% for hip fractures. It was also observed that these effects were not calcium dependent, this conclusion was confirmed three years later by Bischoff-Ferrari, who performed a meta-analysis of vitamin D supplementation at a daily dose of 800 IU (10, 20). Conflicting data emerged from studies that analysed the effects of supplementation at higher doses (ranging from 20000 to 50000 IU weekly), which could explain the different respondents' characteristics (21, 22). The results from a cross-sectional study confirmed the existence of correlations between calcitriol levels and bone mineral density and fracture incidence; the limit of the concentration of 25(OH)D for a positive correlation ranged from 20 ng/mL to 36 ng/mL depending on the examined population and the geographical position of participants whose mineral density was measured (23). It is noteworthy that vitamin D supplementation in large annual doses could impair bone health and lead to unexpected effects (23). This evidence should prompt all healthcare professionals to search all literature when considering doses for vitamin D supplementation.

The vast number of non-skeletal effects of vitamin D is explained by the presence of the vitamin D receptor (VDR) in most tissues and organs in the body. Another potential theory, in addition to the vitamin D receptor, is that after the 25(OH)D level reaches a value over 30 ng/ml, there is enough substratum for the local production of the active metabolite $1,25(OH)_2D$ in the colon, skin, prostate, lung and other non-renal tissue. It is now known that the aforementioned metabolite has the ability to inhibit cell proliferation and induce terminal differentiation as well as to decode genetic information for several hundred genes (2).

Cancer diseases are one of the leading causes of mortality in the world, according to the World Health Organisation; 19,3 million new cases are anticipated to emerge per year until 2025. There is evidence that vitamin D contributes to these pathological conditions, which underlines the importance of adequate vitamin D supplementation in this patient population (24). Epidemiological research on the influence of sunlight on cancer has been conducted since 1936 in the USA. Different studies such as case control and prospective and retrospective trials have affirmed the connection between vitamin D and 15 different types of cancer where the anticancer mechanism of vitamin D by VDR is associated with the regulation of proliferation, differentiation, apoptosis and angiogenesis in normal and cancerous cells (25, 26). Data from the study that examined risk from breast cancer, one of the most common disorder of this type, observed a risk reduction for almost 58% at 25(OH)D level >38 ng/ml (27).

Vitamin D impacted to cardiovascular disease prevention due to the availability of VDR on the endothelium and vascular smooth muscle and cardiac muscle cells. The anti-atherosclerotic effects of vitamin D included the inhibition of the foam cell formation and smooth cell proliferation, the expression of adhesion molecules on endothelial cells and the release of inflammatory mediators (28). Vitamin D also affects hypertension, one of the most common non-communicable diseases. Hypotensive effects occur due to the inhibition of the renin-angiotensin system in the juxtaglomerular apparatus of the kidneys i.e. due to the down-regulation of renin gene transcription by $1,25(OH)_2D$. The inhibition of renin expression by calcitriol occurs due to the binding to the transcription factor cAMP-CRE- binding protein, which disables renal transcription. The prevention of primary hyperparathyroidism and the regulation of calcium metabolism are complementary mechanism of the hypotensive effect (29, 30). Clinical experience about this hypotensive is controversial and results from meta-analyses that show a negative correlation between serum calcidiol level and blood pressure. The results of a multicentre clinical trial may provide more detailed information (31).

Studies that evaluated the influence of vitamin D on the development and modification of clinical pathways of autoimmune disorders have presented positive results regarding the significance of vitamin D in cytokine production, in inflammation decreasing and the induction of immune cells. It is well known that this vitamin increased the quantity of Th2 lymphocytes and exerted immunoregulatory and anti-inflammatory effects via the induction of dendritic cell proliferation (32, 33). The influence of vitamin D on autoimmune processes has most often been evaluated in disorders such as diabetes, multiple sclerosis, rheumatoid arthritis and Crohn's disease. A randomised, placebo controlled study revealed that participants with multiple sclerosis who take vitamin D at a daily dose of 1000 IU, apart from an increase in calcidiol levels, had a higher level of transforming growth factor (TGF)-β1, which is an anti-inflammatory cytokine. This result was in accordance with observations in a cohort study in which vitamin D3 at a higher daily dose of 20000 IU, influenced the function of $CD4^+$T cells (34, 35).

The presence of the VDR on beta cells in the pancreas, the stimulatory influence of calcitriol on insulin secretion, the decrease in insulin resistance in muscles as well as the reduction in inflammation that occur during insulin resistance are some of the reasons why vitamin D has potential for diabetes prevention (23).

The two most important factors for the involvement of vitamin D in brain development and physiological brain functions are the existence of 1α-hydroxylase and VDR in the human brain. Cognition, Alzheimer's disease, anxiety, and depression are some of the conditions in which the connection between vitamin D and disease pathophysiology have been examined.

It is well known that the activation and inactivation of vitamin D occur in the brain. The hypothalamus and substantia nigra are the two regions of the brain where 1,25-dyhidroxyvitamin D is produced due to the presence of the CYP27B1 enzyme. The positive effects of vitamin D in the brain could be explained by the presence of the vitamin D receptor in most neurons and certain glia and the impact of vitamin D on gene regulation that influences the expression of nerve grow factor and neurotrophin 3 and that affects neuroimmunomodulation processes (36).

The hypothalamus and limbic system are connected with the pathophysiology of depression, and the presence of vitamin D receptors and hydroxylation enzymes in the aforementioned areas may indicate a connection between vitamin D deficiency and this disorder (37). One constraint of this assertion is the presence of divergent results from studies with different methodological and experimental approaches in subjects with anxiety or depression and healthy participants (38, 39).

Autism, one epidemic condition that is diagnosed in childhood, may be correlated with vitamin D deficiency and hypovitaminosis D during the prenatal period or during early childhood. Hypovitaminosis D is recognized as one of the potential risk factors for autism, and this hypothesis is based on evidence from epidemiological and clinical examinations (40). However, research on this topic is preliminary, and more extensive studies should be conducted in the future (41).

PHARMACOKINETICS

Pharmacokinetic processes of vitamin D are well known because of a large number of studies with radiolabelled vitamin D3 in both humans and animals, which can considerably facilitate the clinical application of vitamin D according to the characteristics of an individual.

The proximal part of the small intestine is the location where the majority of vitamin D absorption occurs. An essential factor for this process is the presence of normal bile

acid secretion due to the lipophilic structure of vitamin D. Bile acid is an indispensable factor for the incorporation of nonpolar molecules of vitamin D into the micelles of bile salt, after which it can be absorbed into the liquid phase. Normal stomach and pancreas secretion and diffusion through the liquid layers are additional factors for this pharmacokinetic step. In compliance with the aforementioned factors, the cause of reduced absorption is clear in intestinal diseases such as biliary obstruction, chronic pancreatitis, Crohn's disease, renal insufficiency, etc. (42, 43). The effect of malabsorption syndrome on vitamin D absorption was evaluated by Satia and co-workers, and their results indicate that vitamin D3 absorption was higher in healthy subjects than in patients with malabsorption disease (44).

The following pharmacokinetic step, distribution, begins after the absorption of exogenous vitamin D in the small intestine. Distribution occurs due to the transfer of absorbed vitamin D into the lymphatic system. Vitamin D is then transported to circulation and binds to the vitamin D binding protein after which the resulting complex continues to the liver. Because the vitamin D binding protein is a key substance for distribution, it should be noted that hepatic impairment, nephrotic syndrome and malnutrition negatively impact distribution while pregnancy and estrogen therapy have contradictory effects (45).

The metabolic pathway of vitamin D consists of two hydroxylation reactions that take place in the liver and kidneys in the presence of cytochrome P450 enzymes that function as oxidases, including 25α-hydroxylase, a 27A1 cytochrome P450 isoform, and 1α-hydroxylase, a CYP27B1 isoform. These reactions are crucial for the creation of a physiologically active vitamin D form. Eexperiments have demonstrated that hydroxylation in the liver proceed by first order kinetics and that 75% of the total amount of vitamin D consumed, whether it is exogenous or endogenous, undergoes first pass metabolism through the liver (8). The final outcome of the first hydroxylation reaction in the liver is the formation of 25-hydroxyvitamin D, which is the inactive form. Enzyme 25α-hydroxylase is also detected in the skin, the kidneys and the intestines but in far less quantities. After calcidiol formation, the vitamin binds to the vitamin D binding protein and is carried to the kidneys where it is filtered and reabsorbed in the proximal renal tubules. The available data established the presence of the cell surface receptors megalin and cubulin, which facilitate the endocytosis of calcidiol-VDBP by renal cells and other cells in the human body (46). Renal hydroxylation of calcidiol by 1α-hydroxylase forms calcitriol, a more polar and biologically active product of the second metabolic reaction. Particularities of these two hydroxylation reactions are not only specific to the type of enzyme but also depend on endocrine mechanisms. An interesting observation is that the formation of 25(OH)D is independent of endocrinology and occurs exclusively in response to the concentration of available vitamin D, this attribute can be another reason for the consensus about a key marker for the determination of vitamin D status in individuals (47).

Apart from the hydroxylation enzymes, 24-hydroxylase is important for vitamin D metabolism, and its role is reflected in the catabolism of both vitamin D metabolites. Metabolic pathways of ergocalciferol and cholecalciferol are intertwine, but studies based on the clinical examination of differences after oral ingestion of these two forms have noted the controversial results.

Vitamin D is excreted predominantly via the bile and faeces, and a smaller portion is excreted through the urine (6).

FACTORS RELATED TO VITAMIN D DEFICIENCY

Because the cutaneous production of vitamin D is a crucial step for the generation of an adequate vitamin D level, it is important to examine overarching factors that can influence this stage. These factors involve the season of the year, geography, clothing style, age, gender, skin type, obesity, sun exposure time, using of sunscreen, etc. (48).

Important factors that have an enormous influence on vitamin D3 synthesis are the time of day when skin is exposed to the sun light, the time of the year and the geographical position, i.e. the latitude because these factors determine the solar zenith angle, and it is well known that a smaller solar zenith angle is linked to intensive UV radiation (49). A combination of factors that can create unfavourable conditions for vitamin D production at some period of the year in a country with a latitude below 35^0 can be present during most of the year, while for higher latitudes value can exist during the winter months. Serbia is a country that extends into the Balkan Peninsula, and the geographical position between 41°53′ and 46°11′ impedes vitamin D production from April to October, (50). Bandeira and co-workers has shown that countries with a lower latitude position exhibit a high prevalence of vitamin D deficiency, from 50% to 97% (51).

In order to assess the real picture of deficiency, many studies were performed at different locations worldwide, and these results were also devastating. In the United Kingdom, the prevalence of vitamin D deficiency in the general population was 87,1%, in Germany, the prevalence was 50%, in Spain, the prevalence was 33,9%, and in Italy, the prevalence was 17% (52-55). A report from the International Osteoporosis Foundation about hypovitaminosis D in Europe reported concentrations of 25(OH)D of < 25 nmol/l in 2 to 30% of the adult population and 75% of the geriatric population (56). Alarming data were shown in a cross-sectional study of the adolescent population in ten European cities where hypovitaminosis D was present in 80% of the evaluated population, and almost 40% of individuals exhibited deficiency (35). Medical students were also part of the hypovitaminosis D framework in several countries such as Saudi Arabia, Spain and Serbia (52, 58, 59). A recently conducted cross-sectional study in the

Table 1. Vitamin D status at the Southeast Europe country

Country	Year of the study conduction	Population	Number of participants	Mean 25(OH)D level	Percentage of vitamin D deficiency patients	Season
Bulgaria[61,62]	2014	Adults patients with chronic hepatitis C viral infection	296	50.40 nmol/l	49%	Winter
	2012	Adults	2032	38.75 nmol/l	21,3%	Winter
Croatia[63,64]	2013	Postmenopausal women	194	49.1 nmol/l	29.6%	N.A.
	2013	Adults with acute coronary Syndrome	60	34.9 nmol/l	76%	N.A.
Romania[65]	From 2012 to 2014	Very young and very old	6631	29.95 ng/ml	26.1%	All seasons
Serbia[60]	2012	Young, healthy adults	86	13.26± 4.86 ng/ml	88.37%	Summer

Shumadia region on healthy medical students showed a significant presence of vitamin D deficiency (60). Several reports about the status of this vitamin from studies conducted in Southeast European countries in various population groups are shown in Table 1 (60-65).

Currently, computer models created by researchers have been used to determine the amount of UVB radiation required to achieve an adequate level of vitamin D in individuals according to their skin type and place of residence, but the existence of the above mentioned factors disable the use of these software programs in clinical practice (14).

Society modernization and health education about skin care and harmful sun influence that can be reduced by using cosmetics products with the appropriate sun protection factor (SPF) have led to the occurrence of a heliophobic attitude in both females and males, which has created suitable conditions for vitamin D deficiency (66). An interesting observation from a descriptive study that was carried out in London was that participants were not aware that sunscreen preparation blocked vitamin D synthesis (67).

Human aging leads to a decreased concentration of 7-dehydrocholesterol, which consequently negatively impacts the process of vitamin D synthesis. Study data indicate that the capacity is reduced to almost a quarter after 70 years compared to young adults (49).

Skin type varies from nation to nation, and dark skin requires much more UVB radiation than lighter skin, which is experimentally affirmed due to the large amount of melanin that absorbs UVB light and consequently decreases the availability of vitamin D3 (68).

A number of studies indicate that gender affects levels of 25(OH)D, whereby higher levels are recorded in men; this is scientifically explained by the greater amount of adipose tissue in a woman's body that can sequester endogenously produced vitamin D and can store this vitamin in humans (69). Nevertheless, research that has examined correlations between vitamin D serum levels and body mass index showed opposing results (70).

Apart from fat cells, vitamin D made in excess can be stored in the muscles, liver or skeleton, which prevents the attainment of a toxic dose during supplementation. This knowledge about fat storage in vitamin D created confusion regarding the sudden release of vitamin D deposits in some situations such as weight loss, for example, whether this leads to a toxic dose in the human body. The results from studies have not confirmed this theory until now, and experimental research performed with radiolabelled vitamin D has shown that the adipose storage process is not indefinite and even this process is associated with the half-life of vitamin D in the whole body (71).

VITAMIN D DEFICIENCY

Vitamin D deficiency is a problem that has existed since the early years of the 19th century with the emergence of the migration of populations from rural to urban regions, and this problem still exists all around the world in accordance with technology, modernization and lifestyle changes. All types of individuals are vulnerable to vitamin D deficiency, not only children as was considered earlier. Deficiency in children is associated with rickets, and deficiency in adults and older individuals is associated with osteomalacia, osteoporosis and consequently bone fracture (9).

Evidence from studies shows the significance of raising awareness about the influence of vitamin D deficiency on health status in the general population; 25-hydroxyvitamin D (25(OH)D) levels that represent normal, physiological, values of vitamin D are linked with risk reduction for the above mentioned diseas-

es are in the range of 30 ng/ml up to 50 ng/ml. Values of 25(OH)D below 20 ng/ml (50 nmol/l) indicate vitamin D deficiency. However, the value of vitamin D that would separate hypovitaminosis D from the optimal status was discussed by a large number of scientists in this field, and they agreed on this value (72). The evaluation of the precise 25(OH)D concentration that separates inadequate (deficiency or insufficiency) concentrations from the optimal concentration is most frequently performed by monitoring the concentrations of parathyroid hormone (PTH) and calcidiol, which are negatively correlated to these two parameters. Experimental findings are in favour of this inverse correlation until the level of calcidiol reaches the range of 75 to 100 nmol/l, after which the level of PTH is stabilized to the reference values (73). An additional criterion that was measured for this calculation includes the increase in intestinal calcium absorption. It has been reported that it was only when the calcidiol concentration reached levels of 50-80 nmol/l that there was an increase in absorption from 45% to 65%, which could be of great clinical significance (74). The deficiency level was defined in agreement with the results of a study in which healthy participants received vitamin D2 supplementation at a weekly dose of 50000 IU for eight weeks, which was followed by a decrease in the PTH level of 35% in subjects who had a level of 25(OH)D of less than 20 ng/ml (75). This cut-off value was defined as a deficiency, and values from 21 to 29 ng/ml (50 to 75 nmol/l) were defined as an insufficiency, while values from 30 to 100 ng/ml (75 to 250 nmol/l) were defined as being at an optimal level. Specified levels are in conformance with recommendations from the American Institute of Medicine (76). The adequacy of the proposed cut-off values for vitamin D status are justified with observations from prospective, clinical studies of participants with different pathological entities such as colorectal carcinoma, diabetes mellitus, etc. (77).

Vitamin D intoxication was identified at a 25(OH)D level of >150 ng/ml (1, 2). Hypercalcaemia is one of the adverse effects of vitamin D use, and it occurs due to increased intestinal calcium absorption and decreased renal excretion because of vitamin D supplementation and bone remodelling (77).

The exact number of people in the world with vitamin D deficiency is difficult to determine because of the inconsistency of cut-off values that some laboratories are using despite recommendations. Current epidemiological data indicate that vitamin D deficiency or insufficiency exists in approximately 1 million people, which is an alarming fact (9). Therefore, most developed countries recognize the scope of this problem and are implementing procedures to improve vitamin D status, including the use of food fortification or explicitly defining supplementation doses for specific groups such as children, adolescents, pregnant women, elderly people, patients with chronic diseases, etc.

GUIDELINES

According to the results of clinical studies, the upper limit of the vitamin D dose that has no connection with harmful effects ranges from 50 μg/day (2000 IU/day) to 100 μg/day, which is a valid recommendation in Europe and North America (18).

Consistent with the scientific evidence presented by Holick et al., a supplemental dose of 10000 IU/daily of vitamin D in individuals without adequate sunlight exposure is safe, but the authors recommend a daily dose of 1000-2000 IU of vitamin D; therapeutic doses are larger (9). Moreover, a recommendation by the same author is that requirements for vitamin D can be met in the Caucasian population by exposing 10-15% of the skin area such as the neck, head, arms and legs to sun for 10-15 min daily or 2-3 times weekly from 10 a.m. to 3 p.m. (50). This statement should be taken with some reservation due to the previously mentioned factors that determine the quantity of synthesized vitamin D.

CONCLUSION

Vitamin D deficiency is a key public health issue worldwide that requires comprehensive analyses to construct international guidelines for vitamin D supplementation that will decrease the proportion of adverse effects of vitamin D deficiency. A sedentary lifestyle and heliophobic behaviour should be starting points that need to be eradicated in order to achieve optimal vitamin D levels in individuals.

The available literature suggests contradictory conclusions about vitamin D supplementation that may confer benefits in humans. Furthermore, more systematic reviews to unify and analyse the results and the validity of numerous studies of vitamin D around the world are needed.

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SYNTHESIS, CHARACTERIZATION, AND CYTOTOXICITY OF BINUCLEAR COPPER(II)-COMPLEXES WITH SOME S-ALKENYL DERIVATIVES OF THIOSALICYLIC ACID

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SINTEZA, KARAKTERIZACIJA I CITOTOKSIČNOST BINUKLEARNIH BAKAR(II)-KOMPLEKSA SA NEKIM S-ALKENIL DERIVATIMA TIOSALICILNE KISELINE

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ABSTRACT

*New complexes of copper(II) with S-alkenyl derivatives of thiosalicylic acid (alkenyl = propenyl-(**L1**), isobutenyl-(**L2**)) have been synthesized and characterized by microanalysis, infrared spectra, magnetic measurements, and by NMR spectra. The cytotoxic activity of two newly synthesized precursor S-alkenyl derivatives of thiosalicylic acid were tested using an MTT colorimetric technique on HCT-116 human colon carcinoma cells. The cytotoxic effect of the copper(II)-complexes were higher compared to the cytotoxicity of the corresponding ligand (for concentrations from 31.25 to 250 μM). Copper(II)-complexes showed a slightly lower cytotoxicity compared to cisplatin. Complexes of copper(II) with S-alkenyl derivatives of thiosalicylic acid (at concentrations from 250 to 1000 μM) had a cytotoxic effect on HCT-116 cells compared to cisplatin.*

Keywords: *S-alkenyl derivatives of thiosalicylic acid, copper(II)-complexes, IR and NMR spectroscopy, cytotoxic activity*

SAŽETAK

*Novi bakar(II)-kompleksi sa nekim S-alkenil derivatima tiosalicilne kiseline (alkenil = propenil-(**L1**), izobutenil-(**L2**)) su sintetisani i okarakterisani na osnovu rezultata mikroanalize, infracrvenih spektara i magnetnih merenja, dok su odgovarajući S-alkenil derivati okarakterisani i na osnovu NMR spektara. Citotoksična aktivnost dva novosintetisana liganda S-alkenil derivata tiosalicilne kiseline je ispitivana pomoću MTT kolorimetrijske tehnike na humanim ćelijama karcinoma debelog creva, HCT-116. Citotoksični efekat bakar(II)-kompleksa je bio veći u poređenju sa citotoksičnošću odgovarajućih liganada (u koncentracijama od 31,25 do 250 μM). Bakar(II)-kompleksi su pokazali neznatno nižu citotoksičnost u poređenju sa cisplatinom. Bakar(II)-kompleksi sa S-alkenil derivatima tiosalicilne kiseline (u koncentracijama od 250 do 1000 μM) imali su citotoksični efekat na HCT-116 ćelijama kao cisplatina.*

Ključne reči: *S-alkenil derivati tiosalicilne kiseline, bakar(II)-kompleksi, IR i NMR spektroskopija, citoksična aktivnost*

ABBREVIATIONS

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
IR - infrared
NMR - nuclear magnetic resonance
Cu - copper
Zn - zinc
VD - Wilson's disease
MD - Menkes disease
ROS - reactive oxygen species
$Cu(NO_3)_2$ - copper(II)-nitrate trihydrate
LiOH - lithium hydroxide
DMSO - dimethyl sulfoxide
DMEM - Dulbecco's Modified Eagle Medium
FBS - fetal bovine serum
EDTA - ethylenediaminetetraacetic acid
PBS - phosphatebuffered saline
BM - bending magnet

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INTRODUCTION

After successful application of "cisplatin" (*cis*-diamminedichloroplatinum(II)) to chemotherapy in cancer patients, the exploration of other metal-complexes as new anticancer agents has continued, to enhance their specificity towards cancer cells, reduce toxic side effects, and prevent the development of resistance in human cancer cells (1-5).

Copper is an essential element for all organisms who live in environments rich in oxygen. This redox active metal converts oxidation state easily from a Cu(I) to Cu(II), and vice versa, as in chemical reactions and in physiological conditions (6). Copper is essential for the function of several enzymes and proteins, such as cytochrome oxidase, Zn, Cu-superoxide dismutase, lysyl oxidase, tyrosinase, and dopamine-b-monooxygenase (1, 6, 7).

The importance of copper is also reflected in the fact that its deficiency leads to congenital genetic diseases, such as Wilson's disease (VD) and Menkes disease (MD) (8). Copper chelation therapy has attracted considerable attention for use in research and treatment of various neurodegenerative disorders (9).

The presence of proteins found in copper has been associated to metabolic changes in cancer cells. The exact role of copper in the treatment of cancer has not been sufficiently clarified (6, 10). It is assumed that copper is involved in the generation of ROS (reactive oxygen species) and the process of angiogenesis by stimulating the proliferation and migration of human endothelial cells (10-12).

Thiosalicylic acid and its derivatives have a wide range of applications. It has been used for the determination of metals (13, 14); as modifiers of graphite paste electrodes (15); as photoinitiators of free radical polymerization (16); in cosmetics (17); in the treatment of dermatological (18), inflammatory, allergic, and respiratory diseases (19); and as Ras-tumour growth inhibitors (20).

Copper(II)-complexes may exert cytotoxic activity on colon carcinoma cell lines. O'Halloran found that a copper complex (Cu[N-salicylidene-(glutamate)$(H_2O)_2$]·H_2O) at concentrations of 50 and 100 μmol/L was shown to have distinct cytotoxic activity in human HT-29 colon cancer cells after 72 hours. Apoptosis was activated by the generation of large quantities of free radicals (21).

Various studies have confirmed the synthesis and structural characterization of different complexes of copper(II) with thiosalicylic acid as a ligand (22, 23). Complexes of copper(II) with the S-alkyl derivatives of thiosalicylic acid showed moderate antimicrobial activity and low antifungal activity (24). With consideration for these effects, new research examining the cytotoxic potential of copper(II) with S-alkyl derivatives of thiosalicylic acid has found that these derivatives exhibit a lower cytotoxicity compared to cisplatin in the human HCT-116 cell line and in murine cell lines CT26 and CT26.CL25 (25).

The first aim of our study was to synthesize two new ligands acting as S-alkenyl derivatives of thiosalicylic acid (alkenyl = propenyl-(**L1**), isobutenyl-(**L2**)), and two corresponding copper(II)-complexes with these ligands. The composition and structure of S-alkenyl derivatives of thiosalicylic acid was assumed on the basis of microanalysis, IR, and NMR spectroscopy. The composition and structure of synthesized complexes was confirmed based on the microanalysis, IR spectroscopy, and magnetic measurements. Another aim of our study was to investigate the cytotoxic potential of copper(II)-complexes with S-alkenyl derivatives of thiosalicylic acid on the human colon cancer cell line HCT-116.

MATERIALS AND METHODS

Materials and measurements

The reagents were obtained commercially and used without further purification. Elemental analyses were conducted on a Vario III CHNOS Elemental Analyser, Elemental Analysensysteme GmbH. For infrared spectra, a Perkin-Elmer FTIR 31725-X spectrophotometer, and KBr pellet technique were employed.

Syntheses

General procedure for the synthesis of S-alkenyl derivatives of thiosalicylic acid

The S-alkenyl derivatives of the thiosalicylic acid ligand (alkenyl = propenyl-(**L1**), isobutenyl-(**L2**)) were prepared (26) by alkylation of thiosalicylic acid using the corresponding alkenyl halides in alkaline water-ethanol solution.

S-propenyl derivative of thiosalicylic acid (S-propenyl-thiosal), (**L1**): M.p. 180-181°C, white powder, IR (KBr, cm^{-1}): 3446, 3075, 2917, 2654, 2557, 1680, 1586, 1562, 1465, 1415, 1314, 1273, 1256, 1153, 1061, 1045, 889, 741, 701, 651, and 551. ^{1}H NMR (200 MHz, $CDCl_3$, δ ppm): 3.21 (d, 2H, CH_2), 5.92 (m, 1H, CH), 4.93 (m, 2H, CH_2) and 7.47-7.91 (m, 4H, Ar). ^{13}C NMR (50 MHz, $CDCl_3$, δ ppm): 38.2 (CH_3), 115.9 (CH_2), 136.5 (CH), 125.3; 125.6; 130.2; 131.7; 133.8; 140.1 (Ar), and 172.0 (COOH).

S-isobutenyl derivative of thiosalicylic acid (S-isobutenyl-thiosal), (**L2**): M.p. 182-183°C, white powder, IR (KBr, cm^{-1}): 3445, 3076, 2967, 2647, 2556, 1676, 1585, 1562, 1463, 1412, 1317, 1272, 1253, 1154, 1061, 1046, 810, 743, 651, and 550. ^{1}H NMR (200 MHz, $CDCl_3$, δ ppm): 1.82 (s, 3H, CH_3), 4.98 (m, 2H, CH_2), 3.44(t, 2H, CH_2), and 7.42-8.30 (m, 4H, Ar). ^{13}C NMR (50 MHz, $CDCl_3$, δ ppm): 22.5 (CH_3), 46.1 (CH_2), 112.1 (CH_2), 125; 126.5; 126.7; 133.2; 134.1; 142.6 (Ar), and 168.1 (COOH).

Preparation of copper(II)-complex with S-propenyl derivative of thiosalicylic acid [Cu_2(S-propenyl-thiosal)$_4$$(H_2O)_2$] (C1)

Copper(II)-nitrate trihydrate (0.1000 g, 0.4139 mmol) was dissolved in 10.0 mL of water on a steam bath, and S-propenyl derivative of thiosalicylate (0.1607 g, 0.8278

mmol) was subsequently added. The reaction mixture was heated for 3 h, and during this period 10.0 ml of LiOH water solution (0.0199 g, 0.8278 mmol) was added in small portions. The solution was filtered and evaporated to a small volume. The blue precipitate of copper(II)-complex was separated by filtration, washed with cold water, and air-dried. Yield: 0.1604 g (81.5%). Anal. Calc. for $[Cu_2(S\text{-propenyl-thiosal})_4(H_2O)_2] = Cu_2C_{41}H_{43}O_{10}S_4$ (Mr = 951.126): C, 51.77; H, 4.56; and S, 13.49. Found: C, 51.54; H, 4.42; and S, 13.29. μ(294 K) = 1.84 μ_B.

IR (KBr, cm^{-1}): 3441, 3076, 2918, 1610, 1549, 1460, 1435, 1400, 1281, 1258, 1229, 1156, 1062, 1044, 846, 744, 695, 658, and 556.

Preparation of copper(II)-complex with S-isobutenyl derivative of thiosalicylic acid [Cu_2(S-isobutenyl-thiosal)$_4(H_2O)_2$] (C2)

Copper(II)-nitrate trihydrate (0.1000 g, 0.4139 mmol) was dissolved in 10.0 mL of water on a steam bath, and S-isobutenyl derivative of thiosalicylate (0.1723 g, 0.8278 mmol) was subsequently added. The reaction mixture was heated for 3 h, and during this period 10.0 mL of LiOH water solution (0.0199 g, 0.8278 mmol) was added in small portions. The solution was filtered and evaporated to a small volume. The blue precipitate of copper(II)-complex was separated by filtration, washed with cold water, and air-dried. Yield: 0.2228 g (82.1%). Anal. Calc. for $[Cu_2(S\text{-isobutenyl-thiosal})_4(H_2O)_2] = Cu_2C_{45}H_{51}O_{10}S_4$ (Mr = 1007.232): C, 53.66; H, 5.10; and S, 12.73. Found: C, 53.47; H, 5.18; and S, 12.64. μ(294 K) = 1.86 μ_B.

IR (KBr, cm^{-1}): 3446, 2969, 2915, 1613, 1589, 1400, 1281, 1257, 1157, 1062, 847, 744, 719, 656, and 510.

Preparation of drug solutions

Complexes were dissolved in 10% dimethylsulfoxide (DMSO) in distilled water at a concentration of 10 mM and filtered through a 0.22 mm Millipore filter. These stock solutions were diluted in culture medium immediately before use. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5- -diphenyl tetrazolium bromide was dissolved (5 mg/mL) in a phosphate buffer saline having a pH of 7.2, and filtered through the 0.22 mm Millipore filter before use. All reagents were purchased from Sigma Chemicals.

Cell culture

HCT-116 cells were kindly provided by Dr Danijela Vignjević (Institute Curie, Paris, France). Cells were maintained in DMEM (Sigma Aldrich, Munich, Germany) supplemented with 10% fetal bovine serum (FBS, Sigma Aldrich, Munich, Germany), penicillin (100 IU/mL), and streptomycin (100 µg/mL) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Subconfluent monolayers in the logarithmic growth phase were harvested by a brief treatment with 0.25% trypsin and 0.02% EDTA in phosphate-buffered saline (PBS, PAA Laboratories GmbH) and washed three times in serum-free PBS. The number of viable cells was determined by trypan blue exclusion.

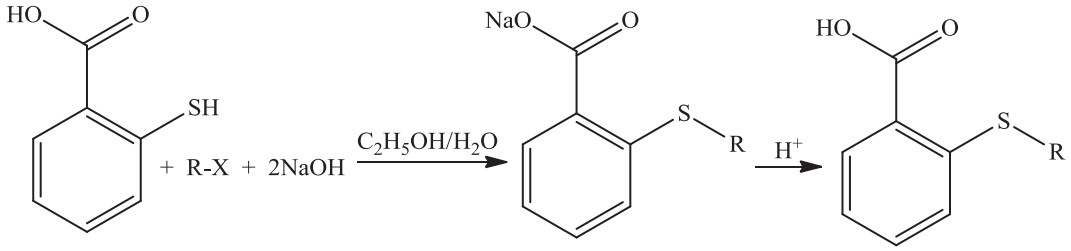
Cytotoxicity assays

The effects of the tested compounds on cell viability were determined using the MTT colorimetric technique (27). HCT-116 cells were diluted with growth medium to $5{\cdot}10^4$ cells/mL, and aliquots ($5{\cdot}10^3$ cells/100 mL) were placed in individual wells in 96-well plates. The next day the medium was exchanged with 100 µL of different compounds, which had been serially diluted 2-fold in the medium to concentrations ranging from 1000 µM to 7.8 µM in growth medium. Each compound was tested in triplicate. Cells were incubated at 37°C in 5% CO_2 for 72 h. After incubation the supernatant was removed, and 15% MTT solution (5 mg/mL in PBS, 10 µL) in DMEM without FBS was added to each well. After an additional 4 h of incubation at 37°C in 5% CO_2, the medium with MTT was removed and DMSO (150 µL) with glycine buffer (20 µL) was added to dissolve the crystals. The plates were shaken for 10 min. The optical density of each well was determined at 595 nm using microplate Zenyth 3100 Multimode detector. The percentage of cytotoxicity was calculated using the formula: % cytotoxicity = 100-((E-B)/(S-B)·100), where B is the background of medium alone, S is total viability/spontaneous death of untreated target cells, and E is experimental well. Each of the tested complexes was evaluated for cytotoxicity in three separate experiments.

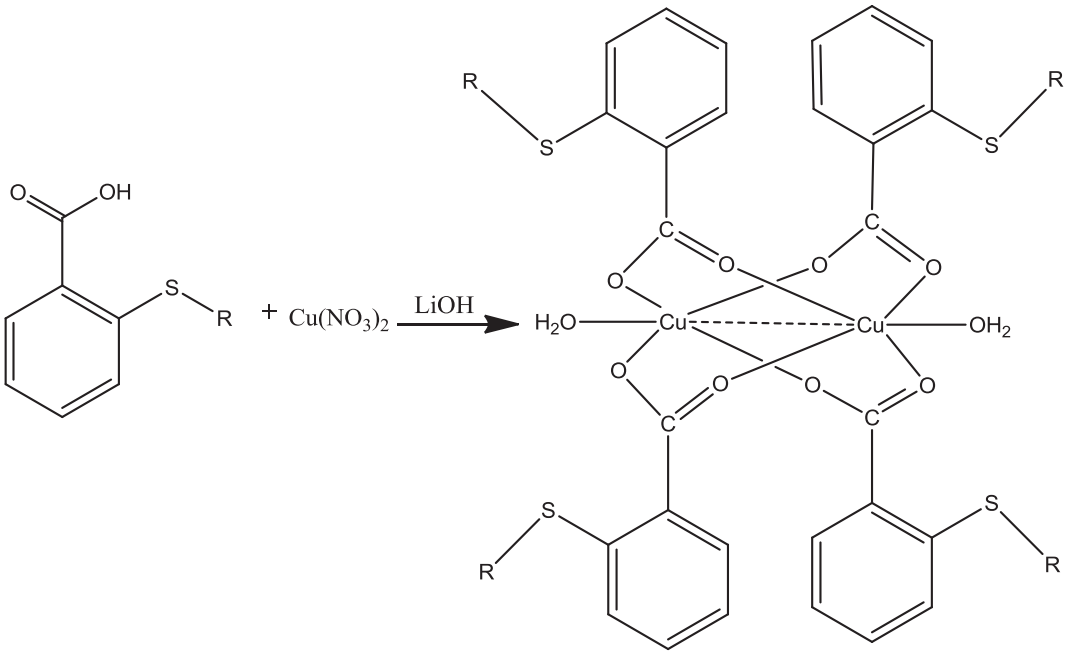
RESULTS AND DISCUSSION

Synthesis and chemical characterization

S-alkenyl (R = propenyl-(**L1**), isobutenyl-(**L2**)) derivatives of thiosalicylic acid were prepared (26) by alkylation of thiosalicylic acid using the corresponding alkenyl halogenides in an alkaline water-ethanol solution (Scheme 1). The corresponding complexes were obtained by a direct



Scheme 1. Synthesis method of S-alkenyl derivates of thiosalicylic acid, R = propenyl-(**L1**), isobutenyl-(**L2**).



Scheme 2. Synthesis of copper(II)-complex with S-alkenyl derivates of thiosalicylic acid, R = propenyl-(**C1**), isobutenyl-(**C2**).

Table 1. The most important infrared bands (cm^{-1}) of the investigated compounds.

Compound	-S-R	-COO⁻ (as)	-COO⁻ (sim)
S-propenyl-thiosal (**L1**)	702(m)	1680(s)	1415(s)
$[Cu_2(S\text{-propenyl-thiosal})_4(H_2O)_2]$ (**C1**)	696(m)	1610(s) 1596(s)	1401(s)
S-isobutenyl-thiosal (**L2**)	702(m)	1676(s)	1412(s)
$[Cu_2(S\text{-isobutenyl-thiosal})_4(H_2O)_2]$ (**C2**)	697(m)	1613(s) 1589(s)	1400(s)

s-strong, m-medium

reaction of copper(II)-nitrate trihydrate with S-alkenyl derivatives of thiosalicylic acid (molar ratio 1:2) in a water solution with satisfactory yields (more than 80%) (Scheme 2).

Infrared spectra of the isolated complexes were measured to find the coordination mode of the S-alkenyl derivatives of thiosalicylic acid. The asymmetric stretching frequencies of the carboxyl group were used to determine whether it was coordinated (the absorption bands are located in the region 1600–1650 cm^{-1}) or uncoordinated (the absorption bands are located in the region 1700–1750 cm^{-1}) to the metal ion (28–30). The infrared spectra of complexes C1 and C2 indicated that the carboxyl groups of S-alkenyl derivatives of thiosalicylic were definitely coordinated to the central copper(II)-complexes.

The isolated $(Cu_2(S\text{-alkenyl-thiosal})_4(H_2O)_2]$ complexes show double sharp and strong asymmetric stretching frequencies of the carboxyl groups of the coordinated S-alkenyl derivatives of thiosalicylic acid to Cu(II)-ion at approximately 1548–1615 cm^{-1} (Table 1). The observed clear double bands for isolated complexes suggest small differences in the coordination of the carboxyl groups of the ligands to the copper(II)-ion. Based on previously published results from structurally similar ligands, we can conclude that there was a coordination of S-alkenyl derivatives of thiosalicylic acid with copper(II)-ions in forming binuclear complexes.

Additionally, the strong sharp single symmetric stretching bands of the coordinated carboxyl groups of the S-alkenyl derivatives of thiosalicylic acid lie in the expected region (approximately 1400 cm^{-1}).

Magnetic measurements

Binuclear copper(II)-complexes (31, 32) are stable in dimeric form. The low value of μ_{eff} at room temperature (approximately 1.86 BM) is indicative of an antiferromagnetic interaction between the two metal centres typical of binuclear carboxylates of copper(II) of the type: $[Cu(R\text{-}COO)_2L]_2$ (33–35). The main factor determining the magnitude of the antiferromagnetic interaction in the dimeric copper(II) carboxylates is the electronic structure of the bridging OCO moiety, as published previously (31, 32–35).

Anticancer activity of copper(II)-complexes

The MTT assay for cell viability shows that the two newly synthesized precursor S-alkenyl derivatives of thiosalicylic acid, as well as their corresponding copper(II)-complexes, exhibit cytotoxic activity in HCT-116 human colon carcinoma cells after 24 hours (Figure 1). The cytotoxic effect of the copper(II)-complexes was higher than the cytotoxicity of the corresponding ligands, especially for concentrations from 31.25 to 250 μM. Copper complexes showed slightly lower cytotoxicity compared to cisplatin. However, higher concentrations of C2 (250-1000 μM) had an almost equal cytotoxic effect on HCT-116 cells, relative to cisplatin.

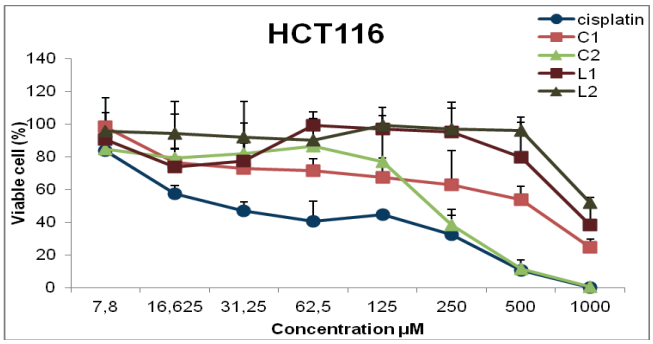


Figure 1. Representative graphs of HCT-116 cell survival after 24 h cell growth in the presence of copper(II) complexes and ligand precursors. Each point represents a mean value and standard deviation of 3 experiments with 3 replicates per dose.

CONCLUSION

The complexes of copper(II) with S-alkenyl derivatives of thiosalicylic acid (alkenyl = propenyl-(**L1**), isobutenyl-(**L2**)) have been synthesized and characterized by microanalysis, infrared spectroscopy, and magnetic measurements. The cytotoxic effect of the copper(II)-complex was higher compared to the cytotoxicity of the corresponding ligands. Copper(II)-complexes showed slightly lower cytotoxicity compared to cisplatin. Complexes with S-alkenyl derivatives of thiosalicylic acid in concentrations from 250 to 1000 μM had a cytotoxic effect on HCT-116 cells that was similar to cisplatin.

Acknowledgement

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THE INFLUENCE OF DIFFERENT TYPES OF PHYSICAL ACTIVITY ON THE REDOX STATUS OF SCUBA DIVERS

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UTICAJ RAZLIČITIH TIPOVA FIZIČKE AKTIVNOSTI NA REDOKS STATUS RONILACA

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ABSTRACT

The effect of scuba diving on ROS production and oxidative stress compared to that of other recreational activities is still poorly understood. The aim of this study was to assess the influence of different types of physical activity on the redox status of scuba divers by testing the pro- and anti-oxidative parameters immediately before and after different types of physical load. The prevalence study included 10 professional police divers. All examinees were male, 32 ± 5.1 years of age, well-trained, and with a minimum of five to a maximum of 20 years of diving experience. The study was divided into three experimental protocols: 1) an exercise test (at atmospheric pressure), 2) an at sea dive (30 meters for 30 minutes), and 3) a dive into river current (10 meters for 30 minutes). Immediately before and after the load test of the divers at atmospheric pressure and immediately before and after the dive, blood samples were taken to determine the values of the following pro-oxidant markers: O_2^-, H_2O_2, NO_2^- and TBARS, as well as antioxidant enzymes (SOD and CAT). A comparison of the results before and after physical activity for all three protocols revealed a significant increase in values for NO_2^-, O_2^-, H_2O_2 and CAT after physical activity. It can be concluded that the values of all oxidative stress markers depend on the season of the year in which the research is conducted or on the frequency of dives and degree of physical exertion during this period of the year.

Keywords: *scuba diving, professional scuba divers, oxidative stress, free radicals*

SAŽETAK

Efekti ronjenja na proizvodnju slobodnih radikala i oksidacioni stres su u odnosu na druge rekreativne sportive malo poznati. Ova studija je imala za cilj da ispita uticaj različitih tipova fizičke aktivnosti na redoks status ronilaca putem merenja vrednosti pro- i anti-oksidacionih parametara neposredno pre i posle različitih vrsti napora. Studija prevalencije je obuhvatila 10 profesionalnih policijskih ronilaca. Svi ispitanici su bili muškarci, 32 ± 5.1 godina, dobro obučeni, sa ronilačkim iskustvom od najmanje pet do najviše 20 godina. Studija je bila podeljena u tri eksperimentalna protokola: 1. test opterećenja na ergo-biciklu (na atmosferskom pritisku), 2. zaron u moru (30 metara u toku 30 minuta), i 3. zaron u rečnoj struji (10 metara u toku 30 minuta). Neposredno pre i posle testa opterećenja ili obe vrste zarona uzimani su uzorci krvi radi određivanja vrednosti pro-oksidanasa: O_2^-, H_2O_2, NO_2^- i TBARS, kao i enzima anti-oksidacione zaštite (SOD i CAT). Poređenjem rezultata pre i posle fizičke aktivnosti u sva tri protokola, pronađena je visoka statistička razlika u vrednostima O_2^-, H_2O_2, NO_2^- i CAT. Može se zaključiti da vrednosti svih markera oksidacionog stresa zavise od godišnjeg doba tokom kojeg je istraživanje sprovedeno ili od frekvencije ronjenja i fizičke aktivnosti u ovom periodu godine.

Ključne reči: *ronjenje, profesionalni ronioci, oksidacioni stres, slobodni radikali*

ABBREVIATIONS

CAT – catalase;
NO_2^- – nitrites;
H_2O_2 – hydrogen peroxide;
O_2^- – superoxide anion radical
ROS – reactive oxygen species;
SOD – superoxide dismutase;
TBARS – thiobarbituric acid reactive substances

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INTRODUCTION

An imbalance between free radical production and antioxidant defence in the human body leads to an oxidative stress state. The negative effects of oxidative stress are associated with the pathophysiology of many diseases and aging. These effects are the consequence of a reduction in resistance to oxidative stress with aging and the accumulation of free radicals. Although it is well known that physical exercise can increase oxidative stress and cause disruption of redox homeostasis (1-4), many studies have shown a beneficial role of the reactive oxygen species (ROS) produced during exercise, which leads to important training adaptations, including an increase in resistance to oxidative stress, angiogenesis, mitochondria biogenesis, and muscle hypertrophy (1).

It is well established that exercise can have both positive and negative effects on oxidative status depending on the exercise load, exercise specificity and the basal physical condition level. However, the effect of scuba diving on ROS production and oxidative stress resistance compared to other recreational activities is still unknown. Furthermore, it remains unclear in which circumstances the practice of scuba diving can be helpful or harmful (5).

Due to the weight of the diving equipment and increased resistance to movement, scuba diving is a demanding physical activity. Furthermore, divers are exposed to changes in environmental conditions that are not usually present in other types of physical activity. In addition to underwater pressure and cold temperatures, these changes include breathing oxygen at an elevated pressure and increased resistance to breathing (6). Intensified physical activity and coldness can lead to the increased production of free radicals. Additionally, hyperoxia as a result of hyperbaric exposure during diving and breathing oxygen at a high pressure could induce oxidative stress (7).

The current data on the influences of scuba diving on oxidative status are controversial; while some studies describe positive effects, other studies suggest undesirable consequences of scuba diving on human health. The main reason for such ambiguous results is that the studies were conducted under different conditions, including differences in the selection of evaluated biomarkers of oxidative status and the time and method of determination (5).

On the basis of the information stated previously, the aim of this study was to assess the influence of different types of physical activity on the redox status of scuba divers.

MATERIALS AND METHODS

Study population

This study included 10 professional police divers who were participating in the training process, which included dive training at the sea and at a river. All examinees were male, 32 ± 5.1 years of age, well-trained, and with a minimum of five to a maximum of 20 years of diving experience. During their diving service, participants were subjected to regular annual medical check-ups by a specialist. During this study, divers had no signs of any acute or chronic disease. They had valid medical documentation that proved they were able to perform the duties of professional divers.

Four weeks prior to blood sampling, the divers were instructed to refrain from any vitamin or antioxidant dietary supplementation. None of the participants reported any eating disorders, had any ongoing or previous injuries (during the last six months), and were not on any medications known to affect oxidative stress. To exclude the influence of different dietary intakes on nitrite levels, all participants were on the same dietary protocol for three days before the study and during the study. This protocol consisted of a prescribed menu for police divers.

All subjects were non-smokers and had normal values of arterial blood pressure, normal echocardiogram, ECG, EEG, and spirometry results, and normal values of fat and sugar in the blood. All divers received an explanation of the study's purposes, risks and benefits and were familiarized with the study's protocol. All subjects gave written consent. The study was approved by the Ethical Committee of the Faculty of Medical Sciences, University of Kragujevac.

Design of the study

The study was divided into three experimental protocols:

1) For the first testing protocol, the parameters of oxidative stress and the antioxidant protection components were determined immediately before and after theload test of the divers at atmospheric pressure. Before the load test, an ECG was performed on each diver. The workload test entailed progressively increasing the workload during a continuous test on a training bike (8). Maximal oxygen uptake (VO_2max) was measured by a direct method with the help of the Fi® Pro apparatus (Cosmed, Italy) (9). Heart rate was measured using a heart rate monitor belt (HR Polar S810, Finland). Assessment of body composition was performed using the bioimpedance method with the machine In Body 720 (Biospace, Korea).

2) Diving protocol 1: Testing was performed at the seaside during a dive training of professional police divers held in May. All divers were dressed in neoprene drysuits and were equipped with open-circuit scuba diving apparatus. The primary cylinders were 18-litre bottles filled with compressed air with an average pressure of 205 bars. The morning medical examination was conducted before the dive and included auscultatory findings from the lungs and measurements of blood pressure and pulse. After arriving at the location, each diving pair made a dive to the specified depth. Each set of divers launched every five minutes. The specified depth was 30 meters and was controlled with either a Suunto or Mares dive computer. The specified duration of the

dive was 30 minutes. During the dive, the divers were instructed to moderate their loads. The descent was at 10 meters/minute, and the ascent was 9 meters/minute, with a three-minute decompression/safety stop at a depth of 3 meters. All 10 divers completed the dive successfully without any sign of decompression sickness. The average air consumption was 56 litres (L) per minute. Air temperature during the examination was 23°C, and sea temperature was 15°C to 19°C.

3) Diving protocol 2: Testing was performed in river currents during a dive training of professional police divers held in May and in November. All divers were dressed in neoprene drysuits and were equipped with open-circuit scuba diving apparatus. The primary cylinders were 18-litre bottles filled with compressed air with an average pressure of 195 bars. The morning medical examination was conducted before the dive and included auscultatory findings from the lungs and measurements of blood pressure and pulse. After arriving at the location, each diver made a dive to the specified depth. The specified depth was 10 meters and was controlled with either a Suunto or Galileo Sol dive computer, which measured the amount of air inhaled and the pulse of the divers. The specified duration of the dive was 30 minutes. During the boat ride to the location of the river current, divers simulated a search of the terrain using the pendulum method. The scuba diver with a life rope tied to the dive boat was a reserve diver. The descent was at 10 meters/minute, and the ascent was 9 meters/minute. All 10 divers completed the dive successfully. The average air consumption was 22 litres (L) per minute. Air temperature during the examination was 23°C, and the river temperature was 12°C to 18°C.

Sample collection protocol

Immediately before and after the load test at atmospheric pressure and immediately before and after the dive, blood samples were taken from the divers to determine levels of the following pro-oxidant markers: superoxide anion radical (O_2^-), (H_2O_2), nitrites (NO_2^-) and the index of lipid peroxidation (TBARS), as well as the activity of antioxidant enzymes (SOD and CAT). Four weeks prior to blood sampling, the divers were instructed to refrain from any vitamin or antioxidant dietary supplementation. None of the participants reported any eating disorders, had any ongoing or previous injuries (during the last six months), and were not on any medications known to affect oxidative stress. To exclude the influence of different dietary intakes on nitrite levels, all participants were on the same dietary protocol for three days before the study and during the study.

Biochemical assays

Blood samples were drawn from the antecubital vein of each diver into Vacutainer test tubes containing sodium citrate anticoagulant. Blood samples were processed and stored immediately. The blood was centrifuged to separate the plasma and red blood cells (RBCs). Biochemical parameters were measured spectrophotometrically.

Index of lipid peroxidation (thiobarbituric acid reactive substances, TBARS) determination

The degree of lipid peroxidation in plasma was estimated by measuring TBARS using 1 % TBA (thiobarbituric acid) in 0.05 NaOH that was incubated with plasma at 100°C for 15 min and read at 530 nm. Distilled water was used as a blank probe. TBA extract was obtained by combining 0.8 ml plasma and 0.4 ml TCA (trichloroacetic acid). Samples were put on ice for 10 min and centrifuged for 15 min at 6000 rpm. This method was described previously (10).

Nitrites (NO_2^-) determination

NO decomposes rapidly to form stable metabolite nitrite/nitrate products. NO_2^- was determined as an index of nitric oxide production using the Griess reagent (11). 0.1 ml 3N PCA (perchloride acid), 0.4 ml 20 mM EDTA (ethylenediaminetetraacetic acid) and 0.2 ml plasma were put on ice for 15 min and then centrifuged for 15 min at 6000 rpm. After pouring off the supernatant, 220 µl K_2CO_3 was added. NO_2^- was measured at 550 nm. Distilled water was used as a blank probe.

Superoxide anion radical (O_2^-) determination

The level of O_2^- was measured using an NBT (nitro blue tetrazolium) reaction in TRIS-buffer combined with plasma samples and read at 530 nm (12).

Hydrogen peroxide (H_2O_2) determination

The protocol for the measurement of H_2O_2 is based on the oxidation of phenol red in the presence of horseradish peroxidase (13). Two hundred microlitres of plasma with 800 µl PRS (Phenol Red Solution) and 10 µl POD (Horseradish Peroxidase) were combined (1:20). The level of H_2O_2 was measured at 610 nm.

Determination of the activities of antioxidant enzymes

Isolated RBCs were washed three times with three volumes of ice-cold 0.9 mmol/l NaCl, and hemolysates containing approximately 50 g Hb/l (14) were used for the determination of CAT activity. CAT activity was determined according to the methods described by Beutler (15). Lysates were diluted with distilled water (1:7 v/v) and treated with chloroform-ethanol (0.6:1 v/v) to remove haemoglobin (16). Subsequently, 50 µl CAT buffer, 100 µl sample and 1 ml 10 mM H_2O_2 were added to the samples. Detection was performed at 360 nm. Distilled water was

used as a blank probe. SOD activity was determined by the epinephrine method of Misra and Fridovich (17). A mixture of 100 µl lysate and 1 ml carbonate buffer was made, following which 100 µl of epinephrine was added. Detection was performed at 470 nm.

Statistical analysis

The statistical analysis was performed using SPSS 19.0 for Windows. The results are expressed as the means ± standard deviation of the means (SD). Data distribution was checked with the Shapiro-Wilk test and depending on its results, the appropriate parametric or non-parametric test was used. The differences between the values of the means from two related samples (before and after the dive) were assessed by Wilcoxon's test, while the difference between two unrelated samples (characteristics of the exercise protocol) was assessed by Student's t-test.

The alpha level for significance was set to $p<0.05$.

RESULTS

All subjects participating in the first protocol had normal ECG findings, while the average value of VO_2max was 42.4 l/kg. The results of aerobic capacity were marked as excellent in 1 subject, very good in 5 subjects and good in 4 subjects. The average air consumption was 133.6 l/min.

The effects of different types of physical activity on TBARS concentration

No significant differences in the TBARS values before or after physical activity were observed among any of the three protocols (groups) ($p>0.05$). TBARS dynamics were also unchanged before and after physical loading within all of the protocol groups ($p>0.05$).

The effects of different types of physical activity on NO_2^- concentration

Before physical activity, NO_2^- values were significantly higher in the exercise test group compared to sea and river dive groups ($p<0.01$). After physical loading, values were the highest in the sea dive group and lowest in the river dive group ($p<0.01$). When comparing NO_2^- values within each protocol group, this marker was increased in the sea dive protocol group ($p<0.01$), and no significant change in NO_2^- was noted in the exercise test and river dive protocol groups ($p>0.05$).

The effects of different types of physical activity on O_2^- concentration

Before physical activity, O_2^- values were significantly higher in the river dive group compared to the sea dive and exercise test groups ($p<0.01$). After physical loading, values were the highest in the sea dive group and the lowest in the exercise test group ($p<0.01$). When comparing O_2^- values within each protocol (group), we observed that this parameter was decreased after physical effort in the river dive protocol group ($p<0.05$), increased in the sea dive group ($p<0.05$), and remained unchanged in the exercise test protocol group ($p>0.05$).

The effects of different types of physical activity on H_2O_2 concentration

The values of H_2O_2 were significantly higher in the sea dive group compared to the river dive and exercise test groups, both before and after physical activity ($p<0.01$). When comparing H_2O_2 values within each protocol (group), this marker was increased after physical loading in the sea dive protocol group ($p<0.05$), but no significant change in H_2O_2 was observed in the river dive and exercise test protocol groups ($p>0.05$).

The effects of different types of physical activity on CAT values

The CAT values were significantly higher in the exercise test protocol group compared to the sea dive ($p<0.05$) and river dive protocol groups, ($p<0.01$) both before and after physical activity. Values of this enzyme were unchanged before and after physical loading in all of the protocol groups ($p>0.05$).

The effects of different types of physical activity on SOD values

In contrast to the previous results, SOD values after physical activity were significantly higher in the river and exercise test protocol groups compared to the sea dive group ($p<0.01$). Furthermore, significant differences were not observed between the exercise test and river dive protocol groups ($p>0.05$). Values of this enzyme remained unchanged before and after physical effort in all of the protocol groups ($p>0.05$).

DISCUSSION

It is well established that exercise can have both positive and negative effects on oxidative status depending on exercise load, exercise specificity and the basal physical condition level. However, compared to other sport activities, data referring to the effect of scuba diving on redox status are insufficient. Furthermore, it remains unclear in which circumstances scuba diving can be helpful or harmful (5). Therefore, the aim of the present investigation was to assess the influence of different types of physical activity on the redox status of scuba divers.

In our study, the first protocol required the divers to participate in a stress test on a training bike at atmospheric pressure. In the second protocol, the divers performed diving under the sea at 30 meters for 30 minutes. Because of the weight of the diving equipment and an increased resistance to movement, the dive was considered to be a very demanding physical activity. In the third protocol, the divers participated in shallow diving (up to 10 meters) in

river currents, in which a diver experiences a physical load in the form of the resistance of the river current.

While in the water current, a diver's body cools faster and thus increases power consumption, which reduces the working capacity of the divers. Reduced visibility decreases the diver's orientation and timely response. The power consumption in the diver during the activity is expected to increase most of the pro-oxidative parameters and reduce antioxidant protection.

It is important to emphasize that the research involving the second protocol was conducted between May and June during regular training at the sea, which occurred after the winter break in training dives. The first protocol was conducted during June and July after returning from the diving training at the sea. Finally, after returning from regular training at the sea, the research involving the third protocol was conducted, which consisted of diving in river currents, during the period from October to mid-November of the same year.

The comparison of the TBARS results before and after physical activity in all three protocols showed no statistically significant differences (Fig. 1). The most likely explanation for this finding is that divers were individually well-trained and developed strong antioxidant systems that did not allow the occurrence of lipid degradation. the In contrast, highly significant differences were observed in the values of other pro-oxidant markers, namely, NO_2^-, O_2^- and H_2O_2. These results are most likely the consequence of external factors such as training frequency, dive frequency, diet, and the period during which the research was conducted.

When comparing the results before and after physical activity in each protocol, we observed that NO_2^- release increased after the sea dive, remained unchanged in the river dive and decreased during the stress exercise test (Fig. 2). These findings most likely resulted from the physical activity that was performed in the course of these protocols. Moreover, considering the research period for these protocols, the values of NO_2^- may be the result of adaptation of the organism due to the frequent number of dives made during regular training at the sea and during the diving training in inland waters.

The difference in O_2^- release both before and after physical activity in all three protocols was statistically significant. This finding may indicate that various types of exercise have different influences on the formation of O_2^-. The lowest values of O_2^- were found before the effort and at the end of the season of frequent dives, which indicates a possible adaptation to increased O_2^- release. However, these results should be considered with caution, considering the short half-life and high reactivity of O_2^- (18).

When comparing the values of this parameter for each protocol, an increase in the O_2^- production was observed during a sea dive to 30 meters for 30 minutes, but a reduction in its release was observed during a river dive (Fig. 3). This finding is probably caused by the lower depth of the dive in the river compared to the sea dive. We have attempted to compare our results with data from the available literature, but unfortunately, we did not find any related studies published in this field.

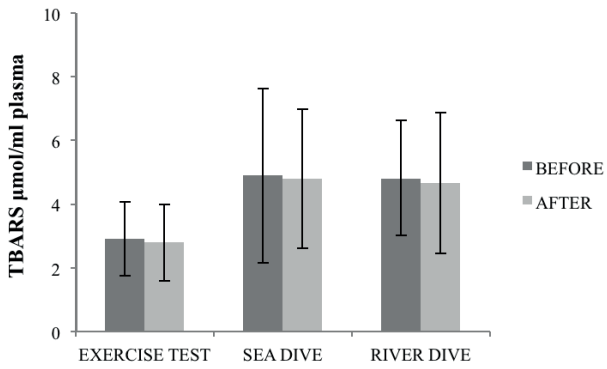

Figure 1. TBARS values before and after physical activity in all three protocols. P values less than 0.05 were considered to be significant (*p<0.05; **p<0.01).

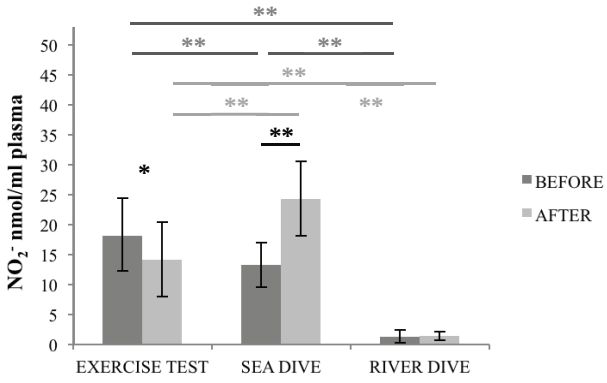

Figure 2. NO_2^- values before and after physical activity in all three protocols. P values less than 0.05 were considered to be significant (*p<0.05; **p<0.01).

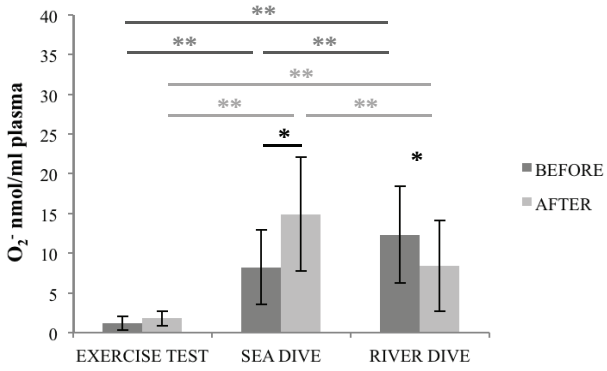

Figure 3. O_2^- values before and after physical activity in all three protocols. P values less than 0.05 were considered to be significant (*p<0.05; **p<0.01).

The values of the results for the H_2O_2 showed highly significant differences in all three protocols before and after exercise (Fig. 4). Similar to previous cases, the above mentioned external factors were likely to have an impact

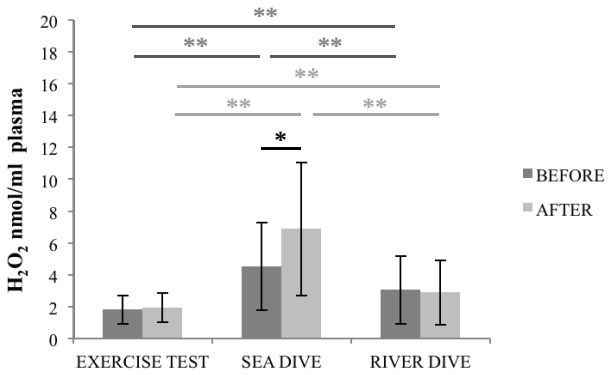

Figure 4. H_2O_2 values before and after physical activity in all three protocols. P values less than 0.05 were considered to be significant (*p<0.05; **p<0.01).

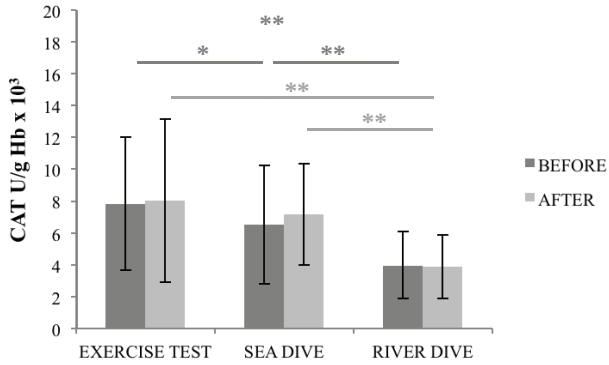

Figure 5. CAT values before and after physical activity in all three protocols. P values less than 0.05 were considered to be significant (*p<0.05; **p<0.01).

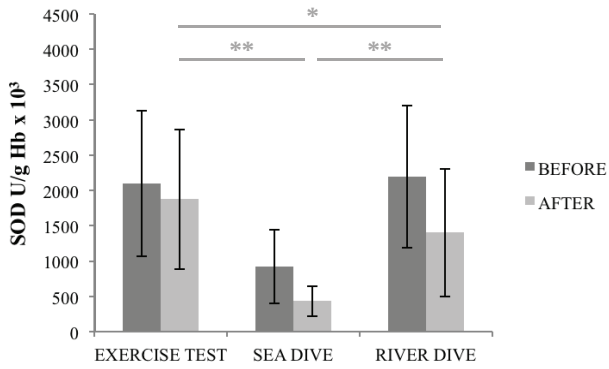

Figure 6. SOD values before and after physical activity in all three protocols P values less than 0.05 were considered to be significant (*p<0.05; **p<0.01).

on these results. The H_2O_2 release increased during the sea dive, while they remained unchanged over the course of a dive in the river current and during the stress test. This result could be a consequence of the greater depth of the sea dive and easier adaptation to physical effort in the river stream and during the stress test.

Moreover, it may be expected that during a dive, the functioning of the antioxidative enzymatic system should change. Thus, we investigated the activity of two major antioxidative enzymes, CAT and SOD, to create the most complete picture of each diver's redox state. SOD catalyses the dismutation of O_2^- to H_2O_2 and allows for better interpretation of results regarding O_2^- and H_2O_2 (19), while CAT decomposes H_2O_2 to H_2O and O_2 (19).

The results for CAT showed highly significant differences in all three protocols before and after exercise, suggesting that external factors are likely to have an impact on the results.

Comparing the results before physical activity in all three protocols, no significant differences in the values of SOD were observed. However, by contrast, when we compared the results after the physical stress loads were applied in all three protocols, highly significant differences in the activity of this enzyme were observed.

If we observe the time period when the research was conducted and the values of SOD, higher values were measured during the first protocol (training at the sea from May to June) and the third protocol (training at the sea in September and October). The increased SOD values in the divers during these training periods seem to be related to a long period of exposure to increased physical effort. The increased SOD values most likely increased the ability of the diver's body to endure the oxidative stress, which in this case could have been caused by different types of physical activities such as sea diving to 30 feet for 30 minutes, diving in the river current and participating in a stress test at atmospheric pressure.

CONCLUSION

Considering that our study involved professional divers who all had several years of diving experience, it can be assumed that due to frequent dives, a variety of diving training methods and well-trained endurance to physical stress, a well-adapted antioxidant protection system already existed in the divers. We can conclude that diving in the sea caused the greatest oxidative damage, which is followed by the weakest antioxidant response. Analysing the results of markers of oxidative stress and antioxidant protection, it can be concluded that values of pro- and anti-oxidants depend on the season of the year in which the research is conducted or on the dive frequency and degree of physical exertion during this period of time.

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USAGE OF INTRAMAMMARY ANTIMICROBIAL VETERINARY MEDICINAL PRODUCTS IN THE REPUBLIC OF SERBIA FROM 2011 TO 2014

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PROMET VETERINARSKIH INTRAMAMARNIH ANTIBIOTIKA U REPUBLICI SRBIJI OD 2011. DO 2014. GODINE

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ABSTRACT

Prudent use of antimicrobial medicine is an imperative in both human and veterinary medicine today. Antibiotic usage in humans and animals has increased over the years, consequently giving rise to antimicrobial resistance in pathogenic microorganisms. Mastitis is one of the most common conditions in bovine species, and intramammary antibacterial medicinal products are used in animal husbandry for mastitis treatment and prophylaxis.

This paper presents the quantities of intramammary antibiotics sold in the Republic of Serbia from 2011 to 2014 based on data reported to the Medicines and Medical Devices Agency of Serbia by Marketing Authorization Holders. Furthermore, we assessed the number of treated cows and compared those results with the reported total number of cows in the same time period, providing information on animal exposure to particular antibiotics.

In 2011 and 2012, beta-lactams were the most commonly used antimicrobials, while beta-lactams in combination with other substances were the most commonly used antibiotics in 2014, with a total of 80,927 treated animals. From 2011 to 2014, 15-25% of cows were treated with intramammary antimicrobials.

Bearing in mind the growing importance of antibiotic resistance in humans and animals, these results reveal the scope of the potential human exposure to antibiotics via consumption of the milk of treated cows.

Keywords: *mastitis, intramammary antibacterial agents, veterinary medicinal product, resistance*

SAŽETAK

Odgovorna primena antimikrobnih lekova danas je jedan od imperativa u humanoj i veterinarskoj medicini. Primena antibiotika kod ljudi i životinja je u porastu, što posledično dovodi do razvoja rezistencije na antibiotike kod patogenih mikroorganizama. Mastitis je jedno od najčešćih stanja kod goveda, te se intramamarni antibiotski lekovi primenjuju u lečenju i profilaksi mastitisa.

Ovaj rad prezentuje količine intramamarnih antibiotika koje su se nalazile u prometu u Republici Srbiji u periodu od 2011. do 2014. godine, a na osnovu podataka dostavljenih Agenciji za lekove i medicinska sredstva Srbije od strane nosilaca dozvole za lek. Pored toga, ovaj rad procenjuje broj tretiranih krava u istom vremenskom periodu, čime pruža podatke o izloženosti životinja pojedinim antibioticima.

U 2011. i 2012. godini najčešće su primenjivani beta-laktamski antibiotici, dok su tokom 2014. godine najčešće primenjivani beta-laktamski antibiotici u kombinaciji sa drugim supstancama sa 80927 lečenih životinja. Između 15% i 25% krava lečeno je intramamarnim antibioticima u periodu od 2011. do 2014. godine.

Imajući u vidu sve veći značaj rezistencije na antibiotike kod ljudi i životinja, ovi rezultati mogu ukazati na obim potencijalnog izlaganja ljudi antibioticima putem korišćenja mleka lečenih krava.

Ključne reči: *mastitis; intramamarni antibiotik, veterinarski lek, rezistencija*

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INTRODUCTION

People, animals, and the environment represent an interconnected, dynamic system. One health initiative aims towards better understanding and addressing contemporary health issues created by the convergence of humans, animals, and environmental domains (1). Antimicrobial medicinal products are essential for treating infections in both humans and animals. Globally, an estimated 50% of all antimicrobials are used in veterinary medicine. Additionally, in animals antibiotic use is the primary contributor to the selection and spread of antimicrobial resistance. Animals and humans share the same antibiotic resistance mechanisms (2). Antimicrobial resistance is regarded as one of the major threats to the future health of individuals and populations, and national and international organizations have repeatedly underlined an urgent need for action (3). It is essential to monitor trends in the consumption of antibiotics to identify the risk factors that contribute to the spreading of the resistance (4).

Mastitis is a major challenge to the worldwide dairy industry (5). Modern farming practice widely employs intramammary antimicrobial medicines in dairy production since intramammary infections are one of the most common conditions in bovine species (6). Mastitis is an inflammation of the mammary gland, usually caused by pathogenic microorganisms (7). The disease is followed by a complex series of events leading to reduced synthetic activity, compositional changes, and elevated somatic cell count, all affecting milk production and quality. *Escherichia coli* and *Streptococcus uberis* are the microorganisms most frequently isolated in bovine mastitis (5).

Intramammary antibiotics are used both for the treatment and prevention of mastitis in cattle. In cows with clinical mastitis, the treatment outcome depends on the host, bacterial and management factors as well as on the selected antimicrobial medicine (8). When used for mastitis treatment, immediate release formulations are administered during lactation. Such medicines are administered only into the affected teat, and milk should be discarded during a brief time period, usually for several days. In contrast, long-acting formulations are administered during the dry period into all quarters. These medicines are used as a prophylactic treatment of mastitis in herds where subclinical mastitis is present. Long-acting preparations have long withdrawal periods, which can last as long as 60 or more days, depending on the time between dry-off and calving.

Allergy to antimicrobial medicines, especially to β-lactams, is a well-established adverse effect in human medicine. Sensitive persons can be exposed to antimicrobials through food, especially milk and dairy products (9). Not only does this instigate a human health hazard, but the residues of antimicrobial medicines in milk can be a problem in the dairy industry (10) since antibiotics disable the fermentation of microorganisms used in manufacturing of dairy products such as cheese and yogurt. Therefore, the judicious use of antimicrobials in food-producing animals, compliance with prescribed withdrawal periods and strict monitoring of drug residues are essential for the safety of both humans and animals.

In this paper, we presented the quantities of intramammary antibiotics sold in the Republic of Serbia. Furthermore, we estimated the number of cows treated with these medicines and compared this number with the reported total number of cows.

MATERIALS AND METHODS

The Medicines and Medical Devices Agency of Serbia annually collects information on medicinal sales from Marketing Authorization Holders. Those quantities are presented according to ATCvet classification of the World Health Organization (11). A review of such information for 2014, prepared for the general public, is available on the Agency's website (12).

ATCvet classification has become the gold standard for international drug utilization research. It classifies active substances according to system organ class, therapeutic indication and chemical properties. The European Medicines Agency published the Principles on assignment of defined daily dose for animals (DDDvet) in 2015 and defined the course dose for animals (DCDvet) (13). Since DDDvet and DCDvet are technical units, they are intended for the investigation of medicinal consumption. When medicinal products are used during lactation, they are used for the treatment of the affected quarter, and the treatment lasts for two to four days; therefore, such products have been assigned only with DDDvet. When intramammary medicines are administered at dry-off, they are administered concomitantly into all quarters as a single treatment, and they have been assigned with DCDvet only. However, we chose not to present our findings in accordance with these principles because we could not calculate the number of animals exposed to intramammary antimicrobials in the Serbian market in such a manner. DDDvet and DCDvet are calculated on the basis of the usual posology for certain active substances and do not reflect individual variations in authorized posology for medicines containing the same active substances. Therefore, we used data from authorized Summaries of Product Characteristics for each of these products and calculated the number of treatments. On the basis of these data, we estimated the number of cows exposed to intramammary antimicrobial treatment and compared those figures with the total number of cows in the Republic of Serbia, as reported by the Statistical Office of the Republic of Serbia (14). In this way, we could determine the approximate number of treated cows for a certain year, reducing possible errors arising from variations in posology to a minimum. All calculations were performed in Microsoft Office Excel 2007.

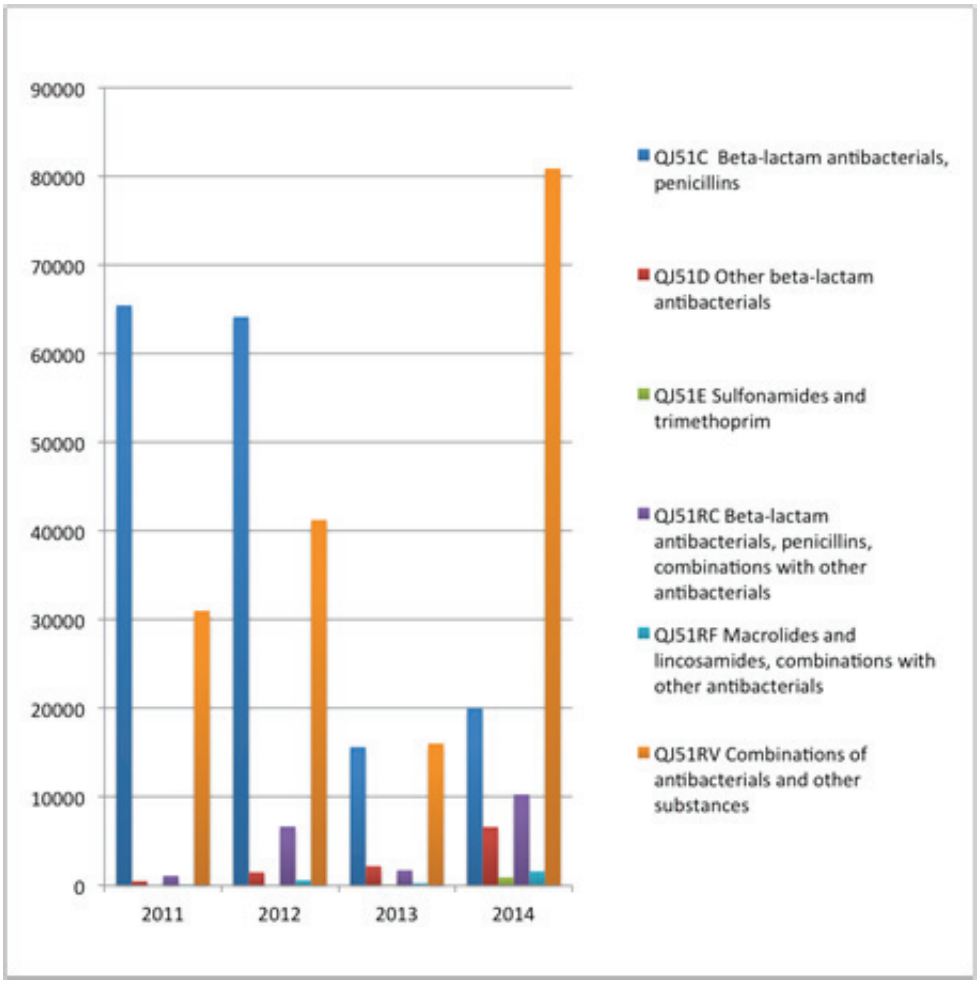

Figure 1: Number of packages sold per ATCvet code

RESULTS

From 2011 to 2014, in the Republic of Serbia, there were between 19 and 25 intramammary antimicrobial medicines with Marketing Authorization (15,16). In the same time period, there were between 451,000 and 509,000 cows in the Republic of Serbia (Table 1). Figure 1 presents the number of packages sold per ATCvet code. Beta-lactam antibiotics from the penicillin group accounted for most packages sold in 2011 and 2012 (65,489 and 64,180 packages, respectively), while antibacterials in combination with other substances were the most used intramammary medicinal products in 2013 and 2014 (16,033 and 80,927 packages, respectively).

Figure 2 presents the number of treated cows in the Republic of Serbia, calculated based on information available from the authorized Summary of Product Characteristics. The total number of treated cows varies between 68,900 animals in 2013 and 120,473 animals in 2012, most of them treated during lactation (44,777 animals in 2013 and 90,129 animals in 2012). As shown in this figure, the number of treated cows corresponded to the number of cows treated during lactation, but the number of cows treated during the dry period gradually declined, from 28,235 in 2011 to 14,110 in 2014. Additionally, we observed

Table 1: Number of registered intramammary antimicrobials and number of cows in 2011-2014

	Number of registered intramammary antimicrobials	Number of cows
2011	21	509000
2012	25	480000
2013	19	451000
2014	24	460000

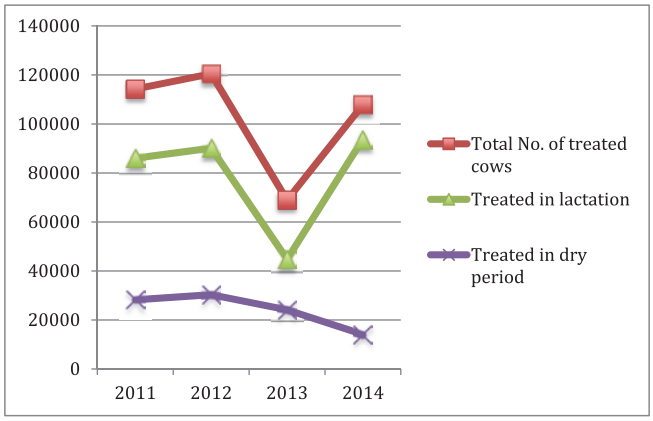

Figure 2: Number of treated cows in RS

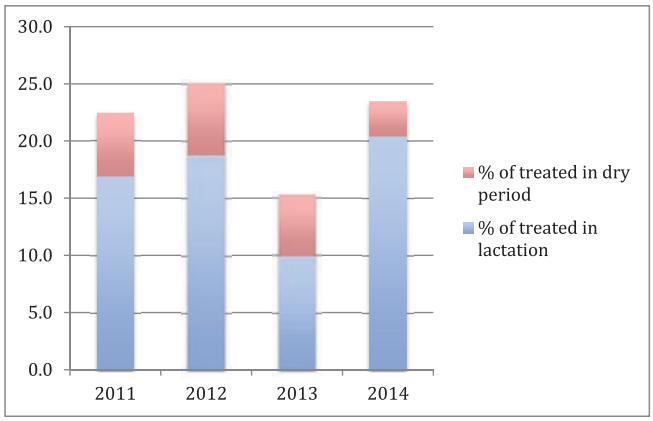

Figure 3: Percentage of treated cows in comparison to total number of cows in RS

that variation in the number of treatments between 2012 and 2013 was the result of variation in the sales of products used during lactation and not in the dry period.

As presented in Figure 3, the percentage of cows treated with intramammary antibiotics compared to the total number of cows in the Republic of Serbia varied between 15% and 25%. This meant that possibly 15% to 25% of milk might contain residues resulting from administration of intramammary antibiotics. This is not the final percentage because a number of animals received parenteral antimicrobial treatment. The percentage of cows treated during the dry period was approximately 5% over the first three years of monitoring (5.3%-6.3%), but the percentage declined to just above 3% in 2014.

The number of treatments per ATCvet group is presented in Figure 4. Beta-lactams, used as a single agent or in combination with other substances, were the most used intramammary products in the Republic of Serbia. In 2014, 80,927 cows were treated with beta-lactams in combination with other substances (ATCvet code QJ51RV). Sulphonamides and trimetoprim were the least used intramammary antibiotics, with only 119 treatments in 2013 and 952 treatments in 2014. Macrolides and lincosamides were also infrequently used, with 103 treatments in 2011, increasing to 1626 treatments in 2014.

DISCUSSION

As our research showed, up to 25% of cows in the Republic of Serbia were treated with intramammary antibiotics over one year. Most of those antibiotics were beta-lactams, whose sensitizing potential is well known. Therefore, prudent use of intramammary antimicrobials is necessary for preserving the health of both animals and humans.

Finding that beta-lactams are the most commonly used intramammary products in the Republic of Serbia was in line with findings from the Danish Integrated Antimicrobial Resistance Monitoring and Research Program (DANMAP) (4), whose report stated that beta-lactama-

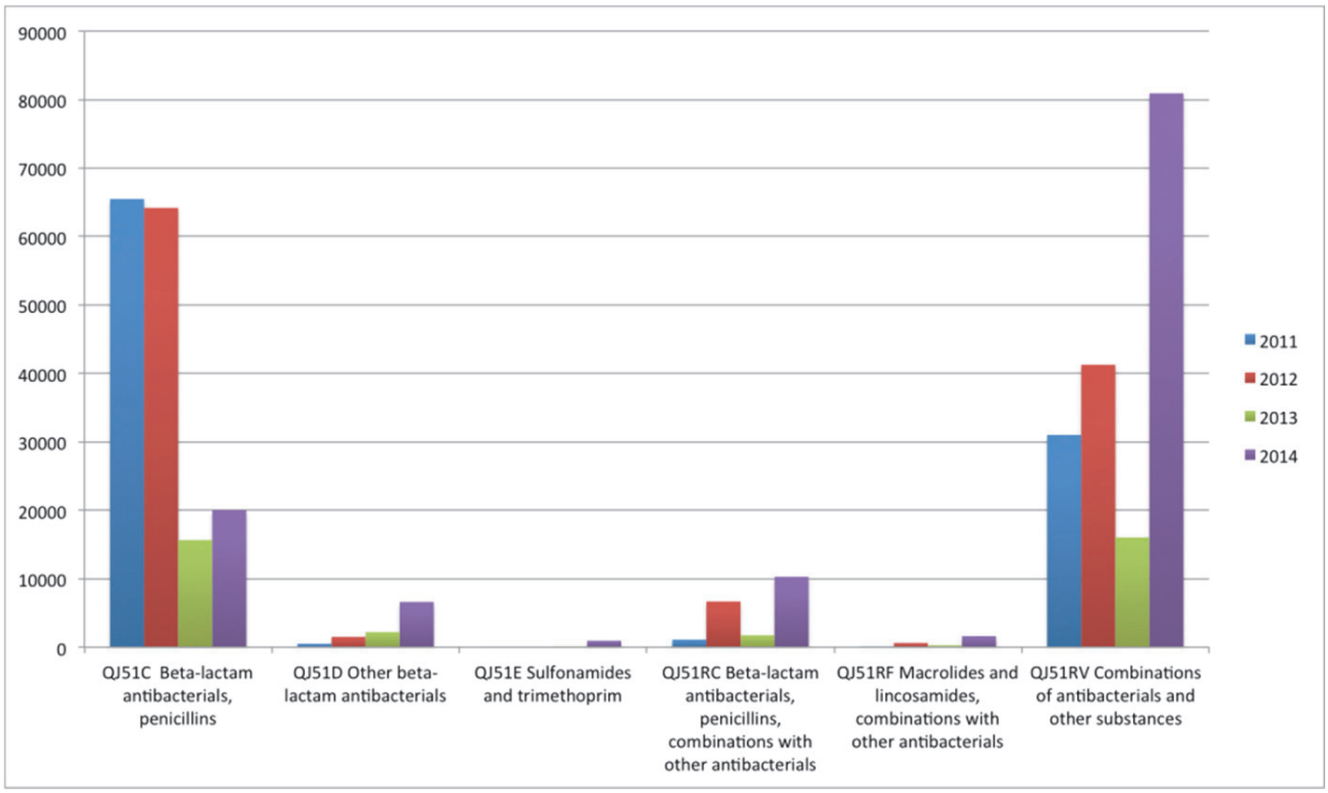

Figure 4: Number of treatments per ATCvet group

se-sensitive penicillin accounted for the majority of antimicrobial consumption in Denmark for the reported time period (2005-2014). It is likely that the results of "milk quality campaign", conducted by the Danish Cattle Association (Agriculture and Food Council) since 2010, aimed to reduce treatment of clinical mastitis by 50%. Furthermore, Order (DK) 785/2010 provided legal regulations on the use of antimicrobial agents for mastitis in cattle (recommending using simple penicillin).

As shown in Figure 1, the number of sold packages varied greatly. This variation could be explained by the fact that Marketing Authorization holders report the number of sold packages of medicinal products to the Medicines and Medical Devices Agency of Serbia on a yearly basis. Larger quantities of medicinal product sold during one year might be the cause of smaller quantities of the same medicinal product sold during the next year. These differences can be observed for 2012 and 2013, when large quantities were on the market in 2012, followed by significantly smaller quantities in 2013.

It was also shown that beta-lactam antibacterials (ATCvet group QJ51C) were the most frequently used intramammary antibiotics in 2011 and 2012, which significantly differs from data for 2013 and 2014, when most packages sold were from group QJ51RV, combinations of antibacterials and other substances. Those other substances are usually steroidal hormones used to reduce inflammation. The reason for this switch from beta-lactams to combination therapies is likely due to because one large domestic manufacturer discontinued the manufacturing of veterinary medicines, including intramammary ones, and other medicine manufacturers took over the market.

Furthermore, the number of sold packages does not reflect the number of treated cows since some medicinal packages contain 20 or more intramammary injectors and others contain a single injector in the package. The posology also differs, and such information is available in the Summary of Product Characteristics (SmPC) for each registered product. For that reason, to determine the exposure of the animal population to intramammary antibiotics, it was necessary to calculate the number of treated cows to estimate the exposure of animals to such medicines. However, these figures are not final and represent only an approximate value since they are based on medicinal quantities reported by Marketing Authorization Holders. Collecting data from veterinary institutions and end users could provide more accurate information on medicine usage.

These results provide important information about exposure of cattle to certain antibiotics and, therefore, direct future research towards investigating a linkage between antibiotic resistance and administration of intramammary veterinary medicinal products. Beta-lactams are the most frequently used intramammary antimicrobials in the Republic of Serbia and, therefore, their residues represent a major concern that needs to be closely monitored and further investigated.

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TEMPORAL VARIATIONS OF STROKE OCCURENCE

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VREMENSKE VARIJACIJE UČESTALOSTI MOŽDANOG UDARA U KLINIČKOM CENTRU KRAGUJEVAC

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ABSTRACT

Stroke is one of leading causes of death worldwide. Different frequency of stroke occurence is observed in days of the week and months in the year, and incidence of stroke has irregular time pattern. We analyzed 516 patients who had acute stroke and were treated in Clinic of Neurology, Clinical Center Kragujevac from January 1, 2013 to January 1, 2014, mean age 72,11±11,52. Statistical analysis is conducted out using the SPSS software version 20.0. We used descriptive statistic, student T-test, chi-square or Fisher exact test.

Friday is day we found the most IS and all stroke types occurences, and Wednesday is day we found the most IS in men. We found the most strokes in women younger than 65 years on Wednesday, but in women older than 65 years on Friday. Monday is day with the most admissions to hospital for patients with IS, and we observed that there is average delay in the refering to the doctor for 1.80±1.44 days. Friday is the day with the most ICH symptom beginings and the most admissions to the hospital, and Saturday is the day with the least symptom beginings and admissions to the hospital in the case of IS and ICH. The most IS occured in winter (in Decembar), and the least in summer (in August). The most ICH occured in May, and the least in July and October.

We confirmed that there is a significant weekly variability in the IS symptom onset day.

Keywords: *stroke, variations, occurence, time pattern, symptom onset*

SAŽETAK

Moždani udari su jedan od vodećih uzroka smrtnosti širom sveta. Različita učestalost pojave moždanog udara je praćena prema danima u nedelji i mesecima u godini, i primećeno je da njihova incidenca ima nepravilan vremenski obrazac. Analizirali smo 516 pacijenata koji su imali moždani udar i lečeni u Klinici za neurologiju Kliničkog centra u Kragujevcu od 1. januara 2013. do 1. januara 2014. godine, čija je prosečna starost bila 72,11±11,52 godine. Statistička obrada podataka je sprovedena pomoću SPSS softvera, verzija 20.0. Korišćena je deskriptivna statistika, studentov T-test, Hi-kvadrat test i Fišerov test. Petak je dan kada se dešava najveći broj moždanih udara svih tipova, a sredom su najčešći ishemijski moždani udari kod muškaraca. Žene mlađe od 65 godina češće oboljevaju od moždanog udara sredom, a starije od 65 godina petkom. Najveći broj pacijenata sa ishemijskim moždanim udarom je hospitalizovano ponedeljkom u Kliniku za neurologiju, i utvrđeno je da postoji kašnjenje u vremenu hospitalizacije u odnosu na početak simptoma moždanog udara koje iznosi u proseku 1.80±1.44 dana. Najveći broj pacijenata sa hemoragijskim moždanim udarom je hospitalizovano petkom, istog dana kada su se javili prvi simptomi bolesti. Subotom se dešava najmanji broj ishemijskih i hemoragijskih moždanih udara, i to je dan kada je zabeležen najmanji broj hospitalizacija. Najviše ishemijskih moždanih nastaje u zimskom periodu, u decembru, a najmanje tokom leta, u avgustu. Najviše hemoragijskih moždanih udara je zabeleženo u maju, a najmanje u julu i oktobru. Zaključeno je da postoji značajna vremenska varijabilnost u učestalosti moždanih udara.

Ključne reči: *moždani udar, varijacije, učestalost, vremenski obrazac, početak simptoma*

ABBREVIATIONS

IS - ischemic stroke,
ICH - intracerebral hemorrhage,
SAH- subarachnoid hemorrhage.

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INTRODUCTION

Stroke is one of leading causes of death worldwide (1, 2, 3). Studies were conducted in many countries and reported that incidence of stroke has irregular time pattern, and that different frequency of occurence is observed in days of the week and in months of the year (4, 5). Analysis on temporal pattern of stroke occurence can help in clarification of mehanisms that we can consider as trigers of this diseases occurence (2, 4, 6, 7).

Ischemic stroke (IS), intracerebral hemorrhage (ICH) and subarachnoid hemorrhage (SAH) are differnt in etiology, and consequently, it is expected they have different incidence according to time variations (8).

Different occurence of all stroke types is found among men and women of different age (3). Some studies showed Sunday is the day with the lowest incidence of stroke (9, 10). Some of studies found that Monday is the day with the highest IS incidence (2, 4), another found it is Wednesday (3). Monday is the day when occured one-third of total ICH in both genders (5), some of them showed that the most frequent occurence of IS were obseved on Monday in men. Also, the most ICH were observed on Monday and the most IS were observed on days of weekend in women (6). In patients younger than 60 was observed higher frequency of all stroke types on Mondays compared to weekend days (5).

Previous studies showed differencies in time of delay of admission to hospital for IS, some of them found it was 8.8 hours, other 3 hours and other more than 24 hours (11, 12, 13).

Some studies showed that stroke occurence was more frequent on weekdays than on weekends (6, 7, 9, 14).

Some studies showed the highest incidence of IS in January and the lowest incidence for women in August, and for men in June (15). Other studies showed the highest incidence in January for all stroke types (15), and in Septembar (16), while the highest incidence of ICH is in February but the lowest in August. (5)

The incidence was higher in the summer season for IS (16), other studies showed the highest incidence of IS during the winter (5), or in autumn (17).

The aim of this study was to analyze weekly and monthly variations in incidence of IS, ICH and SAH. Knowledge of temporal variations can be important for healt care profesionals and for organisation of work in stroke units.

MATERIAL AND METHODS

This is opservational and cohort, preliminary, retrospective, one-year study. We observed all patients who had acute stroke in 2013 and were treated in Clinic of Neurology, Clinical Center Kragujevac.

Diagnosis of stroke is determined by anamnesis, neurological examination and appropriate diagnostic procedures, involving computed tomography (CT) or magnetic resonance imaging (MRI).

Table 1. Distribution of patients by gender and diagnosis

diagnosis	both gender	male	female
IS	415	209	206
ICH	88	44	44
SAH	13	7	6
Total	516	260	256

We analized 516 patients (table 1).

We collected general demografic data (gender, age), date of symptom onset and date of admission to the Clinic of Neurology. According to stroke type patients were divided into three groups (IS, ICH, SAH), and based on anamnesis of former stroke were divided into two groups (first stroke and previous stroke).

Patients were divided into seven groups acording to the day of symptom onset (Monday, Tuesday, Wednesday, Thursday, Friday, Saturday and Sunday), also they were divided into twelve groups acording to month of the year (January, February, March, April, May, Jun, July, August, September, October, November and December), and into four groups acording to season (winter, spring, summer and autumn).

Descriptive statistic is used to present distribution of patients by days, months and seasons. We used student T-test to compare mean age of patients. Comparing categorical variables is carried out using a chi-square or Fisher exact test. Distribution of patients is examined separately for each stroke types. Statistically significant is $p < 0.05$. Statistical analysis is carried out using the SPSS software version 20.0.

RESULTS

From January 1, 2013 to January 1, 2014 we registered 516 patients with all stroke types, 260 (50,4%) male and 256 (49,6%) female. Mean age of all patients by diagnosis and gender is shown in table 2.

We found that male patients were significantly younger than female patients in total group, and also in group with IS.

There were 430 patients with first stroke (83,3%), and 86 patients (16,7%) with previous stroke. We observed day of symptom onset in 489 patients (94,76%), and in 27 patients (5,23%) there was no reliable data about time of symptom onset.

Table 2. Mean age for patients by diagnosis, and gender

diagnosis	both gender	gender		*p*
		male	female	
IS	72,94±11,22	69,80±12,60	76,13±8,53	0.000
ICH	69,83±11,94	66,61±11,03	73,05±12,08	0.011
SAH	60,77±11,06	59,14±12,79	62,67±9,44	0.589
Total	72,11±11,52	68.98±12.476	75.29±9.489	0.000

Table 3. Distribution of patient's first stroke symptoms according to the stroke type, day of the week, gender, and age

diagnosis	day of the week	gender		age		total
		male	female	≤65 years	>65 years	
Ischemic stroke	Monday	30	27	18	39	57
	Tuesday	33	29	9	53	62
	Wednesday	35	27	16	46	62
	Thursday	16	24	9	31	40
	Friday	32	39	15	56	71
	Saturday	16	18	5	29	34
	Sunday	32	34	13	53	66
	total	194	198	85	307	392
	p	0,693		0,326		0,02
Intracerebralhemorrhage	Monday	7	6	4	9	13
	Tuesday	10	7	8	9	17
	Wednesday	6	5	3	8	11
	Thursday	3	6	1	8	9
	Friday	9	9	5	13	18
	Saturday	0	5	0	5	5
	Sunday	7	4	6	5	11
	total	42	42	27	57	84
	p	0,276		0,188		0,118
Subarachnoid hemorrhage	Monday	1	0	1	0	1
	Tuesday	2	0	1	1	2
	Wednesday	1	3	3	1	4
	Thursday	0	0	0	0	0
	Friday	1	2	3	0	3
	Saturday	2	0	2	0	2
	Sunday	0	1	0	1	1
	total	7	6	10	3	13
	p	0,199		0,310		0,676
All stroke types	Monday	38	33	23	48	71
	Tuesday	45	36	18	63	81
	Wednesday	42	35	22	55	77
	Thursday	19	30	10	39	49
	Friday	42	50	23	69	92
	Saturday	18	23	7	34	41
	Sunday	39	39	19	59	78
	total	243	246	122	367	489
	p	0,967		0,560		0,000

In the table 3 we showed distribution of stroke occurence by gender and age in patiens with all stroke types, considering to time of symptom onset.

For the day of the week of stroke onset, statistic significantly difference was found for all stroke types (χ^2=28.642, df 6, p=0.000). The most stroke occurences were observed on Friday (17.8%), and the least on Saturday (7.9%).

We also found statistic significantly difference for IS occurence (χ^2=20.321, df 6, p=0.02). Most IS were found on Friday (18.1%), and the least on Satrday (8.2%). Statistic significance didn't reach for ICH occurence, although we found that most ICH occured on Friday and the least on Saturday. We found no statistic significance for SAH because of small sample.

There was statistic significantly difference of men who had IS by the day of symptom onset (χ^2=14.340, df 6, *p*=0,026). For the most of men first symptoms began on Wednesday (16.7%), and for the least on Thursday (7,7%) and Saturday (7,7%). Statistic significance wasn't found in men who had ICH (χ^2=4.286, df 5, *p*=0.509), and SAH (χ^2=0.857, df 4, *p*=0.931).

We found no statistic significant difference in the day of symptom onset for women regardless of stroke type – for IS (χ^2=9.737, df 6, *p*=0.136), for ICH (χ^2=2.667, df 6, *p*=0.849), and for SAH (χ^2=1.000, df 2, *p*=0.607). According to results Friday was day with the most IS (19.7%) and ICH occurence (21.4%).

We found statistic significantly difference in women older and yonger than 65 years (χ^2=13.520, df 6, *p*=0.035). Most women younger than 65 years noticed symptom begining on Wednesday, but women older than 65 years noticed first symptoms mostly on Friday. We found that the most male younger

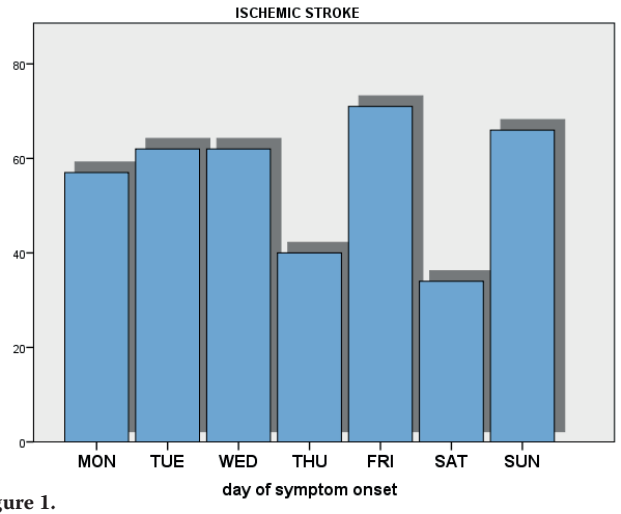
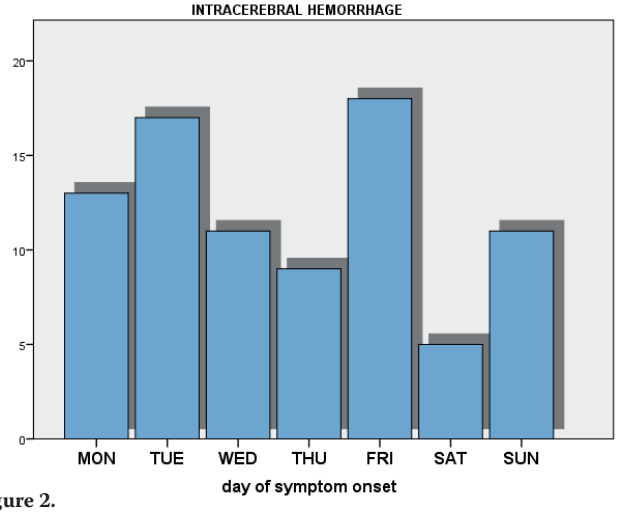


Figure 1.



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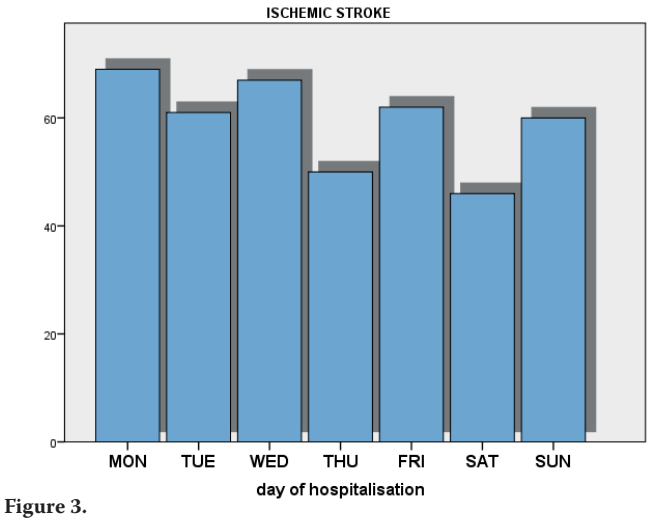

Figure 3.

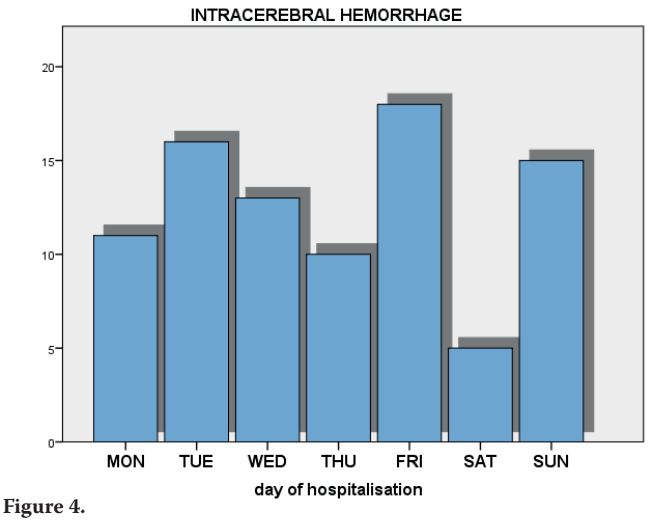

Figure 4.

than 65 years had symptom onset on Monday, but older than 65 years mostly had symptom begining on Friday, but there was no significant difference (χ^2=6.086, df 6, p=0.414).

According to our results, the most patients with IS and ICH had symptom onset on Friday (Fig. 1, 2). The most hospitalizations were realised on Monday for IS (Fig. 3). The most patients with ICH were hospitalized on Friday (Fig. 4), and that is also a day with most symptom onsets for ICH were observed. Saturday is a day when we noticed the smallest number of stroke onsets, and the smallest number of hospitalized patients who had IS and ICH (Fig. 1, 2 , 3, 4).

According to how many days passed between the date of symptom onset and the date of hospitalisation, we figured our that in total of 489 patients who we could get reliable data abuot the day of first symptoms, 389 patients (79,6%) were hospitalized in the day of symptoms beginning, 57 patients (11.7%) were hospitalized one day after the day of symptoms begining, and 19 patients (3.9%) two days after. We excluded six cases who were hospitalized seven or more days after first symptom, and average delay for remaining 94 patients is 1,78±1,34.

Patients with IS had average delay 1.80±1.44, and patients with ICH 1.71±0.726. There is no statistically significant difference between those two groups of patients (p=0.833). We observed that of total 392 patients with IS 84 patients had delayed hospitalisation (21.42%), and of total 79 patients with ICH there were 14 patients who had delayed admission to hospital (17.72%).

We found no statistically significant differenties among weekend and weekday distribution of occurence for all stroke types ($\chi^{2=}$0.943, df 2, p=0.624).

In the table 4 we showed distribution of IS occurence by month and season, gender and age. Statistic analysis by all stroke types showed no significant variations in seasons when disease occured – for IS ($\chi^{2=}$6.619, df 3, p=0.085), ICH ($\chi^{2=}$4,273, df 3, p=0.233), and for SAH ($\chi^{2=}$2,077, df 3, p=0.557). There are no significant variations in months – for IS ($\chi^{2=}$11.449, df 11, p=0.406), ICH ($\chi^{2=}$8.545, df 11, p=0.664) and for SAH ($\chi^{2=}$2,923, df 8, p=0.939). Although statistic significance did not reach, we noticed that there were the most IS in winter (30.12%), in Decembar (11.32%), and the least in summer (21.68%), in August (6.26%). The most ICH occured in May (13.63%), and the least in July (4.54%) and October (4.54%).

DISCUSSION

Studies around the world were conducted to determine the variability in the occurrence of stroke according to the

Table 4. Distribution of patients with IS by month and season of stroke occurence, gender and age

IS	gender		age		total
month	M	F	≤65	>65	
December	25	22	12	35	47
January	19	18	10	27	37
February	19	22	8	33	41
Winter	63	62	30	95	125
p	0,929		0,796		0.544
March	39	19	20	7	39
Apryl	35	15	20	10	35
May	29	19	10	6	29
Spring	103	53	50	23	103
p	0,768		0,531		0.478
June	31	16	15	6	31
July	31	14	17	8	31
August	26	18	8	8	26
Summer	90	49	41	24	90
p	0,399		0,715		0.753
September	29	12	17	7	29
October	32	16	16	8	32
November	37	16	21	5	37
Autumn	97	44	53	19	97
p	0.361		0,218		0.607

day of the week. We found that Friday was a day when the most patients with IS had symptom onset. Monday was a day with the most hospitalized patients with IS. For patients with ICH Friday was day with most symptom onsets and most hospitalisations. In this sample were not considered patients with SAH because of their small number.

The most of studies shows that Monday is the day with the highest incidence (2, 4, 8, 9), and our results showed the most hospitalized patients on Monday, but Friday is a day with most symptom onsets. According to results of some studies Sunday is the day with the lowest incidence of stroke (9, 10), but we found Saturday as a day when occured the least number of patients with symptom onset and the least number of hospitalized patients with IS and ICH.

Some studies showed a different occurence of all stroke types among men and women of different age. The most patients with IS in study conducted in Nis, Serbia, was found on Wednesday (3). Monday is the day when occured one-third of total ICH in both genders (5), but we found that Friday is leading for symptom onset and day of hospitalisations in patients with ICH. Wednesday is day when occured most IS in men in uor study, while some studies showed that the most frequent occurence of IS were obseved on Monday in men. The most ICH were observed on Monday and the most IS were observed on days of weekend in women (6). According to our results Friday was day with the most IS occurence in women. Frequency of all stroke types was higher on Mondays compared to weekend days in patients younger than 60 (5). Our results showed that in patients younger than 65 years, symptom onset is mostly noticed on Wednesday in women, and on Monday in men. Patients older than 65 years mostly had symptom onset on Friday.

We found that 20.4% of all patients were not hospitalized on the day of symptom onset, but they were hospitalized in next days. Comparing our results with other studies, there is a divergence in delay times of admission to hospital. Some of them found time of delay 8.8 hours for ischaemic stroke , other 3 hours (11, 12). In Minesota study results showed that half patients arrived within 3 hours of symptom onset, and 90% arrived within 24 hours. Patients with approximated delay times had longer delays, and less than 40% of these patients were hospitalized within 24 hours of symptom onset. They explain this by the ethnicity, which involves different cultural patterns. (13).

According to some studies stroke occurence was more frequent on weekdays than on weekends (6), and usually on Mondays (7, 9, 14). Some studies found no difference in the frequency of occuring of all stroke types by days of the week (2), some of them found no difference for ICH and SAH (4, 7). We found no statistical signifant differenties among weekend and weekday distribution for all stroke types.

Some studies showed that the highest incidence of IS was in January, but in our study most IS occured in December although we found no statistic signifficantly difference. We found the least incidence in August for both gender, and other studies found the lowest incidence for women in August, and for men in June (15). Other studies showed the highest incidence in January for all stroke types (15), and in Septembar (16). The highest incidence of ICH is found in February but the lowest in August (5), and we found that May is month with the most ICH, and July and October with the least ICH occurences.

The incidence was higher in the summer season compared to the winter for IS, while the difference has not been established for hemorrhagic stroke (16). Other studies showed the highest incidence of IS during the winter (5), as we found, another in autumn (17).

In our country, it is necessary for health promotion strategies to improve community awareness of early symptoms of stroke. Constantly informing the public of the need to seek medical help promptly after stroke onset and using an ambulance and direct transportation to the hospital. Also it is necessary to have more effective in-hospital organization in order to improve availability of effective acute treatment options to stroke patients (14).

Those are only a preliminary results of our study whose limitation is decreased power to detect variabilities because of small sample and short duration of research. It shold be confirmed with future investigations and higher number of patients.

In conclusion, we confirmed that there is a significant weekly variability in the IS symptom onset day.

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FATIGUE IN PATIENTS WITH AUTOIMMUNE THYROID DISEASES

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ZAMOR KOD PACIJENATA SA AUTOIMUNSKIM BOLESTIMA ŠTITASTE ŽLEZDE

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ABSTRACT

Fatigue is a common feature in a wide variety of chronic inflammatory and autoimmune diseases, but fatigue in autoimmune thyroid disease (AITD) has not been investigated so far. The aim of this study was to examine fatigue in patients with AITD and to analyse the correlation between fatigue and the serum concentrations of thyroid antibodies, thyroid function and depression. This cross-sectional clinical study included 62 patients with increased concentrations of thyroperoxidase antibodies (TPOAbs) as confirmation of AITD and 52 healthy individuals who were negative for thyroid antibodies; all controls were euthyroid. Thyroid antibodies, free thyroxine and thyroid-stimulating hormone were measured in the sera of all subjects. The Fatigue Severity Scale was used to measure the severity of fatigue; the level of depression was measured by the Beck Depression Inventory. Eight (12.9%) patients had evident fatigue, 7 (11.3%) patients had fatigue limit values, and 47 (75.8%) patients had no fatigue. The frequency of fatigue was highly significant and almost three times higher in the AITD patients compared to the control group, in which only 2 (3.8%) patients had evident fatigue. The majority of patients with fatigue had normal thyroid function, and only one (1.6%) patient had overt hypothyroidism. Seven (11.3%) patients had both fatigue and depression, whereas one (1.6%) patient had fatigue without depression. We did not find significant correlations between fatigue and the concentrations of thyroid antibodies, but we found statistically significant correlations between fatigue and depression in AITD patients.

Keywords: *antibodies, depression, fatigue, thyroid disease, thyroid function*

SAŽETAK

Zamor je simptom koji prati mnoge hronične inflamatorne i autoimunske bolesti, međutim, učestalost zamora kod osoba sa autoimunskim bolestima štitaste žlezde (engl. autoimmune thyroid disease, AITD) do sada nije ispitivana. Cilj ovog rada je da se ispita postojanje zamora kod osoba sa AITD i da se analizira da li je težina zamora povezana sa serumskim koncentracijama anti-tireoidnih antitela, tireoidnom funkcijom i/ili depresijom. Kod svih ispitanika izmerene su serumske koncentracije anti-tireoidnih autoantitela, slobodnog tiroksina i tireostimulišućeg hormona. U eksperimentalnu grupu su uključena 62 pacijenta sa AITD i povećanom koncentracijom autoantitela specifičnih za tireoidnu peroksidazu (engl. thyroperoxidase antibodies, TPOAbs), a u kontrolnu grupu 52 eutireoidna ispitanika bez povećanih koncentracija TPOAbs. Za procenu stepena zamora korišćena je Skala težine zamora, a nivo depresije meren je primenom Beck-ove skale depresivnosti. Kod osam (12,9%) pacijenata sa AITD utvrđeno je evidentno postojanje zamora, 7 (11,3%) pacijenata sa AITD imalo je granične vrednosti, dok kod 47 (75,8%) pacijenata sa AITD nije utvrđeno postojanje zamora. Zamor je skoro tri puta učestaliji kod pacijenata sa AITD u odnosu na kontrolnu grupu, u kojoj je zamor utvrđen kod 2 (3,8%) ispitanika. Kod većine pacijenata sa zamorom tireoidna funkcija je bila normalna, a samo jedan pacijent (1,6%) imao je tešku hipotireozu. Kod sedam (11.3%) pacijenata sa AITD zamor je bio udružen sa depresijom, a samo jedan (1,6%) pacijent imao je zamor bez depresije. Korelacija između stepena zamora i koncentracije anti-tireoidnih antitela nije pokazana, dok statistički značajna korelacija između zamora i depresije kod pacijenata sa AITD postoji.

Ključne reči: *antitela, depresija, zamor, bolesti štitaste žlezde, tireoidna funkcija*

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INTRODUCTION

Fatigue is defined as an overwhelming sense of tiredness, a lack of energy and a feeling of exhaustion (1, 2). Fatigue is a common feature of a wide variety of diseases, including chronic inflammatory, infectious, neurological, and psychiatric diseases, and cancer (2, 3). The overall prevalence of chronic fatigue depends on the type of instrument used to measure fatigue (4). In many cases, fatigue is associated with inflammation, including both acute infectious and chronic inflammatory disorders. Fatigue is a common feature among patients with multiple sclerosis (5), rheumatoid arthritis (RA) (6), systemic lupus erythematodes (SLE) (7), primary Sjögren's syndrome (8) and autoimmune thrombocytopenia (9).

To our knowledge, this is the first study of fatigue in patients with autoimmune thyroid disease (AITD). The aim of this study is to investigate fatigue in patients with AITD and to determine whether there is a correlation between the symptoms of fatigue and the serum concentrations of thyroid antibodies (Abs) or with the thyroid function. Because depression is strongly associated with fatigue and thus may affect the measurement of fatigue as a confounding factor (2), an additional aim of this study is to examine the prevalence of depression in patients with AITD and to determine whether fatigue and depression in patients with AITD are associated.

MATERIALS AND METHODS

Study population

The research was conducted at the Centre of Nuclear Medicine and the Clinic of Neurology, Clinical Centre Kragujevac, in accordance with the Declaration of Helsinki (2000) of the World Medical Association. This study was approved by the Ethics Committee of the Clinical Centre Kragujevac.

The study included 62 patients who showed increased concentrations of thyroperoxidase antibodies (TPOAbs) as confirmation of AITD. After the finding of increased TPOAbs concentrations, patients were informed about the study protocol, and consent was obtained from each subject before they were included in further testing. The control group consisted of 52 healthy individuals who were negative for thyroid antibodies, and all controls were euthyroid.

Methods

The concentrations of thyroglobulin antibodies (TgAbs) and thyroid function (free thyroxine and thyrotropin) were evaluated in all study participants. All blood samples had been obtained originally for diagnostic purposes. Blood samples (10 ml) from each subject were taken by venepuncture, and the serum was separated by centrifugation at 2000 rpm for 15 minutes. The sera were divided into separate tubes for each analysis, stored frozen at -20°C, then thawed and assayed.

The concentration of TPOAbs was determined by a radioligand assay (TPO-Ab-CT, *Cis-Biointernational*, France) according to the manufacturer's instructions. The lower detection limit for this assay was 8 U/ml. The measured TPOAb concentrations were analysed towards the value of 130 U/ml, whereas autoantibody concentrations higher than 130 U/mL were considered "increased".

The concentration of TgAb was determined by a competitive "one-step" radioimmunoassay (TgAb I step, *Cis-Biointernational*, France). The method was calibrated against the WHO First International Reference Preparation CRM 65/93 and had an analytical detection limit of 6.0 IU/ml. Autoantibody concentrations higher than 30 IU/mL were considered "increased".

The concentration of free thyroxine (fT4) was determined by a radioimmunoassay (*Cis-Biointernational*, France). The detection limit for this assay was 0.5 pg/ml, and the reference range was 7-18 pg/ml.

The concentration of thyrotropin (TSH) was determined by an immunoradiometric assay (IRMA TSH, Zemun, Serbia), with a detection limit of 0.056 mIU/L and reference range of 0.3-5.5 mIU/L.

The Fatigue Severity Scale (FSS) (10), which focuses on the physical symptoms of fatigue, was used to assess the severity of fatigue. FSS contains a total of 9 questions, and all subjects rated their responses on the scale from 1 to 7, where 1 represents disagreement and 7 represents complete agreement with the statement. The subjects were assigned to one of two groups on the basis of FSS scores. One group included patients with fatigue (F), which was identified by an FSS score of at least 5, and the other group included patients without fatigue (WF), which was identified by an FSS score of 4 or less. Patients with FSS scores between 4.1 and 4.9 were classified in the marginal group. Patients in the marginal group were excluded from the between-group analyses, although their scores were included in the correlational analysis of fatigue severity. Patients diagnosed with chronic systemic connective tissue diseases, anaemia, tumours, liver disease or kidney disease were excluded from the study. Patients who had taken psychoactive medication (e.g., steroids, amantadine or antidepressants) in the previous two months that might have affected fatigue were also excluded.

Depression was diagnosed using the DSM-IV criteria for depressive symptoms (American 1994), and the level of depression was measured by using the Beck Depression Inventory (BDI) (12). This scale contains 21 questions that refer to the patient's mood in the last 4 weeks. The measured dimensions include cognitive, somatic and motivational aspects of depression.

Statistical analysis

The data were analysed using descriptive statistics, the Mann-Whitney test, Spearman correlation, the Kruskal-Wallis test and binary logistic regression. Statistical sig-

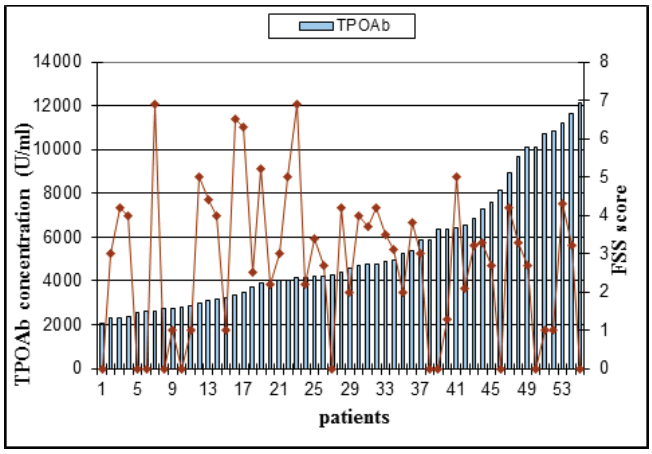

Figure 1. TPOAbs and fatigue in patients with AITD

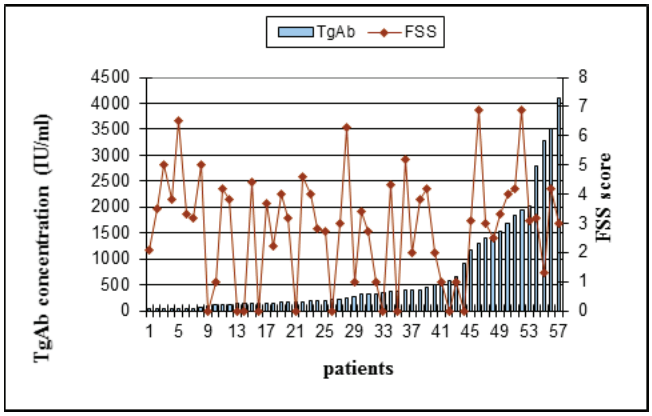

Figure 2. TgAbs and fatigue in patients with AITD

nificance was accepted when the probability (p) was less than or equal to 0.05. SPSS 10.0 and the Microsoft EXCEL programme were used for the analyses.

RESULTS

Fatigue and thyroid autoantibodies

The study included 62 patients (60 women, 96.8%; 2 men, 3.2%) with autoimmune thyroid disease. The mean age was 51.6 (SD 12.45) years. The youngest patient was 22 years old, and the eldest was 75 years old. All patients had increased serum concentrations of TPO antibodies, and the average value of TPOAb was 4891 (SD 2921) U/mL (minimal concentration 280 U/ml, maximum 12125 U/ml). The control group consisted of 52 healthy subjects without TPOAbs and TgAb (46 women, 88.5%; 6 men, 11.5%). The mean age was 45 (SD 10.46) years.

From a total of 62 patients, eight (12.9%) patients had evident fatigue (FSS>5), 7 (11.3%) patients had fatigue limit values (FSS=4.1-4.9) and 47 (75.8%) patients had no fatigue (FSS≤4). The fatigue frequency was highly significant ($p<0.01$), and almost three times higher in AITD patients compared to the control group, in which only 2 (3.8%) subjects had evident fatigue. The Kruskal-Wallis test showed no significant difference in the concentration of TPOAbs between patients with and without fatigue (pTPOAb=0.733). There was no significant correlation between the concentration of TPOAbs and fatigue (pFSS=0.338, Spearman coefficient rFSS= -0.124). The relationship between TPOAbs and fatigue in all 62 patients is shown in Figure 1.

The majority of the patients (57/62, 91.9%) had an increased concentration of TgAb, with an average TgAb value of 679 (SD 936) IU/ml (minimal concentration 36 IU/ml, maximum 4112 IU/ml). There was no significant correlation between the concentration of TgAbs and fatigue (pFSS= 0.773, Spearman coefficient rFSS= +0.037). The relationship between TgAbs and fatigue in all patients is shown in Figure 2.

Fatigue and thyroid function

The testing revealed that 49 (79%) subjects had normal thyroid function, 5 (8.1%) patients had subclinical hypothyroidism, 3 (4.8%) patients had overt hypothyroidism, 2 (3.2%) had subclinical hyperthyroidism and 3 (4.8%) patients had overt hyperthyroidism (Table 1). Table 1 shows that the majority (7/62, 11.3%) of patients with fatigue at the time of testing had normal thyroid function, whereas only 1/62 (1.6%) patients had overt hypothyroidism. When

Table 1. Fatigue and thyroid function in patients with AITD

Thyroid function/ fatigue	Without fatigue (FSS ≤ 4)	Fatigue limit values (FSS = 4.1-4.9)	With fatigue (FSS ≥ 5)	Total
Euthyroidism	36 (58%)	6 (9.7%)	7 (11.3%)	49 (79%)
Subclinical hypothyroidism	5 (8.1%)	0	0	5 (8.1%)
Overt hypothyroidism	1 (1.6%)	1 (1.6%)	1 (1.6%)	3 (4.8%)
Subclinical hyperthyroidism	2 (3.2%)	0	0	2 (3.2%)
Overt hyperthyroidism	3 (4.8%)	0	0	3 (4.8%)
Total	47 (75.8%)	7 (11.3%)	8 (12.9%)	62 (100%)

Table 2. Fatigue and depression in patients with AITD

Depression/ Fatigue	Without fatigue (FSS ≤ 4)	Fatigue limit values (FSS = 4.1-4.9)	With fatigue (FSS ≥ 5)	Total
With depression	21 (33.9%)	3 (4.8%)	7 (11.3%)	31 (50%)
Without depression	26 (41.9%)	4 (6.5%)	1 (1.6%)	31 (50%)
Total	47 (75.8%)	7 (11.3%)	8 (12.9%)	62 (100%)

the relationships of fatigue and the concentration of free thyroxine were analysed, a significant correlation was revealed between the degree of fatigue and the concentration of fT4 (pFSS= 0.040, Spearman's coefficient r=-0.261), meaning that as the concentrations of fT4 increase the fatigue level decreases. However, the linear regression revealed that the strength of connections between fT4 and FSS was rather weak (p=0.047).

Fatigue and depression

Half of the AITD patients (31, 50%) had depression (BDI>9), where none of the subjects in the control group showed evidence of depression. Among the ATID patients, 7/62 (11.3%) had fatigue and depression, whereas only one (1.6%) had fatigue but no depression (this patient had normal thyroid function) (Table 2). There was a significant correlation between fatigue and depression among our AITD patients (p=0.007, Spearman's coefficient r=0.342).

DISCUSSION

This study examined the frequency of fatigue in patients with AITD and analysed whether the occurrence of fatigue among these patients was correlated with the serum concentration of thyroid antibodies or thyroid function. To our knowledge, no such study has been performed until now.

We have shown that 8 (12.9%) patients with AITD had fatigue, and this was almost three times the number of healthy control group subjects with fatigue. According to data reported in the literature, fatigue is a common problem with a prevalence that varies depending on the definition, duration, setting (13, 14), and study population. Therefore, fatigue is present in 6% of the control subjects without cancer, in comparison with more than 33% of patients with cancer (3). The frequency of fatigue among patients in primary care varies among studies; frequencies of 11.3% (4), 19% (15), or from 10% to 40% have been reported (13, 14). However, it should be noted that a portion of primary care patients have a disease that is accompanied by fatigue (e.g., systemic connective tissue diseases, anaemia, tumours, liver and kidney diseases, patients on psychoactive medication), and such patients were excluded from our study.

However, if the prevalence of fatigue in our patients with AITD is compared with data in the literature related to chronic inflammatory connective tissue diseases, then the fatigue among our patients is less frequent than that among patients with SLE, RA or primary Sjögren's syndrome. The prevalence of fatigue is as high as 81% in patients with systemic lupus erythematosus (7), 42% in patients with RA (6), 68% in patients with Sjögren's syndrome (16) and 37% in patients with immune thrombocytopenic purpura (9). The majority of patients with multiple sclerosis (53–87%) have fatigue (17).

In our study, we have shown that the occurrence and severity of fatigue does not depend on the concentration of TPOAbs or on the concentration of TgAbs. Although multiple mechanisms are involved in the damage of the thyroid tissue in AITD, especially cellular immunity (18-20), high concentrations of thyroid antibodies in the serum of patients with AITD indicate the activity of the autoimmune process (19). Therefore, the fatigue in our patients with AITD is not correlated with disease activity. Similar results have been obtained in the majority of studies related to fatigue in autoimmune diseases. The relationship between fatigue and activity of SLE is controversial (21-23), while fatigue in RA was related to pain and functioning but not inflammation (6). Given that patients with AITD have not expressed incapacitating clinical symptoms, especially if the thyroid function is unchanged, then a similar mechanism cannot be a cause of fatigue in our patients.

Thyroid dysfunctions may be accompanied by numerous neurological (24, 25) and psychiatric disorders (26, 27), the most well-known being cognitive impairment and depression in hypothyroid patients (28, 29). Considering that the majority of subjects with fatigue in our study had normal thyroid function, we could preliminarily conclude that hypothyroidism alone cannot account for the fatigue in our study. When we tested the relationship between fatigue and the concentration of free thyroxine, we found that there was a statistically significant correlation between the degree of fatigue in our patients and the concentration of fT4; therefore, the fatigue level decreased as the concentration of fT4 increased. However, the strength of the correlation between fT4 and FSS is weak (p=0.047). Regardless of this result, hypothyroidism is less likely an aetiology of fatigue in our AITD patients. A similar finding was reported in a recently published study of Louwerens et al. (30), in which autoimmune hypothyroid patients had significantly higher levels of fatigue compared to the patients with differentiated thyroid carcinoma, but it could not be attributed to clinical or thyroid hormone parameters.

There are numerous reports in the literature on the associated occurrence of depression and AITD (29, 31). Giv-

en that depression may affect the measurement of fatigue as a confounding factor (2), we examined the frequency of depression in patients with AITD, as well as in the healthy control group. In addition, we analysed whether the occurrence of fatigue and depression was associated. We showed that 50% of patients had depression, whereas 11.3% patients had fatigue and depression; only one (1.6%) patient had fatigue but no depression. In the control euthyroid group, none of the subjects had depression, although two subjects had evident fatigue. There was a significant correlation between fatigue and depression in our AITD patients. While a number of previously published studies found no association between fatigue and depression in patients with multiple sclerosis (32, 33), the majority of recently published studies found that depression had a significant impact on the occurrence of fatigue (34, 35). Although depression was more common than fatigue in our AITD patients, the positive correlation between the two variables indicates that the occurrence of fatigue and depression are associated. However, this does not necessarily mean that depression in our study is a confounding factor that affects the measurement of fatigue. The possibility that the fatigue and depression in AITD were caused by some other still insufficiently clarified mechanism cannot be excluded.

In conclusion, the frequency of fatigue was highly significant and almost three times higher in our AITD patients compared to the healthy subjects in the control group. The majority of patients with fatigue had normal thyroid function. We did not find significant correlations between fatigue and the concentrations of thyroid antibodies. There was a statistically significant correlation between fatigue and depression in our AITD patients.

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BURNOUT, DEPRESSION AND PROACTIVE COPING IN UNDERGROUND COAL MINERS IN SERBIA – PILOT PROJECT

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SINDROM SAGOREVANJA, DEPRESIJA I PROAKTIVNO PREVLADAVANJE KOD RUDARA RUDNIKA UGLJA U SRBIJI – PILOT PROJEKAT

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ABSTRACT

Mining is unsurprisingly considered a high-risk occupation because it involves continuous hard labour under highly demanding and stressful conditions. Many of these work stressors can impair individuals' well-being in both a physiological and psychological sense. The aims of this study were to assess the prevalence of burnout and depressive symptoms and to evaluate aspects of proactive coping among underground coal miners in Serbia. The study involved 46 male underground coal miners. Burnout was measured with the Copenhagen Burnout Inventory, depression was assessed with the Patient Health Questionnaire-9, and level of proactive coping was measured with the Proactive Coping Inventory. The results showed a low level of burnout syndrome among the underground coal miners (12.46±4.879). Depression was slightly above the minimum (1.2±2.094), and the majority of the participants had no symptoms of depression (93.5%). Overall, the underground coal miners' ability to proactively cope with work stress was very good (42.17±6.567). This is in contrast to the findings of the few previous international studies and is a good basis for further research using a larger sample in Serbia.

Keywords: *underground coal miners, burnout, depression, proactive coping*

SAŽETAK

Rudarstvo je neiznenađujuce smatrano profesijom visokog rizika s obzirom na to da podrazumeva obavljanje teškog rada u kontinuitetu, pod visoko zahtevnim i stresnim uslovima. Veliki broj ovih stresora na poslu može da naruši zdravlje pojedinca kako u fiziološkom tako i u psihološkom smislu. Cilj ovog istraživanja je bio pokušaj da se proceni prevalence sindroma sagorevanja i depresivnih simptoma, i da se oceni u kojoj meri se rudari u Srbiji prilagođavaju svom radnom okruženju. Studija je obuhvatila 46 rudara podzemne eksloatacije uglja. Sindrom sagorevanja je meren uz pomoc Kopenhagen upinika o izgaranju, depresija je procenjena uz pomoc Upitnika o zdravlju pacijenata-9, a nivo proaktivnog prevladavanja meren je Skalom proaktivnog prevladavanja. Rezultati su pokazali da je sindrom sagorevanja kod rudara podzemne eksloatacije na niskom nivou (12.46±4.879). Depresivnost je bila malo iznad minimuma (1.2±2.094), odnosno vecina ispitanika nije imala simptome depresije (93, 5%). Ukupno proaktivno prevladavanje kod rudara podzemne eksloataije uglja je veoma dobro (42.17±6.567). Ovo je u suprotnosti sa veoma malo sprovedenih internacionalnih studija, a i dobra je osnova za dalje istraživanje na vecem uzorku u Srbiji.

Ključne reči: *rudari podzemne eksploatacije uglja, sindrom sagorevanja, depresija, proaktivno prevladavanje*

ABBREVIATIONS

CBI - Copenhagen Burnout Inventory
DSM-V - Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition
MBI - Maslach Burnout Inventory
PCI - Proactive Coping Inventory
PHQ-9 - Patient Health Questionnaire-9

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INTRODUCTION

Psychological well-being is an increasingly important issue that has, considerable implications for economic productivity, social cohesion, and individual health and happiness as well as and for the development of mental health disorders. The health of workers is important in determining the ability of employees to maintain productivity. Coping with work stress and managing depressive symptoms are crucial factors in enhancing mental health in the workplace, which may, in turn, boost the performance and even profits of organizations. Given that our country is in transition, the current technological situation in the mining industry and the industry's lack of consistency with world standards can influence the mental health well-being of employees.

Coal miners are exposed to a number of demanding daily job tasks and are among the workers with the highest incidence rate of occupational injury (1). Coal miners perform drilling and blasting operations; use chain and belt conveyors for coal hauling; work in confined spaces with narrow corridors, high ground pressures, and the potential for roof caveing; and work in the presence of the methane and oxidation processes in coal. Employees are also exposed to high temperatures in excess of 28° Celsius, highly unionized environments (2, 3), noise, vibration, poor air quality, high humidity and cramped work spaces (4). Such a population group is interesting because it operates 24/7 and is associated with significant psychosocial risk factors, including extended roster periods, shift work and high production demands (5).

A number of these stressors at work can impair an individual's well-being in both a physiological and psychological sense. The experience of occupational stress has been linked to negative individual outcomes such as high rates of depression and anxiety, burnout, secondary traumatic stress and reduced work performance (6). Underground coal miners have been found to suffer from more severe mental health problems than surface workers from the same coal mine (7). Thus, more attention should be paid to the protection of the mental health of underground miners.

Burnout syndrome in the workplace is characterized by mental or emotional exhaustion and fatigue, with a greater emphasis on psychological rather than physical symptoms. People suffering from burnout are excitable and overexcited, constantly tense, impulsive, and reserved; they often resort to alcohol or drugs; and they may express sadness, pessimism, emotional rigidity, hypersensitivity, helplessness, and despair. They may feel malaise and have undefined physical pains for longer periods of time (headaches, back pain, insomnia, upset stomach, etc.) (8). Given these symptoms of burnout, it is inevitable that the consequences will be manifested through negative work attitudes, low levels of performance (9, 10), low productivity, absenteeism and job turnover (11, 12), and these results have been found in all types of organizations.

Based on the nature of burnout, it is possible that working conditions may affect whether the state of burnout progresses into a clinically significant condition such as depression (12, 13). Depressed workers have high rates of absenteeism, presenteeism and even work cessation and turnover intention (14), which can be increased due to job tension, as well as decreased job satisfaction that can be exacerbated by consistently heavy workloads (11). They are more likely to abuse alcohol and drugs than those without depression (15). Moreover, the risk of an occupational injury experience is higher among workers who report depressive symptoms. If not recognized or treated, depression can profoundly impair workers' quality of life (4).

A potent mechanism for burnout and depression prevention is proactive coping. According to Schwarzer's proactive coping theory, proactive individuals aspire to improve themselves to avoid future adverse life situations rather than reacting passively (16). By engaging in proactive coping, individuals can recognize potential difficulties in their environments and address them before burnout occurs (17); thus, by developing a better ability to engage in proactive coping, one tends to experience fewer workplace stressors (18).

The aims of this study were to assess the prevalence of burnout and depressive symptoms and to evaluate aspects of proactive coping among underground coal miners in Serbia.

SUBJECTS AND METHODS

Study Design and Sample. This pilot project was designed as a cross-sectional survey and was conducted in a coal mine, Bogovina, in eastern Serbia, one of nine mines under the public company Resavica, which is involved in underground coal exploitation. The participants completed a set of self-administered questionnaires anonymously in the presence of researchers at the time of their health check-ups. The subjects were 52 male underground coal miners, 46 of whom returned valid questionnaires and were included in the study. The subjects gave signed written consent to participate in the study. They were given instructions for completing the questionnaire and the assurance that the study results would be used only for the purpose of writing a scientific paper. The participants did not write their names on their questionnaire answer sheets; therefore, their anonymity was guaranteed. The collected demographic characteristics included age, level of education, marital status and length of service.

The *semi-structured questionnaire* comprises five questions related to the injuries of miners and their colleagues, choice of workplace and impressions regarding risk and stress on the workplace; answer choices are "Yes" or "No".

The Copenhagen Burnout Inventory (CBI). This inventory was used because, in contrast to other burnout measures such as the Maslach Burnout Inventory (MBI), the CBI focuses exclusively on the dimension of exhaustion as

the core characteristic of burnout (19). The seven work-related questions address the frustration and exhaustion associated with work. All of the questionnaire items have five response categories: always or to a very high degree, often or to a high degree, sometimes or somewhat, seldom or to a low degree, and never/almost never or to a very low degree. The responses in the present study were scaled from 0 to 100 for individual scores, and the scores were calculated by determining the means of the items in the scale. Total scores were also calculated: scores lower than 50 indicated low burnout (< 50), and scores higher than 50 (>50) indicated high burnout (9, 20-22).

The Patient Health Questionnaire-9 (PHQ-9). This inventory consists of nine questions based on the criteria for the diagnosis of major depressive disorder in the DSM-V. The subjects were asked to indicate the frequency of occurrence of each symptom over the past two weeks on a 4-point Likert scale (0: not at all; 1: several days; 2: more than half the days; and 3: nearly every day) (23). The scores for the nine questions were summed to give a total score ranging from 0 to 27, with higher scores indicating more severe depressive symptoms. Based on the total score, patients were categorized as having minimal depression (score 0–4), mild depression (score 5–9), moderate depression (score 10–14), moderately severe depression (score 15–19) or severe depression (score 20–27).

The Proactive Coping Scale. This scale is a part of the Proactive Coping Inventory (PCI) (24). It is a one of seven subscales of the PCI inventory; it consists of 14 items and has been translated into the Serbian language. This subscale combines autonomous goal setting with self-regulatory goal attainment cognition and behaviour. Typical items on the Proactive Coping Scale include: "I turn obstacles into positive experiences" and "After attaining a goal, I look for another, more challenging one". In scoring the responses, 1 is assigned to "not at all true", 2 to "barely true", 3 to "somewhat true" and 4 to "completely true". The total score represents the general level of proactive coping, with higher scores indicating more proactive coping.

Statistical analysis: The obtained research data were statistically processed using SPSS v. 18 for Windows. A descriptive analysis was performed to examine the sample and the answer for every question separately and to measure average values for the scales. The variable results are presented as the means, minimums, maximums and standard deviations. The reliability of the scales was measured using the Cronbach's alpha coefficient. An independent-samples t-test was applied to compare the average values of specific inventories with the questions from the semi-structured interviews, whereas the arithmetic means of three or more variables were compared using an analysis of variance (ANOVA). Pearson's correlation coefficient (r) was used to scale the direction and strength of a linear relationship between two variables. The level of significance was $p < 0.05$ in the applied analytical methods.

RESULTS

The internal consistency obtained for our sample with regard to the Copenhagen Burnout Inventory was good (α=.823), and it was low but acceptable (α=.77) for the Patient Health Questionnaire – 9 and the Proactive Coping Scale (α=.73).

This research included 46 underground coal miners, all of whom were male. The mean age of the participants was 43.7±7.47 years. The total years of service were 25.78±6.728, and the years of service as a miner in the current workplace were 15.65±9.243. Most of the participants had a high school diploma 54.3%, 43.5% had finished elementary school and 2.2% had finished college. The largest proportion was married (93.5%), and 6.5% were single; none of the miners was divorced (Table 1).

Most of them (63,0%) had been not injured in the workplace in such a way that required them to go on sick leave, and 52.2% of the miners had attended to the injuries of colleagues in the workplace. Most of them were employed in the mine because they had no other workplace from which to choose (52%). Half of them thought that their workplace was stressful and 52% thought that their workplace was highly risky (Table 2).

Table 1. Demographic data of coal miners

		Mean	s.d.
Age		43.78	7.474
Total years of service		25.78	6.828
Years of service in the current workplace		15.65	9.243
		N (%)	
Education	elementary school	20 (43.5%)	
	high school diploma	25 (54.3%)	
	college	1 (2.2%)	
Marital status	single	3 (6.5%)	
	married	43 (93.5%)	
	divorced	0 (0%)	

s.d. – standard deviation; N – number of subjects

Table 2. Semi-structured interview analysis

Questions	Answers	
	Yes N (%)	No N (%)
Have you been injured in the workplace in a way that required you to go on sick leave?	17 (37%)	29 (63%)
Have you attended to the injuries of colleagues in the workplace?	24 (52%)	22 (47.8)
Are you employed here because you have no other workplace from which to choose?	24 (52%)	22 (47.8)
Do you think that your workplace is highly risky?	24 (52%)	22 (47.8)
Do you feel that your workplace is stressful?	23 (50%)	23 (50%)

N – number of subjects

Table 3. Descriptive statistics of the items in the burnout, depression and proactive coping scales

	N	Min.	Max.	Mean	s.d.
Burnout	46	7	26	12.46	4.870
Depression	46	0	10	1.20	2.094
Proactive coping	46	27	56	42.17	6.567

N – number of subjects; Min. – minimum; Max. – maximum; s.d. – standard deviation;

In Table 3, the total scores for the burnout, depression and proactive coping scales are presented using descriptive statistics. The results showed that burnout was present at a low level in underground coal miners (12.46±4.879). Depression was slightly above the minimum (1.2±2.094). Overall, the underground coal miners' ability to proactively cope with work stress was very good (42.17±6.567).

By analysing the PHQ-9 scores for depression severity using descriptive statistics, we found that the majority of the underground coal miners had no symptoms of depression (93.5%); mild depression scores were found in 4.3% of the miners, and 1 (2.2%) underground coal miner showed symptoms of moderate depression (Fig. 1).

The results from the questionnaire used for the self-assessment of proactive coping ability are presented for each question and all of the respondents in Table 4. The results obtained by analysing the data from the questionnaire indicated that the majority of the re-

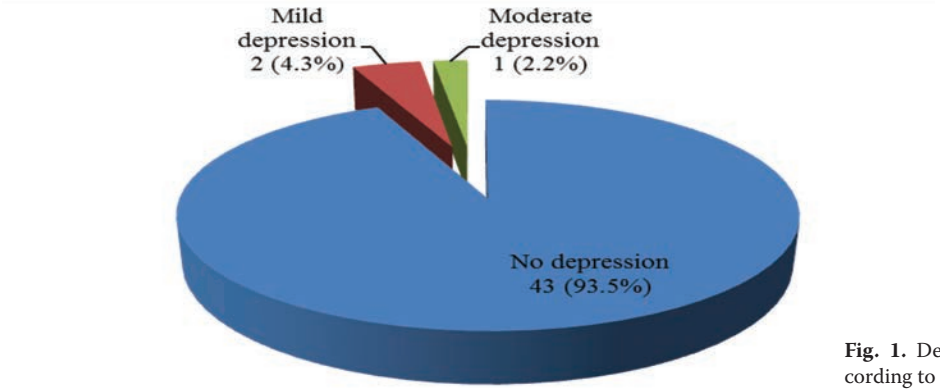

Fig. 1. Depression severity according to the PHQ-9 inventory

Table 4. Proactive Coping Inventory data

Questions		Answers N (%)			
		"not at all true"	"barely true"	"somewhat true"	"completely true"
1	I am a "take charge" person	3 (6.5%)	0 (0%)	6 (13.0%)	37 (80.4%)
2	I try to let things work out on their own	19 (41.3%)	18 (39.1%)	3 (6.5%)	6 (13.0%)
3	After attaining a goal, I look for another, more challenging one	3 (6.5%)	9 (19.6%)	10 (21.7%)	24 (52.2%)
4	I like challenges and beating the odds	20 (43.5%)	1 (2.2%)	8 (17.4%)	17 (37.0%)
5	I visualise my dreams and try to achieve them	3 (6.5%)	3 (6.5%)	15 (32.6%)	25 (37.0%)
6	Despite numerous setbacks, I usually succeed in getting what I want	4 (8.7%)	8 (17.4%)	18 (39.1%)	16 (34.8%)
7	I try to pinpoint what I need to succeed	4 (8.7%)	5 (10.9%)	10 (21.7%)	27 (58.7%)
8	I always try to find a way to work around obstacles; nothing really stops me	2 (4.3%)	4 (8.7%)	22 (47.8%)	18 (39.1%)
9	I often see myself failing so I don't get my hopes up too high	10 (21.7%)	16 (34.8%)	14 (30.4%)	6 (13.0%)
10	When I apply for a position, I imagine myself filling it	4 (8.7%)	4 (8.7%)	14 (30.4%)	24 (52.2%)
11	I turn obstacles into positive experiences	5 (10.9%)	2 (4.3%)	13 (28.3%)	26 (56.5%)
12	If someone tells me I can't do something, you can be sure I will do it	5 (10.9%)	11 (23.9%)	19 (41.3%)	11 (23.9%)
13	When I experience a problem, I take the initiative in resolving it	3 (6.5%)	2 (4.3%)	4 (8.7%)	37 (80.4%)
14	When I have a problem, I usually see myself in a no-win situation	21 (45.7%)	4 (8.7%)	8 (17.4%)	13 (28.3%)

N – number of subjects

Table 5. Correlation analysis between variables

Correlations						
Pearson correlation	Age	Total years of service	Years of service in the current workplace	Proactive coping	Depression	Burnout
Age	1	.686**	.138	.128	.143	-.155
Total years of service	.686**	1	.249	-.063	.298*	-.179
Years of service in the current workplace	.138	.249	1	-.204	.339*	-.215
Proactive coping	.128	-.063	-.204	1	-.368*	-.089
Depression	.143	.298*	.339*	-.368*	1	-.159
Burnout	-.155	-.179	-.215	-.089	-.159	1

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).

spondents were "take charge" individuals (37, 80.4%) and that very few of them did not try to find a way to work around obstacles (2, 4.3%).

The results of the correlation analysis of the total scores for each scale are shown in Table 5. The results showed that coal miners became more depressed with more total years of service and more years of service in the current workplace. In contrast, coal miners with higher levels of proactive coping were less depressed. In addition, no significant correlations were found between burnout and age, burnout and total years of service and burnout and years of service in the current workplace, or between depression and burnout (sig. at 0.05 and 0.01).

An independent-samples T-test was used in the comparison of the measurements of depression, burnout and proactive coping based on questions from the semi-structured interviews. Statistically significant differences ($p<0.05$) were found only for the underground coal miners who were employed in the mine because they had no choice of another workplace; no significant differences were found for the participants who were employed in the mine by choice.

Using an ANOVA test, we compared the measurements of depression, burnout and proactive coping with the participants' levels of education and found no influence from the level of education on burnout, depression or proactive coping.

DISCUSSION

This study showed that burnout syndrome in underground coal miners is at a low level and that the majority of participants did not exhibit depressive symptoms. Overall, underground coal miners' proactive coping levels are very good.

The study of mental health in the mining industry is a useful proxy for the health status of such employees and helps to identify occupational health hazards. The health of workers is important in determining the ability of employees to maintain productivity. Hence, underground coal miners are especially prone to occupational health problems and are among the workers with the highest incidence rates of occupational injury (1) because they spend every work hour in a demanding work environment that may have an impact on their well-being.

Very few studies devoted to the issues studied in this paper were found in the available databases. It is therefore difficult to compare the obtained test results with those of other related studies of these aspects of mental health in underground coal miners.

Researchers from South Africa examined the organizational burnout of employees in various working positions in the mining industry. A study by Horn C.R. showed low to moderate levels of exhaustion, cynicism and reduced professional efficacy as subdimensions of burnout among mid-level managers in an underground coal mine (25). Van der Walt et al found that the predictors of burnout were job demands, overload and a lack of advancement opportunities in the workplace (26). Another study conducted by Roets H. showed that a weak sense of coherence combined with job stress was associated with all three components of burnout (27).

Uysal H.T. and Kesim E. measured organizational burnout in blue-collar workers employed by Turkish Hard Coal Enterprise. They found that the burnout levels of workers were not significantly different according to their working positions (casual workers, technical staff, engineers); however, there were significant differences with regard to the miners' working locations (underground or above ground) and places (different coal mines in Turkish Hard Coal Enterprise) (28).

The underground coal miners in our research showed lower levels of burnout syndrome than workers in other professions in Serbia. However, studies have found high scores for both personal and work-related burnout among manufacturing workers in the food industry in Serbia (29). Moderate to high levels of burnout syndrome were found among orthopaedic surgeons (30), general practitioners (30, 31) and psychiatrists (31). Stanetić K, et al's study results showed that levels of stress and emotional exhaustion increase with length of service and age. In that study, the highest levels of stress and emotional exhaustion were

found among the oldest physicians with the greatest length of service, whereas the lowest levels were found among the youngest physicians with the shortest length of service (8). In contrast, our research showed no correlations between length of service and burnout and age and burnout.

Lui L, et al showed that 62.8% of underground coal miners in China suffered from depression (4). This is in contrast to our research, in which 93.5% of the coal miners had no symptoms of depression. Another Chinese study indicated that workers in the mining industry had symptoms of depression and anxiety and that underground coal miners were found to suffer from more severe mental health problems than surface workers from the same coal mine (32). Marchand A. examined mental health among different occupations in Canada and found that workers reported poorer mental health in twenty selected occupations; the mining industry was among the occupations with lowest prevalence of mental health risks in the sample (33). Similar results were presented by McLean, who conducted qualitative research on 10 residents mine workers in Australia (34).

Our results showed that level of depression increased with length of service and was higher for those who were employed in the mine because they had no other choice of workplace. On the other hand, those who coped better with stress were less depressive. Proactive coping was found to be negatively correlated with depression, which indicates that miners with high levels of proactive coping ability have low levels of depression.

Regardless of the features of their demanding workplace, underground coal miners` proactive coping ability is high. Our results showed that the miners saw themselves as "take charge" individuals; therefore, it can be said that they perceive difficult situations as challenges rather than as threats. This refers to the belief that they have the ability overcome the difficulties of their work environment, which may involve making a plan of action (16). Many of the respondents (80.4%) declared that they take the initiative in resolving a problem when they encounter it. The same per cent stated that they do not let things work out on their own, which confirms that underground coal miners are prepared to take action. Successful action is more likely to be taken if one feels confident and prepared enough to control challenges or threats (16). Respondents considered their workplace highly risky and stressful, but the majority stated that they could easily turn obstacles into positive experience (84.8%) and that nothing could stop them from finding a way to work around obstacles (86.9%). The demonstrated high level of proactive coping confirms their empowerment to face future adverse situations and react proactively to solve problems. They showed equally high levels of self-determination in goal setting behaviours: they saw how to successfully achieve desirable goals and, after that, how to look for another, more engaging goal. Proactive coping consists of the accumulation of various resources and the attainment of skills such as organization, planning, goal-setting and mental simulation (17).

Even though slightly more than a half of the examined coal miners reported that they were employed in the mine because they could not find a job of their own choice, there was no statistically significant difference in proactive coping between those workers and workers who were employed in the mine by choice. However, there was a statistically significant difference between these two groups in terms of depression; we found that that group of miners who were employed in the mine because they could not find a job of their own choice were significantly more depressed than the other group. Overall, despite the influence of job choice on their mood changes, the underground coal miners very proactively performed their activities in a demanding work environment and thus reported lower levels of burnout (16). Challenges stimulate vitality, which leads to positive outcomes and indicates healthy functioning (16). The positive moods of these underground coal miners may help them to perceive obstacles as challenges and to overcome workplace dangers.

The limitations of the study relate to its cross section design and its small sample size as well as to having beenand because the sample was drawn from one coal mine. Because it was conducted during the miners' health check-ups, they may have modified their answers to appear be healthy and complain less. In addition, there was no control group with which to compare the results. Despite these limitations, this study serves as a starting point for developing further initiatives in this area that consider cultural specifics such as rural environments and continuous work throughout generations.

CONCLUSION

The results of this pilot project showed that among underground coal miners, there is a very low level of burnout, depression is absent and the level of proactive coping is high. The results relating to well-being in these aspects of underground coal miner's mental health, which are in contrast to previously conducted international studies, are a good basis for further research in the field. Considering that the obtained results were inconsistent with the expected results, the research variables should be expanded and examined further to determine how they influence miners' mental health. Further research is needed using a bigger sample that includes other coal mines in Serbia.

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ATTITUDES OF MEDICAL AND PHARMACY STUDENTS TOWARDS PATIENTS SUFFERING FROM SCHIZOPHRENIA

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STAVOVI STUDENATA MEDICINE I FARMACIJE PREMA PACIJENTIMA OBOLELIM OD SHIZOFRENIJE

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ABSTRACT

Research over the past twenty years has shown that the attitudes of health care workers and students towards people who are suffering from schizophrenia have become more negative. The aim of our study was to investigate the attitudes of medical and pharmacy students towards patients with schizophrenia and explore the differences in attitudes between study groups and students in different years. Materials and methods: Second- and fifth-year medical and pharmacy students from the Faculty of Medical Sciences at the University of Kragujevac were included in an observational, prospective, cross-sectional study. The sample consisted of 113 students from the pharmacy and medical schools who were chosen via random sampling. The students completed a two-part questionnaire. The first part contained questions about sociodemographic characteristics, whereas the second part was a translated version of the Mental Illness: Clinician's Attitudes (MICA) v4 scale. Results: There is a statistically significant difference ($p<0.05$) in the attitudes towards people with schizophrenia between second- and fifth-year medical and pharmacy students (with lower scores in both groups in fifth-year students). Of the total number of students who had lower summed scores on the Likert scale, 51.3% had previously finished medical high school, whereas 28.3% had previously finished regular high school. Conclusion: Our results showed a statistically significant difference in attitudes towards people with schizophrenia between second- and fifth-year students as well as a difference related to previous high school education. This stresses the importance of levels of knowledge about schizophrenia to reducing the stigmatization of patients who suffer from this disorder.

Keywords: *attitude, schizophrenia, students, medicine, pharmacy*

SAŽETAK

Istraživanja ukazuju da su u poslednjih dvadeset godina stavovi javnosti, ali i zdravstvenih radnika i studenata prema obolelima od shizofrenije postali negativniji. Cilj rada je da se ispitaju stavovi studenata medicine i farmacije prema pacijentima obolelim od shizofrenije i da li postoje razlike u stavovima između studijskih grupa kao i između studenata različitih godina studija. Materijal i metod: Opservacionom prospektivnom studijom preseka obuhvaćeni su studenti druge i pete godine farmacije i medicine Fakulteta medicinskih nauka Univerziteta u Kragujevcu. Uzorak je činilo 113 ispitanika, koji su birani metodom slučajnog uzorka. Ispitanici su popunjavali upitnik koji je u prvom delu sadržao pitanja o sociodemografskim karakteristikama, a drugi deo predstavlja prevedenu verziju Mental Illness: Clinician's Attitudes (MICA) v4 scale. Rezultati: Naši rezultati pokazuju da postoji statistički značajna razlika ($p<0.05$) u stavovima prema osobama obolelim od shizofrenije između studenata druge i pete godine medicine i farmacije, pri čemu su niži skorovi kod studenata pete godine u obe grupe. Od ukupnog broja studenata koji su imali niže vrednosti sumarnog skora na Likertovoj skali za procenu stavova studenata prema osobama obolelim od shizofrenije, 51,3% bili su studenti koji su završili srednju medicinsku školu, dok su 28,3% bili studenti koji su završili gimnaziju. Zaključak: Postoji statistički značajna razlika u stavovima o obolelima od shizofrenije između studenata druge i pete godine studija i u odnosu na završenu srednju školu ispitanika. Naše istraživanje nedvosmisleno ukazuje da je nivo znanja o shizofreniji bitan za smanjenje stigmatizacije obolelih od ove bolesti.

Ključne reči: *stavovi, shizofrenija, studenti, medicina, farmacija*

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INTRODUCTION

Attitudes are defined as learned susceptibilities to react to a certain thing or instance in a certain way, either positively or negatively (1). In 2007, Tornicroft et al. found that attitudes about a certain occurrence, with inadequate knowledge and behaviour that follows such knowledge, contribute significantly to the development of stigmatization. Stigma that is associated with mental disorders is greatly widespread, which is why it is referred to as the ultimate stigma (2).

Research shows that over the past twenty years, public attitudes towards patients with schizophrenia have become more negative (3). Crisp et al. conducted a study using a questionnaire in which they found that 70% of the people who were questioned held the opinion that people with schizophrenia are dangerous and 80% of them thought that people with schizophrenia are unstable (4). A situation such as this one directly influences the treatment efficiency of schizophrenic patients because their social reintegration is already compromised by negative stereotypes and fear in the general population (5).

Contrary to the expectation that health care workers have no prejudice and negative attitude towards this group of patients, research on this subject shows that their attitude does not differ from the attitude of the general population and is sometimes even more restrictive (6, 7). Given the importance of the relationship between the physician and patient in which a positive attitude towards the patient is required to achieve successful treatment, there is a need for research in the field on attitude of future health care workers towards these patients.

An insufficient number of studies can be found in the literature about the attitude of students towards schizophrenia. One of the first research papers on the topic was written in 1989 by Eker, who explored the attitudes of first-year students in Turkey towards different types of mental disorders (8). The results showed that the most negative scores were found in reports of opinions about paranoid schizophrenia. Similar to findings obtained from the general population, research has shown that medical students are prejudiced towards people with mental disorders, as they state that such patients have a hard time recovering and that difficult psychiatric patients are violent and dangerous (9-11). Pharmacy students have also stated that patients with schizophrenia are unstable and dangerous (12). It is presumed that the way that students are taught about schizophrenia creates their mind-set that these patients are chronic and unstable (13). A study that began in 2013 in seven European countries (Bulgaria, Denmark, England, Ireland, Malta and Slovenia) that included medical and psychology students in one group and students who were indirectly associated with healthcare professions in the other group (14) showed the relevance of this topic today.

In accordance with the viewpoint that the relationship with a psychiatric patient is *via regia* to successful treatment, many studies investigate the positive influence of certain factors on reducing stigma (social contact, knowledge in the field of mental health). It has been shown that social contact can have a positive effect on shifting negative attitudes towards this population of patients (15-17). A number of studies have assessed the influence of knowledge about mental disorders on medical students' attitudes (18-20). One of those studies showed that there are no differences in the attitude towards schizophrenic patients between students at a medical high school and medical university students, with 78% of the students who were questioned agreeing that schizophrenic patients are dangerous and violent. Additionally, 95% of the students who were questioned stated that they did not possess enough knowledge in this field (21).

Few authors in Serbia have addressed the issue of psychiatric patient stigmatization. Stoiljković et al. found that medical students are more prone to stigmatize people who visit a psychiatrist (22). Munjiza et al. published a first-of-its-kind study that analysed stigmatization relative to gender and the interaction with schizophrenic patients separately. This study showed that male and female students were less likely to stigmatize people of the same gender for visiting a psychiatrist (23). One of the defined goals of the research that Totić et al. conducted in 2011 was to investigate the attitudes and behaviour of medical students towards psychiatric patients before and after finishing their psychiatry rotation. In this research, education in psychiatry has involved ambivalence about its effects on stigmatization (i.e., it can enhance or diminish it) (24).

The aim of our study was to investigate the attitudes of medical and pharmacy students towards patients with schizophrenia. We also explored possible differences in attitudes between the study groups and between students in different years.

MATERIAL AND METHODS

Sample

Second- and fifth-year pharmacy and medical students at the Faculty of Medical Sciences at the University of Kragujevac in June and July of 2014 were included in this observational, prospective, cross-sectional study. The sample included 113 questioned students who were sorted into four groups: group I: 28 second-year pharmacy students, group II: 27 second-year medical students, group III: 30 fifth-year pharmacy students and group IV: 28 fifth-year medical students. The subjects were chosen using simple random sample. Simple random sampling was performed using a standard procedure based on the results that were obtained by importing data in previously prepared randomization tables in Excel.

The research is part of a graduation thesis that was accepted by the Scientific Board of The Faculty of Medical Sciences at the University of Kragujevac.

Measurement instruments and variables

Subjects completed a questionnaire that had 25 closed-ended questions that are divided into two parts. The first part of the questionnaire contains 7 questions about the socio-demographic characteristics of the subjects.

The second part of the questionnaire is a translated version of the Mental Illness: Clinician's Attitudes (MICA) v4 scale, which was adapted to investigate medical students' attitudes about mental disorders. It contains 16 closed-ended questions that are answered on a six-point Likert scale (25).

The first step was for one of the authors to translate the MICA v4 into Serbian before another author who was not aware of the original text translated it back into English. All the items proved to be easily translatable, and no problems emerged during the translation procedure. Next, the Serbian version of MICA v4 was used with the population of students.

Before they completed the questionnaire, all the students were given a detailed explanation of the way that the questionnaire should be filled out and were notified that the information that they provided would be kept confidential. They were given a sufficient amount of time to think through and complete the questions independently.

The level of agreement with certain statements was quantified in the second part of the questionnaire using scores ranging from 1 to 6 on a Likert scale. The scores were summed for each subject. For questions 3, 9, 10, 11, 12 and 16, the response option "I completely agree" equated to 1 point, "I agree" equated to 2 points, "I partially agree" equated to 3 points, "I partially disagree" equated to 4 points, "I disagree" equated to 5 points, and "I completely disagree" equated to 6 points. For questions 1, 2, 4, 5, 6, 7, 8, 13, 14 and 15, the response option "I completely agree" equated to 6 points, "I agree" equated to 5 points, "I partially agree" equated to 4 points, "I partially disagree" equated to 3 points, "I disagree" equated to 2 points, and "I completely disagree" equated to 1 point.

The lowest possible score was 16 points, whereas the highest possible score was 96 points. A lower value of the summed scores on the Likert scale indicated a lower level of stigmatization, whereas a higher value indicated a higher level of stigmatization. A summed score of 56 points or more was considered to be the border value, which is the arithmetic mean of the maximal and the minimal scores.

Statistical analysis

The IBM SPSS 20 statistical software package was used for the statistical analyses. The scores were presented as the mean ± the standard deviation. The normality of the data distribution was tested using the Shapiro-Wilk test (the number of subjects was fewer than 50 in one group). Given that the distribution was normal ($p>0.05$), the tests of the differences between the groups were performed using the independent-samples Student's t-test. Descriptive statistics were used to determine the percentage of socio-demographic characteristics in the entire sample. The chi-square test was used to analyse the significance of the differences in these variables.

RESULTS

The socio-demographic characteristics of subjects are presented in Table 1. The majority of subjects were female (76 or 67.3%) and had completed medical high school (66 or 58.4%).

Table 1. Socio-demographic characteristics of the subjects

Variables	Number (%)
Gender	
Male	37 (32.7)
Female	76 (67.3)
High school education	
Medical high school	66 (58.4)
Regular high school	47 (41.6)
Marital status	
Married	1 (0.9)
In a relationship	59 (52.2)
Single	53 (46.9)
Socio-economic status	
I am barely managing to pay for food and bills	11 (9.7)
I have enough for basic needs (food, bills)	15 (13.3)
I can spend money on things besides basic needs – clothes, going out, cinema	41 (36.3)
I have enough for my basic needs and I can afford to spend money on bigger expenses (vacation)	26 (23.0)
I don't have significant financial difficulties	20 (17.7)

The mean values of the summed scores on the Likert scale used to evaluate the attitudes of medical and pharmacy students are shown in Table 2. Based on these values, it can be seen that there is a statistically significant difference in attitudes towards people who are suffering from schizophrenia ($p<0.05$) between second-year pharmacy students and second-year medical students, between second- and fifth-year medical students, and between second- and

Table 2. Mean values of the summed score on the Likert scale to evaluate the attitudes of medical and pharmacy students

Total score	Number of subjects	Mean	Standard deviation
II year of pharmacy (II F)	28	49.43	6.12
II year of medicine (II M)	27	57.19	6.41
V year of pharmacy (V F)	30	40.50	6.53
V year of medicine (V M)	28	42.71	7.71
II F : II M	II M : V M	II F : V F	V F : V M
$p<0.05$	$p<0.005$	$p<0.005$	$p>0.05$

Table 3. Mean values of the summed score on the Likert scale in relation to age and previous high school education

Mean value	Older than 23 years of age	Younger than 22 years of age	p value
Lower values of the summed score (16-56)	48.7	31.0	p<0.001
Higher values of the summed score (57-96)	2.7	17.7	
	Medical high school	Gymnasium school	
Lower values of the summed score (16-56)	51.3	28.3	p=0.015
Higher values of the summed score (57-96)	7.1	13.3	

fifth-year pharmacy students. There was no statistically significant difference in attitudes towards people who are suffering from schizophrenia between fifth-year pharmacy students and fifth-year medical students (p>0.05).

The mean value of the summed scores on the Likert scale used to evaluate the attitudes of second-year pharmacy students was 49.43. In fifth-year pharmacy students, it was 40.50. Among second-year medical students, the mean score was 57.19. In fifth-year medical students, it was 42.71. The results showed that there was a strong negative correlation between the attitudes of students towards people with schizophrenia and the age of the students (r=0.387; p<0.001).

A significant difference in the attitudes of students towards people with schizophrenia was found in relation to the age of the students (χ^2 test, p<0.001). Of the total number of students who had a lower summed score on the Likert scale used to evaluate the attitudes of students towards people suffering from schizophrenia, 48.7% of them were older than 23 years of age, whereas 31% of them were younger than 22 years of age (Table 3).

Additionally, a higher value of the summed score on the Likert scale was noted in 2.7% of the students who were older than 23 years of age and 17.7% of the students who were younger than 22 years of age. Based on these values, it can be concluded that older students (>23 years old) had more positive attitudes towards patients with schizophrenia (Table 3).

The results showed that there was a statistically significant strong correlation between the attitudes of students towards people with schizophrenia and previous high school education (r=0.242; p<0.001). Furthermore, it can be said that previous high school education has a strong influence on students' attitudes towards people with schizophrenia.

The study also showed that the difference in the attitudes of students towards people with schizophrenia that was related to previous high school education was statistically significant (χ^2 test, p=0.019).

Of the total number of students who had a lower value of the summed score on the Likert scale used to evaluate students' attitudes towards people with schizophrenia, 51.3% of them were students who had completed medical high school, whereas 28.3% of them had finished regular high school (Table 3). Higher values of the summed score on the Likert scale were observed in 7.1% of the students who had finished medical high school and 13.3% of the students who had completed regular high school.

Figure 1. Socio-demographic characteristics of the subjects

1. Gender:
a) Male
b) Female
2. Year of birth: _____.
3. Integrative academic studies:
a) Medicine
b) Pharmacy
4. Year of study:
a) II year
b) V year
5. High school education:
a) Grammar school
b) Medical high school
6. Marital status:
a) Married
b) In a relationship
c) Single
7. Socio-economic status:
a) I am barely managing to pay for food and bills
b) I have enough for basic needs (food, bills)
c) I can spend money on things besides basic needs – clothes, going out, cinema
d) I have enough for my basic needs and I can afford to spend money on bigger expenses (vacation)
e) I do not have significant financial difficulties

DISCUSSION

Our research explicitly highlights the importance of education level and knowledge about patients with schizophrenia in reducing the level of stigmatization towards these patients.

The fact that there is a statistically significant difference in the scores on the scale between second-year pharmacy students and second-year medical students is interesting, with a higher stigmatization score found in medical students. These data could be explained by the fact that more pharmacy students had previously completed medical high school compared to the medical students. However, it should be mentioned that in studies that compared the stigmatization of schizophrenic patients among medical nurses, physicians, medical students and patients, the

highest level of stigmatization was found in medical students (11). Most research has shown that education against stigma is efficient (26, 27). Research in Serbia has shown that improving knowledge about mental disorders has a positive influence on changing the attitudes of high school students towards their peers who suffer from mental disorders (28). Similarly, our results show that students who had previously finished medical high school displayed the lowest level of stigmatization. Given these students receive information about mental disorders in medical high school as part of the regular curriculum, we can assume that it is especially important for them to acquire a correct positive attitude towards schizophrenic patients.

Another piece of information that is important is that older medical and pharmacy students display a significantly lower level of stigmatization compared to their younger counterparts. In addition, there is no significant difference in the attitudes between students in the fifth year in either of the study groups. Results such as these might be a consequence of the influence that knowledge that is acquired through practical and theoretical teaching in psychiatry has on students' attitudes. Mas also found a difference in attitudes towards schizophrenic patients between first- and final-year medical students. Specifically, first-year students without previous knowledge in psychiatry had a greater tendency to assess mentally ill people as dangerous and to believe in the power of social distance towards them (29). Another study that was conducted in Italy in 2012 showed that 45% of first-year students compared to 57% of final-year students stated that fear was their dominant emotion when dealing with this type of pathology (30). The influence of education on attitudes towards schizophrenia was also observed in a study that was conducted in Croatia that included medical nurses as well as third- and fourth-year medical students. The authors of this study noted the presence of negative attitudes that were caused by a lack of education in the field of mental disorders among medical nurses as well as third-year medical students (31).

A study that was conducted in China in 2014 examined differences in attitudes towards psychiatry and mental disorders among fourth-year medical students before and after finishing a mandatory educational seminar and psychiatry rotation. After their rotation, an improvement in their attitudes towards psychiatry and mental health was noticed (32).

Similar results were found by German researchers, who noticed a positive effect of a two-week educational programme in psychiatry on the reduction of stigma in medical students towards schizophrenic patients (33).

The influence of age and years of study on the attitudes of students was presented in an American study that examined the attitudes of pharmacy students towards patients with mental disorders. The students were questioned at the beginning (first year) and end (fourth year) of their studies. The results showed a significant reduction in the number of students who wanted to distance themselves socially from patients with mental disorders after a four-year

Figure 2. Mental Illness: Clinician's Attitudes (MICA) v4 scale

Using the scale ranging from 1 to 6, answer the following questions: Score 1 - "I completely agree " Score 2 - "I agree" Score 3 - "I moderately agree" Score 4 - "I moderately disagree" Score 5 - "I disagree" Score 6 - "I completely disagree"
1. I just learn about mental health when I have to and would not bother reading additional material on it.
1 2 3 4 5 6
2. People with severe mental illness can never recover enough to have a good quality of life.
1 2 3 4 5 6
3. Working in the mental health field is just as respectable as other fields of health and social care.
1 2 3 4 5 6
4. If I had a mental illness, I would never admit this to any of my friends because I would fear being treated differently.
1 2 3 4 5 6
5. People with mental illness are dangerous more often than not.
1 2 3 4 5 6
6. Health/social care staff members know more about the lives of people treated for a mental illness than do family members and friends.
1 2 3 4 5 6
7. If I had a mental illness, I would never admit this to my colleagues for fear of being treated differently.
1 2 3 4 5 6
8. Being a health/social care professional in the area of mental health is not like being a real health/social care professional.
1 2 3 4 5 6
9. If a senior colleague instructed me to treat people with mental illness in a disrespectful manner, I would not follow his/her instructions.
1 2 3 4 5 6
10. I feel as comfortable talking to a person with a mental illness as I do talking to a person with physical illness.
1 2 3 4 5 6
11. It is important that any health/social care professional supporting a person with mental illness also ensures that his/her physical health is assessed.
1 2 3 4 5 6
12. The public does not need to be protected from people with mental illness.
1 2 3 4 5 6
13. If a person with a mental illness complained of physical symptoms (such as chest pain), I would attribute it to his/her mental illness.
1 2 3 4 5 6
14. General practitioners should not be expected to complete a thorough assessment for people with psychiatric symptoms because they can be referred to a psychiatrist.
1 2 3 4 5 6
15. I would use the terms "crazy", "nutter", "mad", etc., to describe to colleagues people with mental illness whom I have seen in my work.
1 2 3 4 5 6
16. If a colleague told me that s/he had a mental illness, I would still want to work with him/her.
1 2 3 4 5 6

period (34). Our study also showed that older subjects (23 years of age or older) in their fifth year of their studies display a lower level of stigmatization.

However, there are also studies with divergent results. In Spain, 171 fifth-year medical students at three different universities had a high percentage (93.4%) of positive attitudes towards mental disorders (35). Research that was conducted in Serbia showed that stigmatization towards psychiatric patients increased after they finished their psychiatry rotation (24). Research from 2001 noted that positive attitudinal changes of medical students towards mental disorders are ephemeral and disappear after the final year of studies is completed (36). These differences can be explained by variations in the study design, sample, and measurement instruments.

Nevertheless, the results of most studies imply that students' attitudes can be altered by increasing practical and theoretical knowledge in the field of psychiatry. Taking into consideration the importance of the relationship between the physician and the patient, there is a need to increase the sensitivity to a greater extent in future physicians and pharmacists when working with this group of patients.

The main limitation of this study is that we did not previously validate the Serbian version of the scale.

CONCLUSIONS

Our results have shown that there is a statistically significant difference in the attitudes towards people with schizophrenia between second- and fifth-year medical and pharmacy students, as was found in most previous studies on this topic. Although there are studies that did not find that education on mental disorders is significant to the improvement of attitudes towards people with schizophrenia, the issue of education requires special attention. If we take into consideration the idea that patients with schizophrenia are considered to be "difficult" and can cause a wide range of emotions, medical educators should develop methods that can help medical and pharmacy students to modify their emotions and attitudes, thereby reducing obstacles to working with this population. Future research should examine attitudes about patients with schizophrenia in a larger sample as well as include attitudes about different clinical forms of schizophrenia.

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THROMBOTIC THROMBOCYTOPENIC PURPURA: ETIOPATHOGENESIS, DIAGNOSTICS AND BASIC PRINCIPLES OF TREATMENT

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TROMBOTIČNA TROMBOCITOPENIJSKA PURPURA: ETIOPATOGENEZA, DIJAGNOSTIKA I OSOVNI PRINCIPI LEČENJA

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ABSTRACT

Thrombotic thrombocytopenic purpura (TTP) is a clinical syndrome that manifests with thrombocytopenia, microangiopathic haemolytic anaemia and symptoms and signs of kidney and brain damage, but it rarely involves other organs. The main pathophysiological cause of TTP is diminished metalloproteinase ADAMTS13 activity; the main function of ADAMTS13 is to degrade large multimers of the von Willebrand factor. Diminished activity of ADAMTS13 is caused either by a genetic mutation in the gene that codes ADAMTS13 (congenital TTP) or by antibodies that block ADAMTS13 enzyme activity or accelerate the degradation of ADAMTS13 (acquired TTP). Clinically, TTP presents most frequently with signs and symptoms of brain and kidney damage with concomitant haemorrhagic syndrome. TTP is suspected when a patient presents with a low platelet count, microangiopathic haemolytic anaemia (negative Coombs tests, low haptoglobine concentration, increased serum concentration of indirect bilirubin and lactate dehydrogenase, increased number of schysocytes in peripheral blood) and the typical clinical presentation. A definitive diagnose can be made only by measuring the ADAMTS13 activity. The differential diagnosis in such cases includes both typical and atypical haemolytic uremic syndrome, disseminated intravascular coagulation, HELLP syndrome in pregnant women and other thrombotic microangiopathies. The first line therapy for TTP is plasma exchange. In patients with acquired TTP, in addition to plasma exchange, immunosuppressive medications are used (corticosteroids and rituximab). In patients with hereditary TTP, the administration of fresh frozen plasma is sometimes required.

Keywords: *TTP, ADAMTS13, plasmapheresis, rituximab*

SAŽETAK

Trombotična trombocitopenijska purpura (TTP) je klinički sindrom koji se odlikuje trombocitopenijom, mikroangiopatskom hemoliznom anemijom i simptomima i znacima oštećenja bubrega i mozga, ređe drugih organa. Glavni patofiziološki mehanizam nastanka TTP-a je smanjena aktivnost metaloproteinaze ADAMTS13 čija je osnovna uloga razgradnja velikih multimera von Willebrand-ovog faktora. Smanjena aktivnost metaloproteinaze ADAMTS13 nastaje usled mutacije u genu za ADAMTS13 (urođeni TTP) ili usled nastanka antitela koja blokiraju ili antitela koja ubrzavaju razgradnju ADAMTS13 (stečeni TTP). Klinički se ispoljava znacima i simptomima oštećenja mozga i bubrega, kao i slikom hemoragijskog sindroma. Sumnja na TTP se postavlja na osnovu smanjenog broj trombocita, mikroangiopatske hemolizne anemije (negativan Coombs-ov test, smanjena koncentracija haptoglobina, povećana koncentracija indirektnog bilirubina i laktat dehidrogenaze u serumu, povećan broj šizocita u razmazu periferne krvi) i tipične kliničke slike, a definitivna dijagnoza se postavlja merenjem aktivnosti ADAMTS13. U diferencijalnoj dijagnozi treba isključiti tipični i atipični hemolitičko-uremijski sindrom, diseminovanu inravaskularnu koagulaciju, HELLP sindrom kod trudnica, kao i druge trombotične mikroangiopatije. Prva linija terapije kod kod bolesnika sa TTP-om je plazmafereza. Kod bolesnika sa stečenim oblikom TTP-a, uz plazmaferezu se koriste i imunosupresivni lekovi (kortikosteroidi i rituksimab), a kod bolesnika sa urođenim oblikom TTP-a povremena supstitucija sa sveže smznutom plazmom.

Ključne reči: *TTP, ADAMTS13, plazmafereza, rituksimab*

ABBREVIATIONS

ADAMTS13 - disintegrin and metalloprotease with thrombospondin type 1 repeats, member 13
HIV - human immunodeficiency virus
HUS - haemolytic uremic syndrome
FRETS - fluorescence resonance energy transfer
ELISA - enzyme-linked immunosorbent assay
TTP - thrombotic thrombocytopenic purpura
ULvWF - ultralarge von Willebrand factor multimers
vWF - Von Willebrand factor
PAGE - polyacrylamide gel electrophoresis
SDS - sodium dodecyl sulphate
HIT - heparin induced thrombocytopenia

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INTRODUCTION

Thrombotic thrombocytopenic purpura (TTP) is a rare clinical syndrome characterized by the formation of disseminated platelet aggregates in small blood vessels and consequent microangiopathic haemolytic anaemia. The aggregated platelets can cause ischaemia, hypoxia and abnormal function of the affected organs. The classical clinical pentad of TTP consists of microangiopathic haemolytic anaemia, thrombocytopenia, fluctuating neurological signs, impaired renal function and fever. However, patients with TTP usually do not present with all of these clinical signs (approximately 35% of the patients did not present with signs of neurological dysfunction) (1-3). The renal function in patients suffering from TTP can vary greatly, ranging from acute renal failure to fully preserved renal function, but the most common consequence is a moderate and transient decrease in renal function (4). The thrombocytopenia is usually severe (10-30x10^9/l) due to the sequestration of platelets in microvascular thrombi. The haemolytic anaemia is a consequence of mechanical damage to the red blood cells as they pass through the narrowed microcirculation (1-3).

AETIOLOGY AND PATHOGENESIS OF TTP

The main pathogenetic mechanism of TTP is decreased activity of matrix metalloproteinase ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type 1 repeats, member 13). The main function of ADAMTS13 is splitting the ultra-large von Willebrand factor multimers (ULvWF) into smaller fragments (2, 5).

The von Willebrand factor (vWF) is a protein synthesized mostly by endothelial cells and, in a smaller proportion, by megakaryocytes (6). Its role is essential for initiating primary haemostasis. After blood vessels experience endothelial damage, vWF connects to subendothelial collagen via the A3 domain and enables the adherence of platelets through its A1 domain that binds GPIbα/IX/V platelet's receptor. The globular form hides the ULvWF A2 domain that binds metalloproteinase ADAMTS13. After it is transformed into a string-like form, the A2 domain is revealed and binds to ADAMTS13, which cleaves the ULvWF into monomers (Figure 1). The second part of the vWF is then processed in the small blood vessels where the large shear forces unfolds the globular ULvWF. The third place where ULvWF degradation occurs is in damaged blood vessels, where it binds to the subendothelial collagen via the A3 domain, and the ULvWF unfolds and reveals the A2 and A1 domains (3, 10).

The nomenclature of TTP is not standardized, but the term "TTP" is typically restricted to cases with severe deficiency of ADAMTS13 activity (<10%) (11). Based on the pathogenetic mechanism leading to reduced activity of ADAMTS13, TTP is divided into congenital and acquired (3).

The majority of patients suffer from the acquired, antibody mediated form of TTP (approximately 95% of all cases) (12). Commonly, these antibodies block the primary epitope of ADAMTS13 and inhibit its proteolytic function. However, in approximately 10-15% cases of acquired TTP, the patients have non-inhibitory antibodies, and the ADAMTS13 deficiency is a result of an increased clearance of ADAMTS13-antibody complexes. The inhibitory antibodies are IgG isotypes, predominantly of the IgG4 subclass. The IgG4 subclass is associated with more frequent relapses of the disease. The antibodies may also be from the IgG1, IgG2, IgG3 subclasses, as well as from isotype IgA (13, 14).

The aetiological cause of acquired TTP is often unknown, and that form of TTP is categorized as primary (or idiopathic). In the smaller number of patients with immune dysregulation due to other diseases, the term secondary acquired TTP is used (5). In the literature, there are a few reported cases of enterohaemorrhagic Escherichia coli infection with concomitant thrombotic microangiopathy that were treated as classical haemolytic uremic syndrome (HUS), but the subsequent measurement of the activity of metalloprotease ADAMTS13 revealed its reduced activity (<10%) (15). The impact of human immunodeficiency virus (HIV) as an aetiological factor of TTP remains controversial. Different studies have shown different prevalences of HIV infection in patients with TTP (16, 17). Certain medications have been associated with the development of TTP. Ticlopidine increases the risk of TTP by 200-500-fold (18, 19). On the other hand, drugs such as quinine, calcineurin inhibitors and certain chemotherapeutics can cause thrombotic microangiopathy, but they are not associated with significantly decreased activity of ADAMTS13.

The correlation between TTP and pregnancy is complex. Pregnancy decreases the level of ADAMTS13 by approximately 30% (20). In women with reduced ADAMTS13 activity, pregnancy can trigger the acute onset of TTP. In the postpartum period, the immune tolerance present during pregnancy is terminated, and an acceleration of the existing autoimmune response to ADAMTS13 may occur. According to a regional English TTP registry, 5% of all TTP episodes are related to pregnancy (21). Jiang and colleagues explored the connection between TTP and pregnancy complications in 10 women with acquired TTP in the Oklahoma TTP-HUS Registry. These 10 women had a total of 16 pregnancies. The outcomes of the 16 pregnancies were as follows: 2 pregnancies complicated with the acute onset of TTP, 2 with preeclampsia, 2 ended due to pregnancy loss, and 10 without complications (22). These data suggest that TTP is one of the risk factors for pregnancy complications.

Hereditary TTP is the least common form of the disease, and it occurs as a result of mutations in the ADAMTS13 gene that are located on the long arm of chromosome 9. Heterozygous carriers of ADAMTS13 mutations usually have slightly reduced ADAMTS13 activity in the serum, and a small percentage suffer from TTP. Homozy-

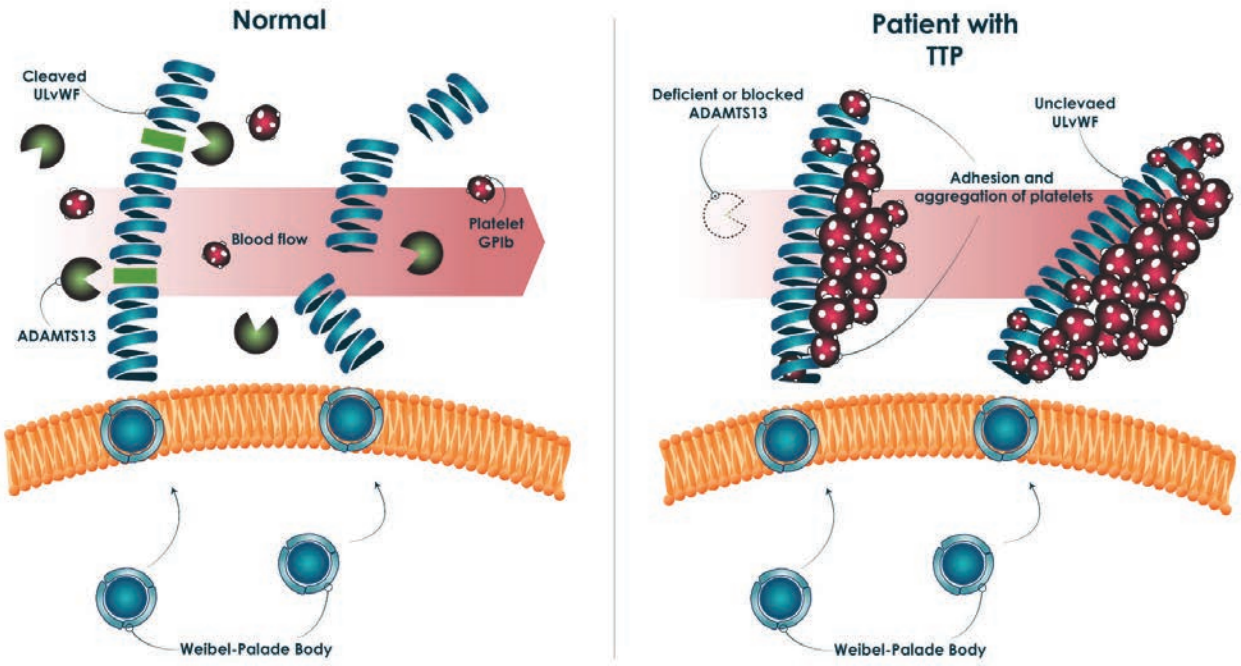

Figure 1. Production and cleavage of ULvWF by ADAMTS13 in a normal subject and in a patient with TTP (original)
Abbreviations: ULvWF - ultralarge von Willebrand factor multimers; TTP - thrombotic thrombocytopenic purpura.

gous carriers of mutations are quite rare; they occur frequently in families with consanguinity. The activity of ADAMTS13 is significantly reduced in homozygous carriers (<5%) (3, 23). There are approximately 140 different mutations of the ADAMTS13 gene, and the most frequently mentioned mutations in the literature are the single base insertion 4143insA in exon 29 and the missense mutation Arg1060Trp in exon 24 (24, 25). About half of the patients with hereditary TTP first exhibited signs and symptoms of the disease during the first year of life. In the other half of TTP patients, the disease occurred between the ages of 20 and 40 years old. Only a few patients had their first attack of TTP after the age of 60 (5).

CLINICAL MANIFESTATION OF TTP

The TTP clinical pentad consisting of thrombocytopenia, microangiopathic haemolytic anaemia, neurological signs and symptoms, renal impairment and fever is not specific to TTP and, in the majority of patients, is not completely manifested. The clinical presentation depends on the organ affected by microvascular ischaemia. Microvascular ischaemia of the central nervous system manifests with headache, somnolence, focal neurological disturbances, convulsions and coma. Renal impairment is usually manifested as intermittent proteinuria and haematuria, and only rarely as acute renal failure. Other, less common clinical manifestations of TTP include nausea, vomiting, abdominal pain, disorders of heart rhythm, congestive heart failure, myocardial ischaemia and, in rare cases, sudden cardiac death (1-5, 26, 27). Bruises and petechial bleeding in the skin, epistaxis, menorrhagia, haematuria or gastrointestinal haemorrhage can arise, but bleeding complication are generally rare despite severe thrombocytopenia, even during the placement of central venous catheters (1-5, 28)

In patients with congenital TTP hyperbilirubinemia, thrombocytopenia and microangiopathic haemolytic anaemia are often identified immediately after birth or in the neonatal period. The frequency of congenital TTP exacerbations varies from several days to several years. Factors that can lead to exacerbation of TTP are fever, infection, diarrhoea, trauma, surgery or pregnancy (1-5, 17).

Thrombocytopenia, due to sequestration of platelets in microthrombi in small blood vessels, is usually severe (10-30x10^9/l). Microangiopathic haemolytic anaemia is obligatory in TTP patients. Laboratory parameters that indicate this type of anaemia are decreased serum levels of haemoglobin (80-100 g/l) and haptoglobine, increased serum levels of indirect bilirubin and lactate dehydrogenase (LDH), the presence of fragmented parts of red blood cells (schistocytes) in the peripheral blood and a negative Coombs tests (Figure 2). Normal serum levels of liver enzymes exclude HELLP syndrome (haemolysis, elevated liver enzymes, low platelet count). The parameters of haemostasis (thrombin time, activated partial thromboplastin time and fibrinogen) are usually normal, which helps differentiate TTP from disseminated intravascular coagulation (1-5).

The diagnosis of TTP should be suspected in the presence of the clinical features and laboratory results described above. It is necessary to measure ADAMTS13 enzyme activity for a definite diagnosis of TTP. The term "TTP" is

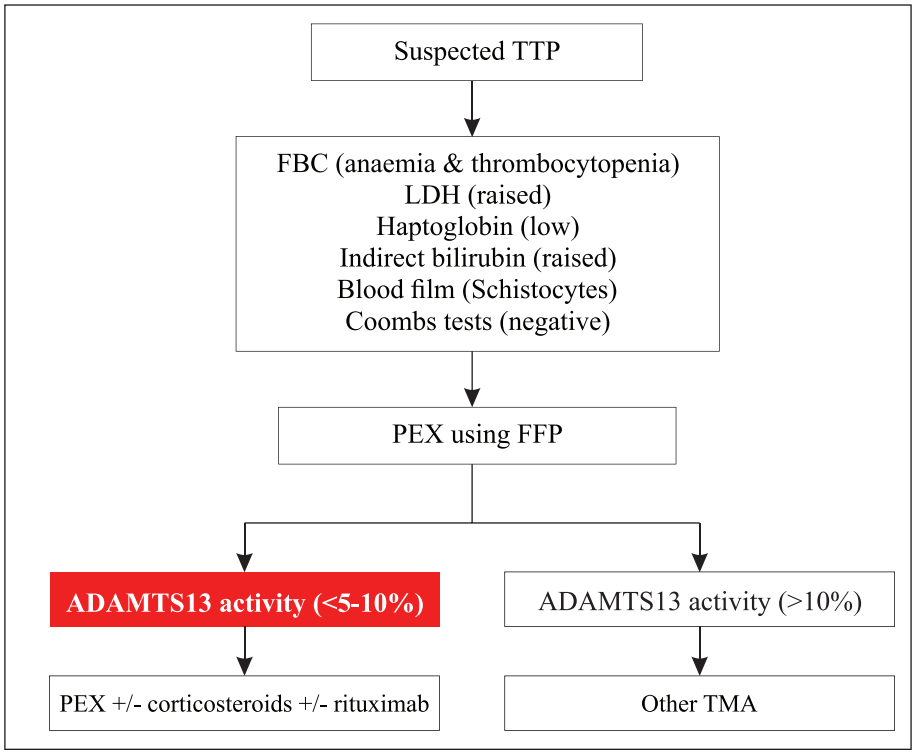

Figure 2. Algorithm for the diagnosis and treatment of TTP (original)
Abbreviations: TTP - thrombotic thrombocytopenic purpura; FBC - full blood count; PEX - plasma exchange; FFP - fresh frozen plasma; TMA – thrombotic microangiopathy

customarily used for thrombotic microangiopathy with measured ADAMTS13 activity less than 5-10% (depending on the type of test). An earlier study demonstrated that ADAMTS13 plasma activity of less than 5–10% appeared to be specific for TTP and helps to differentiate TTP from other thrombotic microangiopathies with a similar clinical appearance (11) (Figure 2). In addition, the serum concentration of the vWF antigen and concentrations of inhibitory and non-inhibitory antibodies to ADAMTS13 can be measured. Based on these tests results, specialists can distinguish congenital from acquired TTP (5, 29).

ADAMTS13 ASSAYS

The plasma assays that measure ADAMTS13 activity are based on the cleavage of plasma-derived or recombinant vWF multimers. Depending on whether the assay measures the substrates of cleavage or the cleavage products themselves, the assays are either direct or indirect measurements, respectively (30, 31).

The direct assays measure the cleavage products of vWF macromolecules, vWF A2 domain, or the small peptides GST-vWF-73 or FRETS-73. Agarose or polyacrylamide gel electrophoresis (PAGE), western blotting, and fluorescence resonance energy transfer (FRETS) techniques are used for the measurement of the cleavage products. There are several direct assays: SDS (sodium dodecyl sulphate) agarose gel electrophoresis and western blotting, SDS-PAGE and western blotting and FRETS Assay (30, 31). The FRETS assay uses the truncated synthetic 73-amino-acid vWF peptide as a substrate for measuring ADAMTS13 activity. This assay is rapid and more sensitive and specific than the others (32).

The indirect assays measure the residual substrate (i.e., vWF) or its disappearance. They include the collagen-binding assay, ristocetin-induced aggregation and the enzyme-linked immunosorbent (ELISA) assays. In the collagen-binding assay, after the incubation of purified vWF with patient's plasma in the presence of 1.5 M urea, and 10 mM $BaCl_2$ for 16 to 24 hours, the amount of residual vWF is determined by its binding to human collagen type III. The bound vWF is quantified by the peroxidase-conjugated anti-vWF immunoglobulin G (IgG) on an ELISA plate, followed by a densitometry measurement (33, 34). The ristocetin-induced aggregation assay is similar to the collagen binding assay, but the residual vWF is measured by ristocetin-induced platelet aggregation using a platelet aggregometer (35). In the ELISA assay, a recombinant vWF fragment is immobilized onto an ELISA plate using an antibody to a tag the vWF. After the incubation of the patient's plasma with an immobilized substrate, an antibody to another tag detects the residual substrate. The photolytic activity of ADAMTS13 is inversely proportional to the residual substrate concentration. This type of assay is relatively simple and sensitive (36).

The above described assays can detect inhibitory antibodies but the FRETS assay appears to be the most sensi-

tive. An ELISA assay along with western blotting using the recombinant ADAMTS13 as an antigen can also be used to detect both inhibitory and non-inhibitory autoantibodies (37).

DIFFERENTIAL DIAGNOSIS OF TTP

In clinical practice, TTP should be differed from both typical and atypical HUS. HUS is a clinical syndrome that is characterized by thrombocytopenia, microangiopathic haemolytic anaemia, small vessel microthrombosis and acute kidney injury (2, 38). Typical HUS is the result of endothelial cell damage, which is caused by the Shiga toxin of the Escherichia coli serotype O157:H7 (2, 38). Atypical HUS is the result of dysfunction of alternative complement pathway regulating proteins, including complement factors I, complement factors B, complement factors H, membrane cofactor protein and complement C3 component (2, 38). To exclude both typical and atypical HUS, the following analyses should be done: microbiological examination of the stool (stool culture for Escherichia coli O157:H7 serotype), anti-lipopolysaccharide antibodies and PCR (polymerase chain reaction) for Shiga toxin, and testing of the alternative complement pathway activity (2, 38).

For the differential diagnosis of TTP, a few more entities should be considered such as disseminated intravascular coagulation, an acquired coagulation disorder which is characterized by a small vessel microthrombosis and secondary haemorrhagic syndrome, often observed in systemic infections and malignancy (38). Antiphospholipid syndrome, especially its rare clinical manifestation called catastrophic antiphospholipid syndrome, can make a differential diagnosis with TTP difficult because this clinical form of antiphospholipid syndrome presents with multi-organ failure due to thrombosis and also with thrombocytopenia and microangiopathic anaemia (39). Occasionally, heparin induced thrombocytopenia (HIT) can mimic TTP because of the organ failure due to thrombosis and consequent thrombocytopenia, but a normal platelet count before the initiation of heparin therapy in HIT and confirmation of the antibodies against the complex of heparin and platelet factor 4 distinguish those two disorders (40). HELLP syndrome should also be considered in pregnant women with thrombocytopenia and haemolytic anaemia. HELLP affects 0.5% to 0.9% of all pregnancies and develops in 10% of patients with preeclampsia. The diagnostic criteria for HELLP syndrome include microangiopathic haemolytic anaemia, a lactate dehydrogenase level >600 U/ml, increased aspartate aminotransferase (≥ 40–70 U/ml, depending on the series), and thrombocytopenia (platelet count < $100x10^9$/l-$150x10^9$/l, depending on the series). Termination of pregnancy does not induce remission of TTP, unlike HELLP syndrome, so it is crucial to differentiate those two diagnoses (41).

MANAGEMENT OF TTP

TTP is an acute, life threatening disease that demands immediate treatment in highly specialized facilities that can perform plasmapheresis.

Plasmapheresis (plasma exchange) is the first-line treatment of patients with acquired and congenital TTP. This process removes the antibodies of ADAMTS13 in patients with acquired TTP. Fresh frozen plasma is used as a substitution for removed plasma during plasmapheresis (Figure 2). Fresh frozen plasma contains metalloproteinase ADAMTS13 and renews its concentration in the serum. Plasmapheresis should be administered within 24 hours of the clinical onset of TTP, and it should be administered daily for 3 consecutive days; during plasmapheresis, 1-1.5 of the plasma volume should be exchanged. In patients who present with neurological deficit and signs of cardiac ischaemia, two plasmapheresis treatments per day are necessary during the first three days of the disease. Daily plasmapheresis should be continued two days after the platelet count gets higher than $150x10^9$/l, and then it should be stopped (29, 42, 43).

During the initial treatment of acquired TTP, in addition to plasmapheresis, immunosuppressive drugs such as corticosteroids and anti-CD20 antibody (rituximab) can be used.

Faster immunosuppression and reduction of anti-ADAMTS13 antibodies is achieved with the administration of corticosteroids. There are no studies that demonstrate the advantage of any corticosteroid in particular, but methylprednisolone is the most commonly used agent at a dose of 10 mg/kg per day. Studies have shown that higher doses of corticosteroids are more efficient than standard ones (1 mg/kg per day) (44). Methylprednisolone is administered daily for three days, right after plasmapheresis, so that only a minimal amount of the drug is removed (29).

Studies have shown that treatment of acquired TTP with rituximab lowers the number of necessary plasmapheresis sessions, shortens the duration of the hospitalization and diminishes the relapse risk. In one observational study, 40 rituximab-treated patients were compared to 40 historical controls who did not receive rituximab and were treated with conventional therapy (plasmapheresis+corticosteroids). The remission rate among the trial group was 93%, and among the controls, it was 95%. When the patients who were admitted to an intensive care unit were excluded, this study showed that the rituximab-treated patients had hospitalizations that were 7 days shorter. There was a statistically significance difference in the percent of relapses and the time to relapse between those two groups. Only 10 percent of the trial cases relapsed, at a median of 27 months, compared to a 57% relapse rate among the historical controls, at a median of 18 months (45). There are no recommendations for the dosage and frequency of administration of rituximab. In the previously mentioned multi-centre study, rituximab was administered at a dosage of 375 mg/m^2, once a week, for 4 weeks in a row. In patients who exhibited a slower response, rituximab treatment was extended to 8 weeks (45).

In addition to its role for the initial treatment of TTP, rituximab is also used for the treatment of refractory and relapsed TTP. An observational study that compared 21 patients with refractory TTP treated with rituximab and 53 historical controls treated with conventional therapy reported that all the patients treated with rituximab experienced platelet count recovery within 35 days compared to only 78% of the control patients (46). The results from 5 other similar studies showed that 83-100% rituximab-treated patients achieved complete remission (47-52). The relapse rates after rituximab treatment ranged from 0% with a median follow-up of 10 months (47) to 33% with a median follow-up of 73 months (52). Patients who are refractory to plasmapheresis, corticosteroids and rituximab may benefit from bortezomib administration (53-55).

Finally, rituximab can be used for prophylactic treatment of patient who experienced a previous acute episode of TTP when their ADAMTS13 activity is <10%, even without clinical signs and symptoms. Several studies have demonstrated that prophylactic treatment with rituximab prolonged relapse-free survival (56, 57).

Rituximab is removed by plasmapheresis, so it should be administered right after a plasma exchange session. During the period of plasmapheresis treatment, rituximab can be used more frequently (every 3-4 days), to keep the serum concentration at a high level (29). Given its high efficacy and safety, rituximab can be recommended as the initial treatment for acquired TTP, treatment of refractory and relapsed TTP and prophylactic treatment.

Although the treatment of TTP has improved significantly in recent years, there is still room for improvement. One of new medications used for the treatment of TTP is recombinant metalloproteinase ADAMTS13. In addition to compensating for the deficiency of ADAMTS13 in patients with congenital TTP, recombinant ADAMTS13 is also resistant to antibody inhibition in most patients with acquired TTP (58). Caplacizumab is a potential new drug for the treatment of TTP. Caplacizumab is an anti vWf-antibody that has shown high efficacy in the treatment of TTP in animal models (59). Caplacizumab binds to the N-terminal end of the A1 region of vWf and inhibits the interaction between vWf and platelets (60).

CONCLUSION

TTP has long been known as a life threatening disease. In the last decade, our understanding of the basic biochemistry of the VWF-ADAMTS13 axis has provided valuable insights into the pathogenesis of TTP. The better understanding of pathogenesis of TTP has led to the development of new therapeutic strategies. In addition, assays that measure ADAMTS13 activity can help differentiate TTP from other thrombotic microangiopathies with a similar clinical appearance. Although the mortality associated with TTP has been appreciably reduced, much remains to be learned, especially about how to treat patients who are refractory to the standard therapies.

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PLANTS FROM THE GENUS *DAPHNE*: A REVIEW OF ITS TRADITIONAL USES, PHYTOCHEMISTRY, BIOLOGICAL AND PHARMACOLOGICAL ACTIVITY

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BILJNE VRSTE RODA *DAPHNE*: PREGLED UPOTREBE U TRADICIONALNOJ MEDICINI, FITOHEMIJA, BIOLOŠKE I FARMAKOLOŠKE AKTIVNOSTI

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ABSTRACT

Plants have an important role in maintaining people's health and improving the quality of human life. They are an important component of people's diet, but they are also used in other spheres of human life as a therapeutic resources, ingredients of cosmetic products, paints and others. The Daphne genus belongs to family Thymeleaceae which includes 44 families with approximately 500 herbal species. The plant species of the genus Daphne are used in the traditional medicine in China and tropical part of Africa for the treatment of various conditions. Previous studies showed significant biological potential of these species as a source of pharmacologically active compounds. This indicates that this genus possess a broad spectrum of biological activity including antimicrobial, antioxidant, analgesic, anti-inflammatory, cytotoxic, anti-ulcerogenic, abortive, hypocholesterolemic and hemostatic effects. Additionally, Daphne plants are the source of valuable bioactive phytochemicals such as coumarins, flavonoids, lignans, steroids and different classes of terpenes. Different parts of the Daphne plants contain specific bioactive metabolites and can represent a source of new, natural, pharmacologically active compounds, which may potentially be used in pharmaceutical, cosmetic and food industries.

Keywords: *Daphne, traditional medicine, pharmacological activity, biological activity, phytochemicals*

SAŽETAK

Biljke imaju bitnu ulogu u održavanju zdravlja ljudi i poboljšanju kvaliteta ljudskog života. One su bitna komponenta ishrane ljudi, ali se koriste i u ostalim sferama ljudskog života nalazeći primenu kao lekovita sredstva, konzervansi, sastojci kozmetičkih preparata, boja i ostalo. Rod Daphne pripada familiji Thymeleaceae koja obuhvata 44 rodova sa približno 500 biljnih vrsta. Biljne vrste roda Daphne se koriste u kineskoj tradicionalnoj medicini i tradicionalnoj medicini tropskog dela afrike u tretmanu različitih oboljenja. Sprovedene studije ukazuju na biopotencijal vrsta iz ovog roda kao izvor farmakološki aktivnih jedinjenja i veliki značaj zbog ispoljavanja širokog spektra medicinskih dejtva i bioloških aktivnosti. Biološke aktivnosti koje ispoljavaju vrste roda Daphne obuhvataju antimikrobnu, antioksidativnu, antiinflamatornu, citotoksičnu, antiulcerogenu, abortivnu i mnoge druge. Ispitivanjem hemijskog sastava vrsta ovog roda utvrđeno je prisustvo različitih klasa sekundarnih metabolite kao što su: kumarini, flavonoidi, lignani, steroidi i različite klase terpena. Ovi konstituenti su povezani sa ispoljavanjem različitih bioloških aktivnosti. Vrste iz roda Daphne mogu predstavljati izvor novih, prirodnih, farmakološki aktivnih jedinjenja, koja mogu naći potencijalnu primenu u farmaceutskoj, kozmetičkoj i prehrambenoj industriji.

Ključne reči: *Daphne, tradicionalna medicina, farmakološka aktivnost, biološka aktivnost, fitohemikalije*

INTRODUCTION

Using herbs for the medical purposes and finding bioactive molecules in plants is an ancient idea. Centuries ago autochthonous plants had been used for the treatment of various diseases. For instance, archeology data exists that Neanderthals, who lived 60 000 years ago, in the site of today's Iran, used high mallow for different purposes (1). These herbs are still widely used in traditional medicine, all around the world. In the past, therapeutic effects of herbs were different – from healing and symptoms relief to toxic effects, and even death. Today, it is estimated that there are 250 000 to 350 000 different species of herbs on Earth. Relatively small percentage (less than 10%) has been used in people's or animal's nutrition, but, probably, much higher number of them was used for medical purposes (2).

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The value of the plants used in traditional medicine for drug discovery

Extraction of particular alkaloids from opium, at the beginning of 19th century, is the crucial event in the development of modern pharmacy. The compounds that were extracted had the same, but much stronger activity than the herbal material which had been used, which paved the way for the use of pure molecules for the treatment of various diseases. From then on, a lot of plants have been used as a source of new natural medicines. The molecules which were extracted from the herbal material, served for the design of new, synthetic medicines, by introducing active chromophores into an existing natural molecules. That is how, for example, from the jaborandi leaf, which was used in Brazilian traditional medicine to induce perspiration, the pilocarpine was isolated and now days is used in medicine as a miotic in the treatment of glaucoma (3). The process of discovery of the new medicines from the natural resources consists of several phases. In the first phase, the data of traditional use of herbal material is being investigated. Consideration of traditional use of a certain herb represents the basis for possible assumption that the herb in question manifests biological and pharmacological activities. If there are any indications of their biological activity, it is necessary to identify the plant and determinate it according to the scientific nomenclature. After conducting relevant tests of biological activity, decision should be made whether to conduct extraction and structural identification of the active constituents which are responsible for manifested activity. Bioactive molecules are being isolated through several cycles of fractionation of extracts. Each fraction's activity is tested and bioactive fractions are further used for the isolation of pure compounds. These molecules, after determining their activity and structure, serve as potentially clinically useful products (4).

The importance of the traditional medicine plants today

Herbs have an important role in maintaining people's health and improving the quality of human life. They are an important component of people's diet, but they are also used in other spheres of human life, finding application as therapeutic resources, ingredients of cosmetic products, paints and others. The use of plants has always been a part of human culture. WHO (World Health Organization) estimates that 80% of human population relies on some of the traditional methods of treatment in the primary health care (5). In some countries, governments advocate the use of autochthonous treatment methods more than the use of expensive imported medicines. In the last 100 years, mass production and use of chemically synthesized drugs are the main part of the health care system. However, a large part of population, especially in the developing countries, still relies on traditional methods of treatment and use of herbal medicines in conducting health care. In such way, for example, in Africa 90% of population relies on traditional methods of treatment, in India 70%, whereas in China, traditional medicine makes 40% of all health care systems, and more than 90% of general hospitals have units for traditional medicine (6-8). However, the use of traditional medicine is not restricted only to developing countries. In the past two decades, interest for traditional treatment methods, with special focus on phytotherapy, is growing in developed countries as well. The research conducted in USA in 2007 showed that more than 35% of adults and around 12% of children are using some of the traditional treatment methods (9, 10). According to the research of National center for complementary and alternative medicine, herbal therapy, with the exception of vitamins and minerals, is most used method of alternative medicine (11).

Secondary plant metabolites as a source of new antimicrobial agents

Infective diseases are still the leading cause of morbidity and mortality around the world, in spite of the major progress of medical technology and scientific findings about infective agents and mechanisms of their development (12). After the discovery of first antibiotics, penicillin, in 1929, there has been a revolution in development of antibiotics in modern medicine. However, in the last couple of decades there has been an increase of global incidence of resistance of microorganisms towards antimicrobial agents (13, 14). Resistance of microorganisms against antibiotics that are currently being used is increasing, so there is the need for continuous findings of new antimicrobial compounds (15). Natural herbal products have been used for treatment of different infective diseases (16-19). Apart from synthetic molecules, natural products are still being considered as the important source for new and innovative therapeutic agents with a wide spectrum of antimicrobial effects (20). Among contemporary antifungal agents more than 35% have natural origin (21). Natural products present a promising source of new antibiotics, antibiotics supplements and disinfectants (22-24). The studies performed in the last couple of decades defined the most important ingredients of plants which have antimicrobial activity. For example, some phenolic compounds exhibited broad spectrum of antimicrobial activity against variety of pathogenic microbes. Generally, since phenolic compounds do not have strong pharmacological effect, they can be used only for prophylaxis and for the treatment of initial stages of diseases (25).

The plant phenols as antioxidants

Previous investigations have shown that phenolic compounds and flavonoids have antioxidant effect in biological systems, mostly because of their redox properties (26). The mechanism of flavonoids activity is based on reduction and neutralization of generated free radical and thus the interest for further study of this compounds remains high (27).

The ability of neutralization of free radicals makes flavonoid compounds important for the therapeutic or prophylactic use, e.g. after infection, inflammation, burns or injury due to radiation exposure (28). The antioxidant activity of phenolic acids is important in the defense mechanisms of biological systems and for the stability of the food. Recent studies have shown that many polyphenol ingredients of the plants are showing much stronger activity than vitamin C and E (28, 29). These results, obtained in *in vitro* studies also show significant protective antioxidant potential *in vivo*.

Polyphenol compounds are strong antioxidants and they have great potential in preventing cellular damage caused by reactive oxygen species and, in that way, they protect organism from cardiovascular, cancerous and other chronic diseases (27, 30, 31). However, the contribution of particular components to overall antioxidant protection is difficult to determine because the manifested activity of the extracts can be the result of synergistic effect of different compounds. In one study, it was recorded that mixtures of lycopene of other herbal polyphenol compounds has better antioxidant effect compared to the effect of individual compounds (32).

Taxonomy, distribution and description of the genus *Daphne*

Plants from the genus *Daphne* are small bushes or short trees with sparse branches (33). Taxonomy of the *Daphne* genus is very complex and complicated because of the existence of great number of species and subspecies. The *Daphne* genus belongs to family *Thymeleaceae* which includes 44 families with approximately 500 plant species (34). The primary center of evolution of this genus was China (33). The genus covers 95 species which are mostly distributed in Europe and certain regions of sub-tropical Asia (35, 36). Until now, in Europe's flora, the presence of 17 species of this genus has been identified (37). The phylogenetic tree of *Daphne* genus is shown in Table 1.

Secondary metabolites present in plants of the *Daphne* genus

The interest for the plants of the *Daphne* genus is increasing because of numerous beneficial medicinal properties of these plants that can be of potential clinical significance. The extracts of the examined species exhibit pharmacological and biological activities and different methods of analysis have identified the active molecules (secondary metabolites) which are responsible for these activities. There are an increasing number of studies aimed to identify specific molecules, improve methods for their synthesis and determine their activity and/or toxicity. The research so far has confirmed the presence of molecules and derivates which belong to different classes of secondary metabolites.

Table 1. The taxonomy classification of the genus *Daphne*

Kingdom	*Plantae*
Division	*Magnoliophyta*
Class	*Magnoliopsida*
Order	*Malvales*
Family	*Thymelaeaceae*
Genus	*Daphne*

The next section of this paper offers a look on the most present secondary metabolites in groups in *Daphne* species, as well as their properties in terms of biological and pharmacological activities.

Coumarins

One of the first metabolites to be isolated (in the 1930s) is coumarin heteroside daphnin, whose presence has been confirmed in several plant species (38). Since then, in *Daphne* species, the presence of about 50 coumarin derivates has been discovered. According to the structure they can be simple coumarins, dimeric and trimeric (Figure 1). Simple coumarins that are found in *Daphne* species is: daphnetin, daphnetin-8-β-glucoside, daphnin, daphnesid, umbelliferone and acetil-umbelliferone (39-43). Some of the dimeric coumarins present in these plants are: rutarensin, demethyldaphnoretin-*7-O*-glucoside, daphnoretin and daphneretusin A (44, 45). Daphnoretin exhibits good antitumor activity. It stops the cell cycle of human osteosarcoma cells in G2 phase and it activates aptoposis over caspase-3 dependent way (46). Daphneretusin B and triumbellin are trimeric coumarin metabolites. Daphneretusin A and B show good antioxidant activities (44).

Biflavonoids

Daphnodorins and similar biflavonoids, genkwanol and daphnorigin, are specific secondary metabolites present in the *Thymelaeceae* family which contain 2,3-functional benzofuran group (47). Daphnodorin A was first isolated from the *D. odora* plant and it has numerous biological effects such as inhibition of α-glucosidase, K+-ATP inhibition, anti-HIV activity, antifungal and insecticidal activity, 12-lypoxygenase and cyclooxygenase inhibitory activity and antitumor activity (48). From the extract of *D. odora*, other daphnodorins have also been isolated (Figure 2). The methanol extract of *D. acutiloba* contains daphnodorin M and H. Spirobiflavonoids, genkwanol, as well as juanhuanin have been isolated from *D. genkwa* and they exhibit cytotoxic properties (49).

Terpenes

Terpenoid secondary metabolites are often found as a constituent of different parts of *Daphne* plants. Mono and sesquiterpenoid compounds are most often found in the

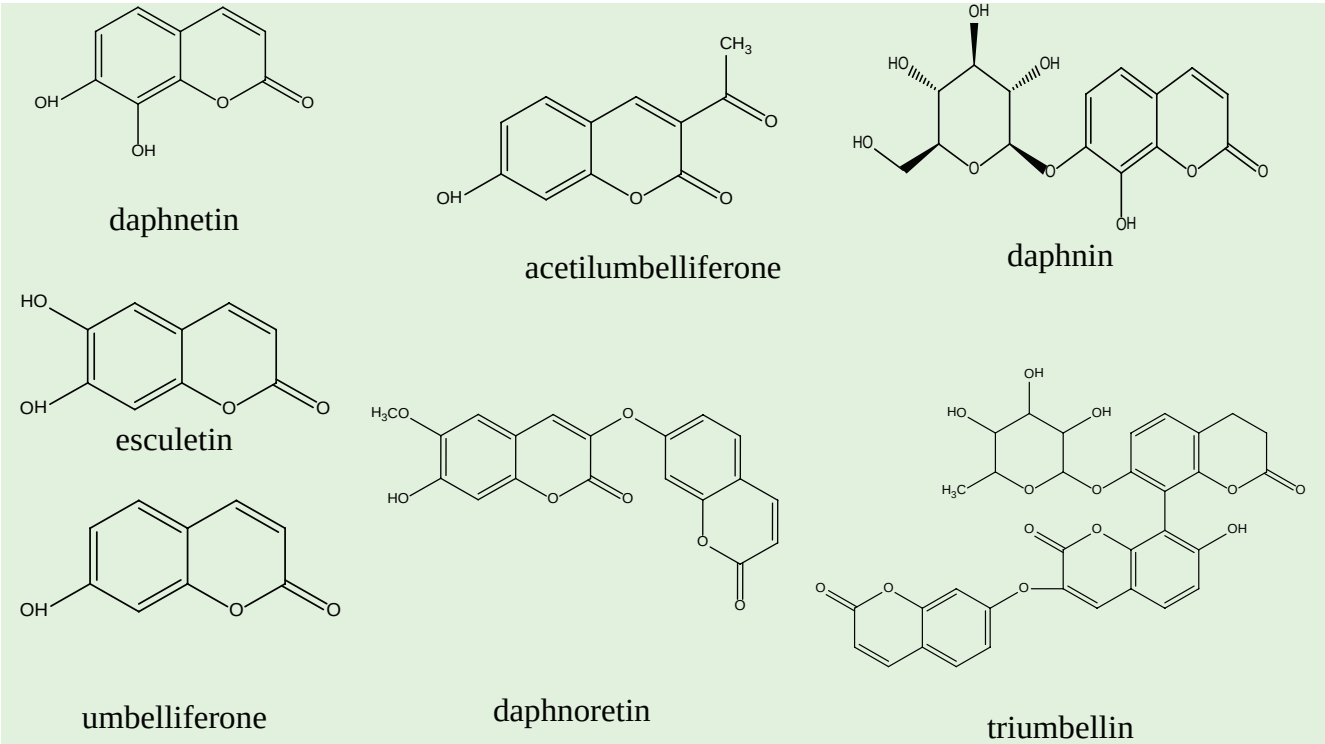

Figure 1. Coumarin derivates in *Daphne* species.

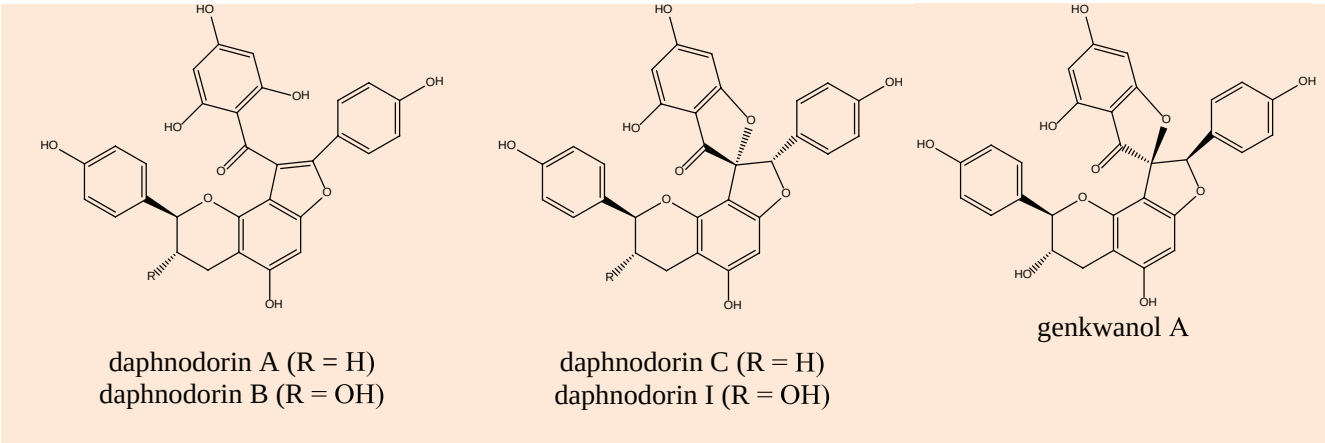

Figure 2. The structures of some biflavonoids isolated from *Daphne* species.

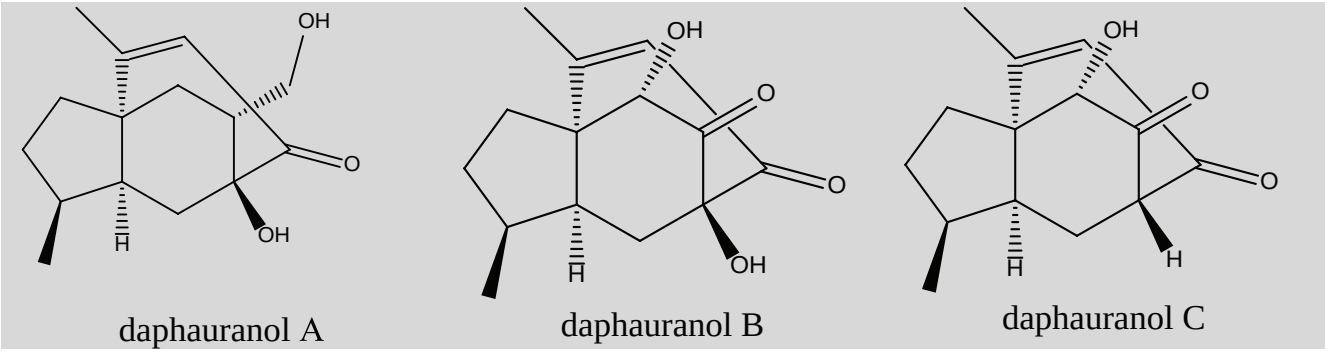

Figure 3. The structures of the sesquiterpenes isolated from *Daphne* species.

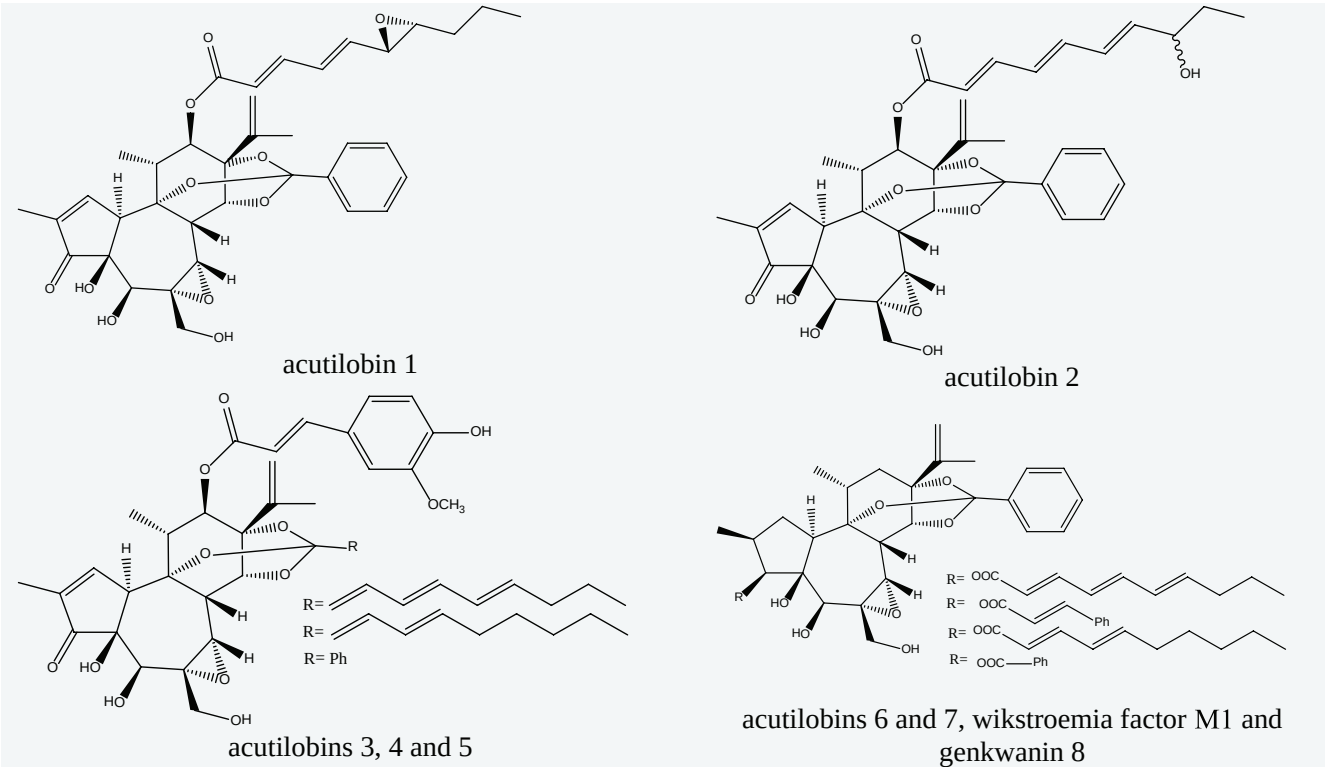

Figure 4. Daphnane-type diterpene esters isolated from *Daphne* species.

composition of aetheric oils obtained from the flowers of these species, giving them a specific smell (50). Diterpenes and triterpenes are found as terpenoid constituents in the extracts obtained from other parts of these plants.

Sesquiterpenes

Daphnauranols are bioactive tricyclic sesquiterpenes which have recently been isolated from *D. aurantiaca* (Figure 3) (51). Their insecticidal activity has been confirmed, so they can be used in the protection of plants from harmful insects as a non-toxic, safe and biodegradable alternative to synthetic pesticides (52).

Daphnane-type diterpene esters

Diterpenoid esters of the daphnan type are primarily isolated from plants that belong to the *Thymeleaceae* family, and only a few of them have been discovered in plants from the *Euphorbiaceae* family. They represent the main type of known plant orthoesters and they exhibit numerous biological activities (53-55). This group of molecules also contains acutilobins, which are found in some *Daphne* plant species, such as *D. acutiloba* (Figure 4). Acutilobins exhibit considerable anti-HIV activity, while the strongest activity is exhibited by acutilobin G. Also, they exhibit a significant cytotoxic activity tested in five human tumor cell lines (56). Juanhuanin is a white amorphous powder isolated from the *D. genkwa* flowers (49). It possesses antitumor activity by inhibiting the growth of lung cancer cells and can find potential use as a chemotherapeutic agent (57). Genkwadaphnin is a daphnan diterpenoid ester which is isolated from *D. genkwa*. It exhibits antineoplastic effects against leukemic cell lines and it induces the apoptosis of skin tumor cells (58).

Triterpenes

Pentacyclic triterpenoids, taraxerol, taraxerone and taraxerol acetate are isolated from *D. papiracea* (59). Additionaly, in different *Daphne* species, the presence of ursolic acid, β-viscol, as well as α and β-amirin were confirmed (Figure 5) (60).

Steroids

The most often molecules of steroid structure found in *Daphne* plant are phytosterols: β-sitosterol and β-sitosterol-β-D-glucoside (Figure 6) (60).

Lignans

Recent research of the chemical composition of the plants of the *Daphne* genus has shown the presence of compounds of the lignan metabolites (Figure 7) such as dihydroxysesamin, sesamin, lariciresinol, pinoresinol and syringaresinol (61, 62). These compounds have been identified in several species of this genus.

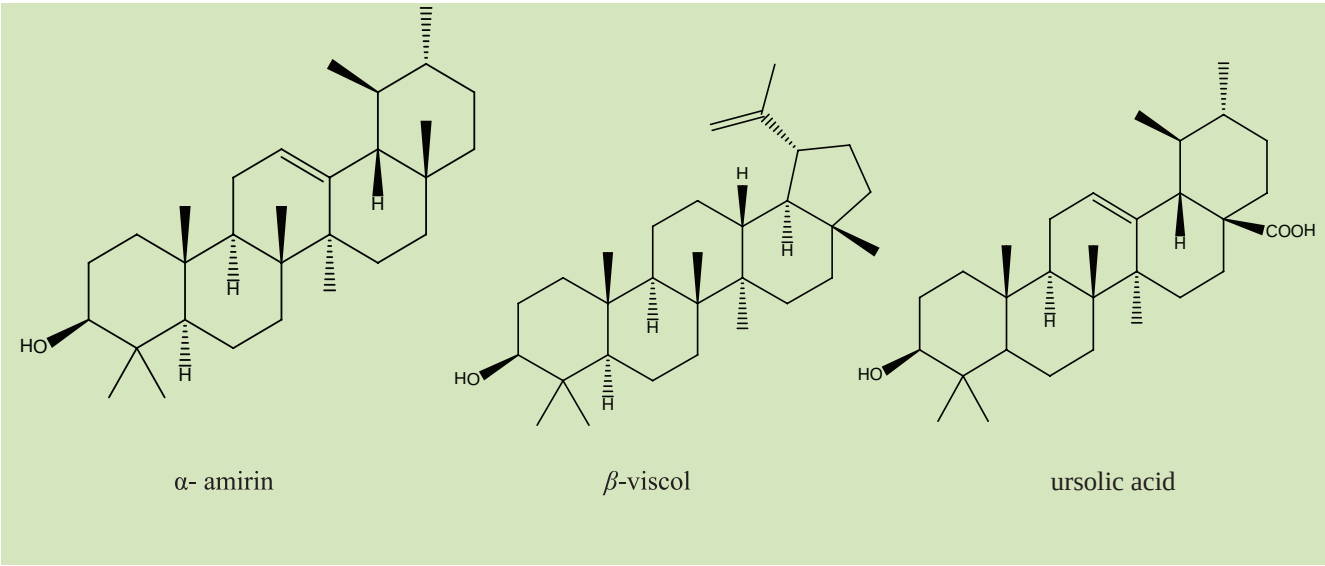

Figure 5. The structures of some triterpenoids isolated from *Daphne* species.

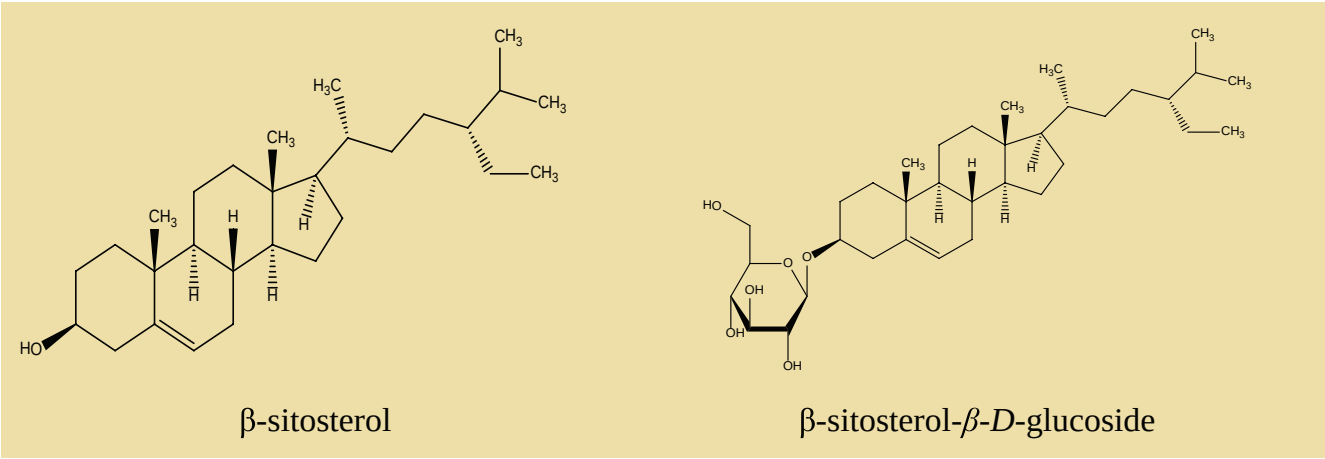

Figure 6. The structures of some phytosterols isolated from *Daphne* species.

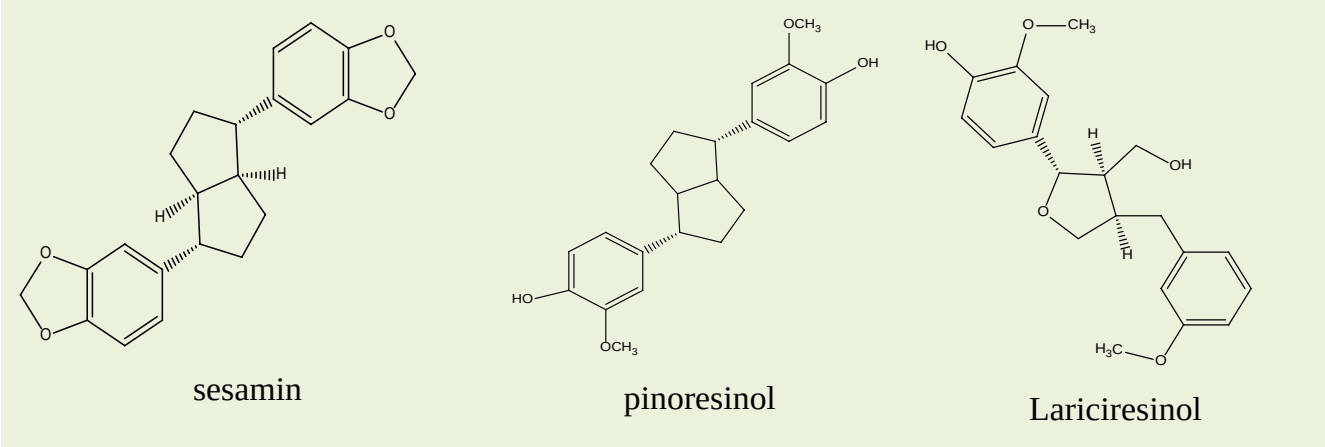

Figure 7. The structure of some lignans isolated from Daphne species.

The plant species of the genus *Daphne*: Pharmacology screening and use in traditional medicine

Plant species of the *Daphne* genus are used in the traditional methods of treatment, especially in Chinese traditional medicine and traditional medicine in tropical part of Africa (63). Although nearly half of the plants of this genus have been investigated in detail, there is still a great possibility for founding new natural bioactive molecules (44).

Daphne oleoides Schreber ssp. Oleoides (Spurge-olive). The *D. oleoides* root is used as a laxative, its cortex and leaves are used in the treatment of skin damage and ulcers (64). The leaves of this plant are used in the treatment of gonorrhea and abscess. The above-ground parts of *D. oleoides* are used in Turkish traditional medicine in the treatment of rheumatic pains, lumbago and fever (65). A study showed that active components isolated from this plant, genkwadafnin and dehydrodaphnetoxin, are primary bioactive components which strongly inhibit cytokines on which macrophage activity is depended (65).

Daphne genkwa Sieb. et Zucc.(Lilac Daphne). The flowers are used as diuretic, anti-tussive, expectorants, anti-carcinogenic and anti-inflammatory agent in Chinese and Korean traditional medicine (66). In Chinese traditional medicine, flowers are used for the relief of the rheumatic symptoms. Latest studies showed that flowers, which mostly contain flavonoid compounds, exhibit anti-inflammatory, analgetic and immunomodulatory activity (67). Flavonoid fraction extracted from the flowers of this plant, which is made of luteolin, apigenin, hydroxy genkwanin and genkwanin shows significant therapeutic effects on arthritis in mice, without obvious adverse effects (68). The methanol extract of flower *D. genkwa* buds exhibits an inhibitory effect on the production of nitrogen monoxide (NO) which has an important role in neurotransmission, blood pressure regulation and cell defense systems (69).

Daphne odora Thunb.(Fragrant Daphne, Winter Daphne). In traditional Chinese medicine, the root of the *D. odora* is used for treating stomachache, bruises and bites of venomous snakes, while the leaves are used for treating abscess and neuralgia (70).

Daphne acutiloba Rehd.. The root and the bark of the *D. acutiloba* plant is used in traditional Chinese medicine under the name "*jin yao dai*" to treat bruises and scrofula (71).

Daphne feddei Levl. This plant which contains daphnane type diterpenes, exhibits immune-stimulating and anti-neoplastic effect (72).

Daphne gnidium L.(Flax-leaved Daphne). In traditional medicine, the leaves of the *D. gnidium* plant are used as a hypoglycemic compound and for treating skin diseases. This plant is traditionally used as a medium for dyeing in the textile industry. However, the use of this plant is considered dangerous because of certain toxic effects. Its use can cause headache, paleness, shivers, swelling of the lips and mouth, convulsions, and even death (73). The analysis of the methanol extracts of the branches has shown that the extract has quite good antimicrobial activity, especially against *Bacillus lentus* and *Escherichia coli* bacteria. Daphnetin, genkwanin and 2,5,7,4'-tetrahydroxy flavanol have shown the strongest effect (39).

Daphne retusa Hemsl. This is a plant from the "*Zhu Shi Ma*" traditional Chinese medicine, and it is used to treat rheumatism and to decrease the swelling and pain in priapism, while its ethanol extract exhibits anti-inflammatory and anti-analgesic activity (74). The results of the toxicity testing of the ethanol extracts on mice have demonstrated a low level of toxicity (75).

Daphne mucronata Royle (Kashmir Daphne). In traditional medicine this plant is used for treating tumors and skin diseases (74). The water-ethanol extract of this plant exhibits cytotoxic activity, especially on breast cancer cell lines. The extract also exhibits antileucemic activity, especially on *U937* cell lines. The ethanol extracts exhibit antimicrobial activity against gram-positive bacteria (*E. coli* and *S. aureus*) (76).

Daphne pontica L. (Twin flowered Daphne). The extracts of different parts of the *D. pontica* plant exhibit anti-inflammatory and antinociceptive activity (77).

Daphne mezereum L. (Mezereon, Paradise plant, February Daphne). The water-alcohol extract of the *D. mezereum* plant has shown antileukemic activity on P-388 lymphocytic cells in mice (78). The active compound mezerein, which has been isolated from this plant, has shown a significant inhibitory effect against P-388 cells and L-1210 type of leukemia in mice in the 50μg dosage (79).

Daphne giraldii Nitsche. Active compounds from this plant exhibits the pharmacological effect of a hemostasis (80).

Daphne altaica Pall. is used in traditional Chinese medicine for treating esophagus and stomach cancer, tracheitis, fever, throat inflammation, snake bites, and it is also used as an antitusic and a diaphoretic agent (81). The extracts of the bark has an antiproliferative effect, so it can be considered as a source of anticancerogenic substances (82).

Daphne papyracea Wall. (Nepali paper plant). The flavonoid compounds found in this plant, exhibit sedative and hypotensive effects (59). Its ethanol extracts exhibit strong cytotoxic effects (83).

Daphne acutiloba Rehd. is used in traditional medicine to treat wounds and bruises (71).

Daphne acuminata Boiss. & Hohen. ex Stocks. The methanol extract of *D. acuminata* exhibits hypotensive and cardiotoxic effects (84).

The extracts of *Daphne* species located in Serbia (*Daphne alpina subsp. alpina, Daphne cneorum* L. (Garland flower or Rose Daphne), *Daphne blagayana* L. Freyer and *Daphne malyana* Blečić) exhibit significant antioxidant and antimicrobial activities. Analysis of the chemical composition of these species has demonstrated the presence of different secondary metabolites that belongs to the groups of coumarins, phenolic acids and flavonoids, which are considered to be responsible for their biological activitiy (37, 40, 41, 85, 86).

The traditional medicinal practices of many cultures utilize parts of several *Daphne* plants species for the treatment of a wide range of disorders. The summary of reported medicinal uses, parts of the plant and form is given in the Table 2.

Table 2. Summary of reported medicinal uses of plant from the genus *Daphne*

Plant	Part	Form	Use	Reference
D. alpina	Root	Tincture	Induction of menses	87
D. genkwa	Buds	Tincture	Abortifacient	88
			Diuretic	89
			Expectorant	90
	Flower, root		Tonic	91
			Malaria	91
			Mushroom poisonig	92
D. giraldii	Rootbark	Tincture	Analgesic antidyne for treatment of hemorrhoids	93
D. gnidium	Bark	Ointment	Vesicant	94
		Tincture	Diuretic	95
			Stimulant	96
	Rooth	Powder	Abortifacient	97
D. lureola	Bark	Powder	Diuretic	55
			Laxative	265
D. mezerum	Bark	Decoction		98
			Anticoaugulant	98
			Scrofula	99
			Siphilis	100
			Vesicant	100
	Root-Bark	Tincture	Laxative	65
		Infusion	Dropsy	31
		Decoction	Rheumatism	
D. odora	Root-Bark	Decoction	Swelling	65
			Toothache	101
			Vermifuge	97
	Root, leaf	Decoction	Sore throat	101
			Wash for smallpox pustules	92
D. oleoides	Leaf	Infusion	Gonorrhea	102
			Abscesses	102
	Leaf	Poultice	Boils	102

CONCLUSION

The data shown in this paper illustrates a wide range of pharmacological and biological activities that the extracts obtained from different parts of the plants of the *Daphne* species exhibit. The presence of different groups of secondary metabolites indicates that these plants might serve as a source of active compounds, some of which with potential pharmaceutical significance. It is also significant that there are still species that have not been examined in detail especially from the aspect of their chemical composition and its exhibition of biological activity, which opens up possibilities for discovering new molecules. Future research should go in the direction of a more detailed and broader chemical characterization of bioactive components, as well as in the direction of the examination of other biological activities, both under *in vitro* and *in vivo* conditions. Plants from the *Daphne* genus may represent a source of new, natural, pharmacologically active compounds, which may potentially be used in pharmaceutical, cosmetic and food industry.

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LIFE-THREATENING PLASMODIUM FALCIPARUM MALARIA IN PATIENT AFTER VISITING ANGOLA-CASE REPORT

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TEŠKA FORMA PLAZMODIJUM FALCIPARUM MALARIJE KOD BOLESNIKA KOJI JE BORAVIO U ANGOLI-PRIKAZ SLUČAJA

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ABSTRACT

Malaria is a potentially life-threatening disease, especially when complicated by a septic shock. It is caused by infection of erythrocytes with protozoan parasites of the genus Plasmodium that are inoculated into the humans by a feeding female anopheline mosquito. Of the four Plasmodia species, infection with Plasmodium (P.) falciparum is oftenassociated with different types of complications and significant mortality. Most imported cases of malaria are not in tourists but in immigrants and their children who have returned to the country of their family's origin to visitfriends and relatives (so-called VFR travelers) and have forgone chemoprophylaxis.

We described a case of a 52 year old patient who came from Angola, an African country with endemic malaria before the occurrence of the first symptoms of the disease. The first symptoms were not recognized by the presence of nonspecific symptoms. Very soon the patient was gone under the hemodynamic unstability that eas followed by shock and high percentage parasitemia of 25%. A global health disorder was developedaccompanied withhemodynamic instability and cerebral dysfunction. He performs pulmonary ventilation disorder and renal failure. Only data from social epidemiological survey of travel to the African country, was sufficient to cast doubt on malaria. The diagnosis was conducted using the standard method - peripheral blood smear. After turning antimalarial drugs, improvement of health status with complete recovery within 10 days was noticed. The only consequence of the disease is persistent hypertension that is sensitive to standard antihypertensive therapy.

Keywords: *P.falciparum malaria, cerebral dysfunction, antimalarial drugs, chemoprophylaxis*

SAŽETAK

Malarija je jedna odnajtežih zaraznih bolesti,posebno ako se komplikuje septičnimšokom.Karakteriše se ponavljanim napadima groznice zbog sinhronizovanog raspadanjaeritrocita, zaraženihjednom ili više vrsta parazita iz rodaPlasmodium. Od četirivrsteroda Plasmodium, infekcijaP. falciparum je često povezana sa različitim komplikacijama i značajnim smrtnim ishodom. Većina importovanih slučajeva malarije nisu turisti, većimigranati ili radnici koji rade u endemskim područjima supsaharske Afrike,koji nisu koristili lekove za hemoprofilaksu.

U ovom radu prikazan je slučaj bolesnika, starosti 52 godine sa teškom kliničkom slikom malarije koji je doputovao iz Angole,gde je malarija endemska bolest.Prvi simptomi bolesti u vidu tempereture i groznice nisu bili pravovremeno prepoznati, tako da u daljoj evoluciji bolesti razvija se hemodinamska nestabilanost , šokno stanje sa multiorganskom insuficijecijomi visokim procentom parazitemije od 25%.Dolazi do razvoja respiratorne i bubrežneinsuficijencije sa razvojem DIK-a uz poremećaj stanja svesti. Podaciiz socio-epidemiološe anamneze o putovanju u afričke zemlje, su ukazali na sumnju na malariju. Dijagnoza je potvrđenaperifernimkrvnimrazmazom kao standardnommetodom. Nakon uključivanja antimalaričnе terapije, uz intenzivnu potpornu terapijuusledilo je poboljšanje zdravstvenog stanja sa potpunim oporavkom u roku od 10 dana. Kao posledica bolesti registrovana je hipertenzija, koja je lečena standardnom antihipertenzivnom terapijom.

Ključne reči: *P. falciparum malaria, cerebralna disfunkcija, antimalarici, hemoprofilaksa*

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INTRODUCTION

Malaria is an important cause of illness in children and adults, especially in malaria's endemic areas. It is caused by infection of erythrocytes with protozoan parasites of the genus Plasmodium that are inoculated into the humans by a feeding female anopheline mosquito. In non endemic areas, this clinical entity is rare and locally acquired infections. Reports from the National Vector Borne Disease Control Programme (NVBDCP) have indicated that around 1.8 million cases of malaria and 1,000 malaria-related deaths occur annually in the country (1). However, the World Health Organization (WHO) estimates that there are about 20 million cases of malaria and 15,000 deaths annually in India. (2)

The signs and symptoms of malaria are nonspecific. Malaria is clinically suspected mostly on the basis of fever or a history of fever. Diagnosis based on clinical features alone has very low specificity and results in over-treatment. Other possible causes of fever and the need for alternative or additional treatment must always be carefully considered. Clinical suspicion of malaria should be confirmed with a parasitological diagnosis.

Of the four Plasmodia's species, infection withPlasmodium (P.) falciparum is oftenassociated with different types of complications and significant mortality. Most imported cases of malaria are not in tourists but in immigrants and their children who have returned to the country of their family's origin to visit friends and relatives (so-called VFR travelers) and have forgone chemoprophylaxis. (3-6)

Malaria is clinically suspected mostly on the basis of fever or a history of fever. Diagnosis based on clinical features alone has very low specificity and results in over-treatment. Clinical suspicion of malaria should be confirmed with a parasitological diagnosis.

The primary objective of antimalarial treatment in severe malaria is to prevent death, but prevention of neurological deficit is also an important objective in treating cerebral malaria. Most of the countries where *P. falciparum* isn't endemic have progressively updated treatment policies from the artemisinin based combination therapies (ACTs); this is the best current treatment for severe form of disease complicated especially for cerebral dysfunction.

CASE REPORT

A previously healthy 52-year-old caucasian man was admitted to the Department of infectious and tropical disease in the Clinical center of Kragujevac, Serbia, due to high fever (39°C), shivering and confusion. Epidemiologically, he left Angola two months before the onset of the first signs of disease. During his two-years' staying in this African country, he used chemo-prophylaxis against malaria, although a disease was endemic in most of the African countries. Specific chemo-prophylaxis was not conductedlast six months before onset of the disease. After the first signs and symptoms were registered, the patient was unsuccessfully treated by macrolide 500 mg daily and aminoglycoside antibiotics. Very soon, reduced exercise tolerance that followed sweating and high fever was noticed.

Laboratory evaluation revealed the white blood cells of 10.4 x 10^9/L , with neutrophils (85.5%), red blood cells of 2.39 x10^{12}/L, hemoglobin of 74 μmol/L, lactate dehydrogenase of 2446 U/L), indicative of ongoing hemolysis. Procalcitonin and C-reactive protein (CRP) were markedly elevated (7.77 μg/L and 99.7 mg/dL, respectively) and the patient showed severe thrombocytopenia (thrombocytes' value of 7x10^9/L) with signs of petechial haemorrhage . In blood analysis, there was a sign of fibrinolysis (D dimer was 15 687). Other blood tests were normal.

The X-ray chest radiograph on admission (PA view) was normal.

The P. falciparum parasites were detected using microscopical examination of a thick blood film and parasitemia of 25% of parasites's infected erythrocytes was noticed.

The somnolence with generalized convulsions were developed several hours after the admission and the patient was admitted in Intensive Care Unit (ICU) of the Clinic. The cerebrospinal fluid (CSF) contained 300 mg per deciliter of protein and 3 100 mg per deciliter of glucose. After he had been admitted in ICU, severe respiratory failure has been detected. The patient was undergone invasive mechanical ventilation with 10 l by minute of pure oxygen therapy. Laboratory findings that suggested on acute respiratory failure were saturation under 86%, oxygen partial pressure of 6.2 kPa and carbon partial pressure of 4.8 kPa. The X-ray chest radiograph after diagnosed respiratory failure showed diffuse emphasized of the lung parenchyma and hila. A mechanical ventilation was immediately initiated under analgesia and sedation with propofol and sufentanil. The patient was breathing spontaneously by means of pressure support of 15 to 20 mmHg and a positive endexspiratory pressure of 5 mmHg. The shock was treated with fluid resuscitation according to the concept of early goal directed therapy.

In the ICU, a central venous catheter was inserted into the right jugular vein and a catheter for pulse contour analysis and continuous hemodynamic measurement was placed into the left femoral artery. A computed tomography (CT) scan of the brain showed no signs of bleeding, ischemia or edema. Due to increased myoglobin levels, increased blood urea nitrogen (value of 49,7 mmol per liter) and creatinine (value of510 μmol per liter), the subsequently developed renal failure, renal replacement therapy was initiated via continuous venovenous hemodiafiltration (CVVHDF).

The drug mefloquine was orally administered in association with artemisinin in dose of 80 mg twice daily. The next day, the parasite load of 25% on admission decreased to 20%. The parasite load decreased to 15% the next day. The extremely high scores (Acute Physiology and Chronic Health Evaluation II, Simplified Acute Physiology Score II, Sequential Organ Failure Assessment) on the day of

the shock improved gradually within thefifth day of orally administered antimalarial drugs. Mechanical ventilation was discontinued on day 7, and continuous dialysis was replaced by intermittent dialysis.The following day the hemodynamic status of the patient stabilized with a raise of SBP to 180 mmHg respectively and the heart rate was 90 beats/min. Laboratory data showed increased thrombocytes ($51x10^9$/L), decreased D-dimer (4854), and improved parameters of renal function.At the time of discharge (after 10 days of hospitalization), renal function had been resumed and thus there was no need for further dialysis. As a consequence of P. falciparum malaria, mild hypertension was registered that was sensitive on standard antihypertensive medications.

DISCUSSION

The consequences of infection with *P. falciparum* range from asymptomatic parasitemia to severe and often fatal malaria. Repeated malaria infections lead to gradual acquisition of immunity in regions where it is endemic, but the mechanisms of antimalarial immunity remain poorly understood. In addition, the reasons why some infections progress from asymptomatic parasitemia to uncomplicated febrile illness and others to severe clinical manifestations are unclear (7).

We described an unusual case of a patient with malaria in which high parasitemia resulted in a positive thick blood film. Many clinical examinations as well as clinical records described the cases ofmalaria with parasitemia being 1.8% to 10% (8, 9). There is no consensus on what constitutes 'hyperparasitaemia'.Hyperparasitaemia in itself does not necessarily have major prognostic significance in semi-immune individuals (individuals living in an endemic area and exposed to malaria several times). The individuals with some antimalaria immunity can often tolerate high parasite counts without severe effects. However, in non-immune travellers, the parasitaemia is often an indicator of potentially severe disease (9), and levels as low as 2% may be considered in some cases as the prelude to severe disease. In our case report a parasitemia amounted to 25%, which is different from many other clinical presentations. At the time of presentation in our emergency room, the patient suffered from severe malaria. He was severely confused and during the first hours his hemodynamic and respiratory status became deteriorated. Despite of his unstable status, the patient showed biochemical features indicating a poor prognosis, amongst others a very high parasite load, respiratory and renal failure as well as disturbance of consciousness.

Procalcitonin, which was found to be pathological during or even prior to sepsis, was extremely high on admission in the ICU. After several hours from admissionthe patient developed a shock. The frequency of shock on admission to a hospital in patients with severe malaria is 7.7% , and is up to 21.5% in hospitals with ICUs, that are specialized in the treatment of infectious diseases (10).

The first symptoms of malaria (most often fever, chills, sweats, headaches, muscle pains, nausea, and vomiting) are often not specific and are also found in other diseases (such as influenza and other common viral infections). Likewise, the physical findings are often not specific (elevated temperature, perspiration, tiredness). These are the reasons why the disease in this patient did not initially recognized. The therapy, thus, was not administered properly in the beginning of the disease. Furthermore, the most important information of relevance for the diagnosis of malaria is the history of travelling to the African country where the malaria is epidemic disease. The social epidemiological survey from the disease's history shows a critical risk factor for the disease. In severe malaria (caused by P. falciparum in our case), clinical findings (confusion, coma, neurologic focal signs, severe anemia, respiratory difficulties) are more striking and may increase the suspicion index for malaria. Therefore the first diagnostic procedure for detection of malaria was done - a thick blood film examination.

The primary objective of antimalarial treatment in severe malaria is to prevent death. In treating cerebral malaria, prevention of neurological deficit is also an important objective. The cause of death in severe P. falciparum malaria is often multi-factorial, but shock is one of the leading causes, a fact that forces us to adapt a quick and sophisticated treatment approach towards the critically ill patient.

Unrousable coma may persist for up to 72 h in children and longer in adults. Long-term neurological sequelae of cerebral malaria have been reported in African children and also in non-immune travellers. Patients who are unconscious should be nursed in the appropriate position, their stomach drained with a nasogastric tube with an endotracheal tube inserted. Regular neurological observations should be recorded. Mechanical ventilation may be necessary to reduce intracranial pressure.

A relationship between the cardiac event and the parasite challenge and/or its treatment seems probable, especially because of the chronology of the event and the absence of an alternative explanation. Thecardiac complications are extremely rare in malaria. To date, myocardial infarction during or after naturally acquired *P. falciparum* infection has not been reported, neither in hundreds of millions of endemic cases, nor in hundreds of thousands military troops that were temporarily deployed to endemic areas. In our case, only cardiovascular complications that raised from malaria was hypertension that was sensitive on standard antihypertensive therapy.

CONCLUSION

Severe imported malaria still carries a relatively high mortality rate, even when treated under optimal conditions in a highly experienced ICU. Although WHO criteria are not all relevant to imported malaria in adults, the presence in the emergency room of any degree of neurologic,

acid-base, circulatory, or pulmonary failure should lead to ICU admission. Bacterial coinfection is not infrequent and may contribute to death. Finally, it should be kept in mind that most of our patients did not take appropriate malaria chemoprophylaxis. Thus, the best way to reduce the number of deaths caused by imported malaria is to improve the quality of prevention.

Every febrile patient with a history of travel to the regions where malaria is endemic (tropical regions for the world, southeast regions for our country) should raise the suspicion of malaria.

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ACCESSORY AURICLES – REPORT OF TWO CASES

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AKCESORNE AURIKULE - PRIKAZ DVA SLUČAJA

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ABSTRACT

Accessory auricle is a rare anomaly with an estimated incidence rate of 0.2-0.5%. The most common form of this malformation is the accessory tragus. It may be a sign of other syndromes, such as oculo-auriculo-vertebral dysplasia (Goldenhar's syndrome). In this paper, we describe two cases of accessory auricle with a focus on diagnosis and surgical treatment.

Keywords: a*ccessory auricles, surgical treatment, congenital syndromes*

SAŽETAK

Akcesorne aurikule su retka anomalija sa incidencom 0,2-0,5%. Najčešći oblik ove malformacije je akcesorni tragus. Može da bude u sklopu pojedinih sindroma, kao što je okuloaurikulovertebralna displazija (Glodenharov sindrom). U ovom radu opisujemo dva bolesnika sa akcesornim aurikulama uz posebnu pažnju na dijagnostiku i hirurški tretman.

Ključne reči: *akcesorne aurikule, hirurški tretman konmgenitalni sindromi*

INTRODUCTION

Accessory auricle (polyotia) is one of the most common congenital anomalies of the ear. This anomaly is the result of a disruption in the development of branchial arc I or II and is thought to be an autosomal dominantly inherited disorder, with an incidence rate of 0.2-0.5%. Accessory auricles usually present unilaterally and are more frequent in male patients. It can be solitary or multiple with different morphology and localization (1-3). The most frequent localization is on the face in front of the tragus. It could be an isolated malformation or be associated with other anomalies, such as cleft lip or palate, hypoplasia of the lower jaw, and eye and spine abnormalities. It could be a component of another syndrome and is best known as Goldenhar's syndrome (oculo-auriculo-vertebral dysplasia). The most common and mildest form of this malformation is accessory tragus. Sometimes, larger accessory auricles may be found on the face or neck as a cartilaginous skeleton covered with skin. This anomaly is rarely seen in other regions, such as the middle ear and the pharynx. The definitive diagnosis of an accessory auricle is achieved by post-excisional histological analysis.

CASE REPORT 1

A 23-year-old male patient was admitted to the Clinical Center Kragujevac in Serbia for surgical treatment of multiple skin tumours that were localized between the tragus and the corner of the mouth (Figure 1). The largest tumour had a fistulous channel and secreted cerumen. There were no

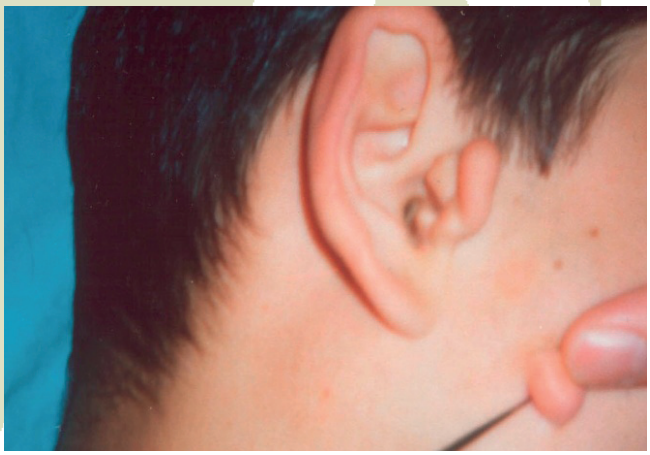

Figure 1 Local findings in case 1 (sound probe in the canal)

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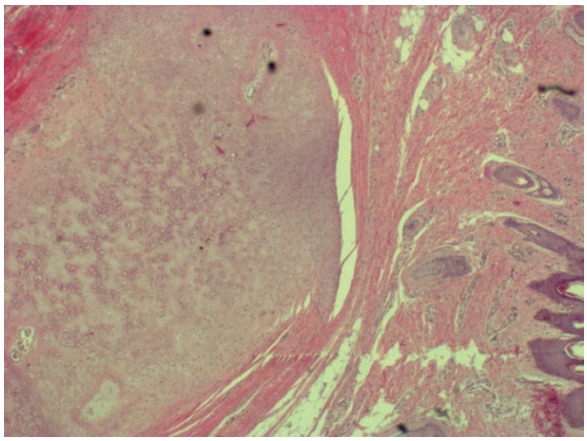

Figure 2 Histopathology (HE, 40X) of the excised accessory auricle of case 1

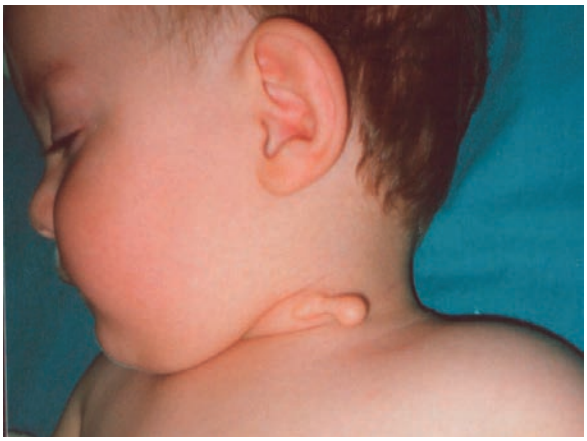

Figure 3 Local findings in case 2

openings of the channels into the mouth. Ophthalmologists and otorhinolaryngologists were consulted, and the patient had no other anomalies. All of the tumours were completely removed together with the fistulous channel to the level of the facial muscles. Histopathology confirmed that this was an inherited anomaly (Figure 2). He was discharged from the hospital on the 3rd postoperative day with no complications, and at the last follow-up examination three months after surgery, there were no signs of recurrence.

CASE REPORT 2

A one-year-old male patient underwent surgery at the Clinical Center Kragujevac in Serbia because of a growth on the left side of the neck that was medial to the anterior edge of the sternocleidomastoid muscle (Figure 3). Ophthalmologists and otorhinolaryngologists were consulted, and the patient had no other anomalies. During surgery, the fistulous channel, which extended to the common carotid artery bifurcation, was completely removed. Histopathological analysis confirmed that the growth was an accessory auricle. The postoperative period was uncomplicated, and the patient was discharged from the clinic on the 3rd postoperative day. There was no recurrence during the three-month observation period.

DISCUSSION

Congenital anomalies of the ear are numerous but relatively rare. Their aetiology is not known. Data in the literature indicate that the incidence of these malformations is 1:6000 and is most prevalent among the Japanese population and the Navajo Indians (4). It is thought that ear malformations occur due to improper fusion of the six auricular hillocks during auricle development. One of the most common congenital anomalies of the ear is the accessory auricle. In most cases, it is a mild malformation that manifests as a supplementary tragus in front of the normal tragus or at the ascending crus of the antihelix. The more severe anomalies are less common, and those with a morphologically formed auricle are referred to as polyotia (5-9). This is a very rare form of malformation and is defined as an accessory auricle that, by its size and morphology, resembles a normal auricle. Accessory auricles are more common in male patients and are unilateral in 90% of cases (10). This anomaly most frequently occurs on the face, but it can be found on other locations such as the lateral side of the neck (11, 12), suprasternal region (13), middle ear (14), Eustachian tube (15), nasopharynx (16, 17, 18), oropharynx (19) and nasal vestibule (20).

Many genetic studies of accessory auricle have indicated that it is an autosomal, dominantly inherited disorder, although there have also been reports of it being acquired from X-linked recessive and autosomal recessive inheritance. One study analysed gene maps of 11 families for the autosomal dominant accessory auricular anomaly (ADAA) and showed that it was an autosomal dominant abnormality with complete penetration, and the isolated gene locus was 14q11.2-q12 (21). According to numerous studies, accessory auricle may be associated with other congenital anomalies. Goldenhar's syndrome is the most common form and is also known as oculo-auriculo-vertebral syndrome (OAV) (22-24). This is a rare syndrome with incomplete ear, nose, soft palate, lip, and lower jaw development, and often occurs with spinal scoliosis and lordosis. Sometimes, there are anomalies of internal organs such as the heart, lungs and kidneys. The incidence of this syndrome in the UK is 1/3,500 to 1/26,000. These anomalies are unilateral in 90% of cases. They are frequently accompanied by limbal dermoid of the eye, accessory tragus, and strabismus. The following syndromes are much rarer: Wolf-Hirschhorn syndrome (WHS) (25, 26), also known as chromosome 4p deletion syndrome; and Pitt-Rogers-Danks syndrome (PRDS) or Pitt's syndrome. The main features are microcephaly, micrognathia, hypertelorism, accessory auricle, short philtrum, prominent glabella, psychophysical retardation, muscular hypotonia, and abnormalities of the heart. Less common features are hypospadias, iris

coloboma, renal abnormalities, and IgA deficiency. Multiple accessory tragus and aplasia cutis congenita are part of the rare Preston Delleman's syndrome (oculocerebrocutaneous syndrome) (27). Townes-Brocks syndrome (TBS) is extremely rare and occurs in only 200 people worldwide. It is the result of mutations in the SALL1 gene and is autosomal dominantly inherited. It is characterized by accessory tragus, inner ear anomalies, and malformations of the anorectal region, heart, kidneys, hands and feet.

Accessory auricle is diagnosed by clinical examination and histopathologic analysis. It is usually treated surgically and usually involves a radical excision into the periphery and below the growth (2, 5). The operative duration is dependent on the severity of the anomaly and the presence of associated anomalies. Occasionally, preoperative fistulography is required. In addition to traditional excision, other surgical methods have been described such as the application of special clips (28), which may be useful for milder forms of the anomaly and for those without a fistula.

The diagnosis of accessory auricle is usually based on clinical examination and sometimes requires fistulography; a consultation to rule out the possibility of a skin tumour is also warranted. Therefore, definitive diagnoses are possible only after histopathological analysis of the completely excised skin lesion. It is important to exclude the associated anomalies, especially in the middle and inner ear as well as the eye. Accessory tragus is a feature of Goldenhar's syndrome, and therefore, otorhinolaryngological and ophthalmological examination is recommended. Occasionally, there is a need for paediatric or genetic consultation. Treatment of this anomaly is surgery (30). Since the rudimentary canal may spread to the deeper facial and neck neurovascular structures, a plastic surgeon and/or otorhinolaryngologist should be consulted.

DECLARATION OF CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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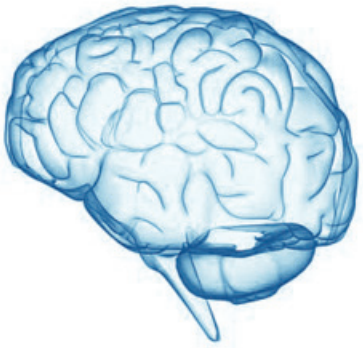

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