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MODULATION OF EPILEPTIC ACTIVITY IN RATS: FOCUS ON SLEEP, PHYSICAL EXERCISE AND NITRIC OXIDE-MEDIATED NEUROTRANSMISSION IN A MODEL OF HOMOCYSTEINE THIOLACTONE-INDUCED SEIZURES

Dragan Hrncic[1], Aleksandra Rasic- Markovic[1], Veselinka Susic[2], Dragan Djuric[1] and Olivera Stanojlovic[1]†
[1]Laboratory of Neurophysiology, Institute of Medical Physiology "Richard Burian", Faculty of Medicine, University of Belgrade, 11000 Belgrade, Serbia
[2]Serbian Academy of Sciences and Arts, 11000 Belgrade, Serbia

MODULACIJA EPILEPTIČNE AKTIVNOSTI KOD PACOVA: SPAVANJE, FIZIČKA AKTIVNOST I NEUROTRANSMISIJA POSREDOVANA AZOT MONOKSIDOM U MODELU EPILEPSIJE IZAZVANE HOMOCISTEIN TIOLAKTONOM

Dragan Hrnčić[1], Aleksandra Rašić- Marković[1], Veselinka Šušić[2], Dragan Djuric[1] i Olivera Stanojlović[1]†
[1]Institut za medicinsku fiziologiju "Rihard Burijan", Medicinski fakultet Univerziteta u Beogradu, Beograd, Srbija,
[2]SANU, 11000 Beograd, Srbija

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ABSTRACT

Epilepsy is a chronic neurological disorder characterised by recurrent epileptic seizures. Understanding the mechanisms by which it initiates and develops, as well as its modulating factors, are of great scientific interest. Experimental models of epilepsy are useful for understanding these mechanisms.

Homocysteine, an amino acid endogenously generated in the body, together with its reactive metabolite, homocysteine thiolactone (HCT), is recognised as a risk factor for a variety of diseases. HCT-induced seizures are a model of generalised epilepsy in which the coexistence of two types of epileptic activity has been documented. The complex interplay between sleep and epilepsy is still only poorly understood. Additionally, the relationship between physical exercise and epilepsy is quite intriguing, especially the mechanism underlying this relationship. The role of nitric oxide (NO)-mediated neurotransmission in the development of epileptic activity is highly debated in the existing scientific literature .

In this review article, we described the modulation of epileptic activity in rats and focused on sleep, physical activity and NO-mediated signalling. First, we explain the characteristics of the experimental models of epileptic activity and the unique features of HCT-induced seizures. Second, the modulating effects of sleep and regular physical exercise training on epileptic activity, along with works from the authors, are discussed. Finally, the anticonvulsive effects of NO that is produced via nNOS and iNOS in HCT-induced seizures are reviewed.

Keywords: *homocysteine, seizures, sleep, physical activity, nitric oxide*

SAŽETAK

Epilepsija je hronično neurološko oboljenje koje karakteriše rekurentna pojava epileptičnih napada. Razumevanje menahizama nastanka i širenja epileptične aktivnosti, kao i foktora modulacije ovih procesa, od izuzetnog je naučno-stručnog značaja. Eksperimentalni modeli epilepsije su značajni za razumevanje upravo ovih mehanizama.

Homocistein, aminokiselina koja se endogeno sintetiše u organizmu, zajedno sa svojim reaktivnim metabolitom homocistein tiolaktonom (HCT), je prepoznat kao faktor rizika za nastanak različitih bolesti. Epilepsije izazvane HCT-om predstavljaju model generalizovane epileptične aktivnosti u kome je pokazana koegzistencija dva tipa napada. Složeni uzajamni odnos između spavanja i epilepsije još uvek nije dovoljno razjašnjen. Takođe, međuodnos fizičke aktivnosti i epilepsije je krajnje interesantan, naročito mehanizmi ovih odnosa. Uloga neurotransmisije posredovane azot monoksidom (NO) u nastanku epileptične aktivnosti je vrlo kontradiktorna u postojećoj literaturi.

Predmet ovog rada bila je modulacija epileptične aktivnosti kod pacova sa posebnim osvrtom na spavanje, fizičku aktivnost i neurotransmisiju posredovanu NO. Najpre su razmotreni koncepti eksperimentalnih modela epileptične aktivnosti sa osobenostima HCT epilepsija. Zatim su diskutovani modulatorni efekti spavanja i regularnog fizičkog veržbanja na epileptičnu aktivnost zajedno sa rezultatima radova autora. Na posletku, dat je osvrt na antikonvulzivnu ulogu NO i doprinos nNOS i iNOS u epileptičnoj aktivnosti izazvanoj HCT-om.

Ključne reči: *homocistein, epilepsije, spavanje, fizička aktivnost, azot monoksid*

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†Corresponding author: Prof. Dr. Olivera Stanojlović, M.D., Ph.D.
Institute of Medical Physiology "Richard Burian", Belgrade University School of Medicine, Višegradska 26/II 11000 Belgrade, Serbia
Tel/ Fax: ++381-11-3607-106, e-mail: solja@afrodita.rcub.bg.ac.rs

INTRODUCTION

Epilepsy is a chronic neurological disorder with an incidence rate of approximately 50 per 100,000 people per year (1). It is characterised by a variety of cellular and molecular alterations of the brain, especially the cerebral cortex, that result in recurrent epileptic seizures (2). Despite extensive improvements in the pharmacotherapy for epilepsy, it remains poorly controlled in almost 40% of patients (3). Therefore, understanding the mechanisms by which it initiates and develops, as well as its modulating factors, are of great scientific interest. Experimental models of epilepsy are useful in these attempts, but no single model system can be useful for all types of epilepsy (4).

Homocysteine is an amino acid that is endogenously generated in the body during methionine metabolism (5). Homocysteine, together with its reactive metabolite, homocysteine thiolactone (HCT), is recognised as a risk factor for a variety of diseases and is one of the most potent excitatory agents in the central nervous system (CNS) (6-9). The primary mechanism of its epileptic properties has been attributed to the activation of glutamate receptors (10). HCT-induced seizures are a model of generalised epilepsy in which the coexistence of two types of epileptic activity has been documented (11). There is a complex interplay between sleep and epilepsy. This interplay is of special interest for neuroscientists because it is still only poorly understood (12,13). Additionally, the relationship between physical exercise and epilepsy is quite intriguing, especially the mechanism underlying this relationship.

Nitric oxide (NO) belongs to the family of gasotransmitters. The role of NO-mediated neurotransmission in the development of epileptic activity is highly debated in the existing scientific literature (14). The results of studies on the role of NO in epileptogenesis have indicated that it depends on the type of NO production, among other factors (15-17).

With these considerations, we described the modulation of epileptic activity in rats while focusing on sleep, physical activity and NO-mediated signalling in this review article. First, we explained the characteristics of experimental models of epileptic activity and the unique features of HCT-induced seizures.

Second, the modulating effects of sleep and regular physical exercise training on epileptic activity, along with works from the authors, are discussed.

Finally, the anticonvulsive effects of NO that is produced via nNOS and iNOS in HCT-induced seizures are reviewed.

Modelling of epileptic activity in animals: unique features of homocysteine thiolactone seizures

Epileptogenesis is defined as a process of by which a neuronal network transforms into a network of synchronised hyperexcitable neurons. Primarily, it is a consequence of an imbalance between inhibitory and excitatory neurotransmission systems, with overstimulation of the latter (1). The main goal in treatment of epilepsies is the reestablishment of the homeostasis between these systems. Therefore, the only way to develop new antiepileptic drugs and treatments is to further understand the process of epileptogenesis and the mechanisms contributing to it.

A variety of experimental models for epilepsy have been developed, and it is highly likely that no single model system could be useful for all types of epilepsy (4). Modelling the epileptic activity in animals involves two main approaches: administering different chemical compounds and electrical stimulation kindling. In our Laboratory for Neurophysiology, different animal models have been developed, including metaphit audiogenic seizures (18-21) and lindane- (22-24) and homocysteine thiolactone-induced seizures (11). In addition to these models, the other commonly used experimental epilepsy models are those induced by factors such as N-methyl-D-aspartate (NMDA), pentylenetetrazol, pilocarpine, kainic acid, and 4-aminopyridine amygdala kindling (for review see 4). These experimental models of generalised epilepsy each have distinct advantages and disadvantages and are suitable for research on epileptogenic mechanisms, as well as for preclinical evaluation of antiepileptic drugs (25,26).

Of the chemically induced seizures, those induced by HCT are of particular interest because of both the properties of homocysteine and its related compounds and some unique features of this model. In particular, Stanojlovic et al. (11) showed that acute administration of HCT to adult rats significantly alters neuronal circuits, leading to epileptogenic activity in the electroencephalogram (EEG) with characteristic spike-and-wave discharges (SWDs) and convulsive episodes in the animal behaviour. SWDs are ictal phenomena accompanied with absence-like behaviour and characterised in the EEG as follows (11): a) spontaneous and generalised, rhythmic 5-7 Hz discharges, b) with a typical spike-wave complex lasting more than 1 s, and c) an amplitude of at least twice the background EEG activity. The convulsive behaviour elicited by HCT includes motor phenomena ranging from lower jaw twitching to tonic whole body convulsions. Therefore, HCT-induced seizures are widely accepted as a suitable model of generalised epilepsy in which the coexistence of two types of epileptic activity, i.e., convulsive and absence-like seizures, has been documented (27-28).

Sleep and epilepsy: bidirectional relationship

Sleep is a cyclic and vital physiological process. On average, it constitutes one-third of human life (29). Electrophysiological studies have shown the existence of two sleep types: rapid eye movement (REM) and non-REM sleep (30). REM sleep is characterised by intensive brain activity, which is similar to the awake state, while the body is relaxed; thus, REM sleep is termed paradoxical sleep.

Non-REM sleep is characterised by delta activity in the EEG (also known as slow-wave sleep, SWS). (31). Tonic events include the suppression of electromyographic activity to the level of atonia; a desynchronizised, high-frequency, low-voltage (20–30 μV) EEG; a high awakening threshold; and reduced body temperature. Phasic events include rapid eye movements; muscle contraction in the eardrum, lip and tongue; muscle movement in the limbs; and respiratory and cardiac changes (32). Numerous findings revealed similarities between rats and humans, justifying the use of rats in preclinical sleep studies (32-34)

The intimate and bidirectional relationship between sleep and epilepsy has been known since the time of Aristotle and Hippocrates (32, 35). The sleep state is known to influence seizure onset, especially in certain epilepsy syndromes. The converse is also true; epilepsy may disrupt sleep, either directly through convulsive nature of disease or indirectly through the effects of antiepileptic drugs (reviewed in (36)). Neuroscientists have remained particularly interested in the complex interplay between sleep and epilepsy because it is still only poorly understood (32).

Although most studies on this issue are based on clinical trials, experimental studies are of great significance for understanding this relationship (13). Most experimental techniques for selective REM sleep deprivation are based on the single platform method of Jouvet (37,38), which was modified by Susic and Markovic (33). Alterations in behaviour upon REM sleep deprivation are mostly a consequence of imbalance in neurotransmitter systems (39). It has been shown that the generalised down-regulation of muscarinic receptors (40), which are necessary for the initiation and coordination of paradoxical sleep, is included in this effect (41). This change also involves dopamine neuronal circuits, inducing the up-regulation of postsynaptic dopamine receptors (42). Moreover, REM sleep deprivation affects the levels of the excitatory amino acid glutamate and of aspartate, which become elevated in the cortex and hippocampus (43).

Sleep modulation and its effects on homocysteine thiolactone seizures

The effects of sleep modulation on the epileptic activity induced by HCT have been recently investigated (44) using the single platform method to selectivity deprive rats of REM sleep. This study showed that selective REM sleep deprivation increased the incidence and number of seizure episodes per rat that are induced by a subconvulsive dose of HCT. Moreover, the REM-sleep-deprived animals showed a shorter latency time to seizures in behaviour and a significant rate of lethality after HCT administration; in contrast, no significant effects were observed on the seizure severity. EEG analysis in the same study showed a significant increase in the number and duration of SWDs and a decrease in latency of its EEG appearance in the REM-sleep-deprived rats upon treatment with a subconvulsive dose of HCT. In the EEG of rats receiving 0.9% NaCl instead of HCT in the corresponding condition, SWD was not registered. Such behavioural and EEG outputs revealed that selective REM sleep deprivation aggravated the HCT-induced process of epileptogenesis.

Physical activity in epileptic patients: should patients participate in exercise and sport activities?

In 1968, the American Medical Association (AMA) recommended that contact sports and physical education should be limited in epileptic patients whose seizure activity is uncontrolled or not controlled sufficiently well. Since then, the recommendations have been constantly revised: the AMA revised them in 1974, and other institutions, such as the American Pediatric Association, have also revised the recommendations. For a long time, there were established beliefs that epilepsy patients should avoid physical exercise and involvement in sports due to the paroxysmal nature of seizure attacks and the higher possibility of head injuries. Therefore, an increase in sedentary lifestyles in epileptic patients is observed in population-based studies (45). Namely, studies based on large population studies showed that epileptic patients are significantly more sedentary than the general population (46-48). It should be noted that these populations displayed differences in the form of physical activity. The dominant activity among epileptic patients was walking for physical exercise, possibly because of the restrictions imposed on driver's license for patients suffering from epilepsy (48). However, regular physical activity is known to improve both physical and mental health and to contribute to improved quality of life and better social integration. Moreover, it generally known that a lack of physical exercise is a risk factor for a variety of disorders, including cardiovascular diseases, obesity, diabetes and many others (49). Therefore, the beneficial aspects of physical exercise could be lost for these patients (50). With these considerations, the relationship between physical exercise and epilepsy is quite intriguing, particularly the mechanism underlying this relationship. Further investigations that elucidate the role of physical activity in epilepsy are of particular interest.

Experimental epilepsy models have been extremely useful in understanding the role of exercise in epilepsy. There are some reports showing aggravation of epileptiform EEG activity upon physical exercise (51,52). Moreover, studies using the kainate model of seizures report that physical activity aggravated neuronal damage in rats (53). However, opposite results are also reported. Initially, the Arida group used the kindling model of epilepsy to determine the effect of physical activity on rats (54). Arida et al. (54) have shown that chronic physical exercise increased the seizure threshold in an amygdala kindling model of epilepsy. The same group also reported that a physical exercise program decreased the seizure incidence in a model of temporal lobe epilepsy (55). Souza et al. (56) used swimming training as a paradigm of physical activity

and showed that this type of physical exercise prevented the neuronal hyperexcitability induced by pentylenetetrazol (PTZ); this change manifested as increased seizure latency and attenuated seizure duration. It has also been reported that aerobic physical exercise beneficially affects pilocarpine seizures (57). The possible beneficial effects of physical exercise on neuronal hyperexcitability have also been reported in some clinical trials (58, 59).

Physical activity in a model of homocysteine thiolactone seizures

Hrncic et al. (60) investigated the effects of regular physical activity on the epileptic activity induced by homocysteine thiolactone using an experimental paradigm of aerobic physical activity on a treadmill. Namely, rats were made to run on treadmill apparatus for 30 minutes once a day for 30 consecutive days while the belt speed was set to 20 m/min with a 0° incline. Physical exercise using this protocol could be considered aerobic (61) because it has been reported that rats reached the maximal lactate steady state (MLSS) at this belt speed.

The results of that study (60) showed that the rats subjected to regular physical exercise training on a treadmill for 30 consecutive days had a significantly prolonged latency time to development of the first seizure sign and a significantly lower number of HCT-induced seizure episodes per rat comparing with the rats that were sedentary during the same period of time. EEG analysis in the same study showed congruent results. Namely, statistical analysis of the SWD appearance in this study showed a significantly lower number of SWDs in the rats subjected to the physical exercise protocol vs. their sedentary mates with no differences in the duration of SWD. These results suggested that physical activity decreased the susceptibility of rats to developing HCT-induced seizures. In the same study, Hrncic et al. showed that this physical exercise protocol partially prevented the elevation of lipid peroxidation after HCT administration and prevented an HCT-induced decrease in SOD and CAT activity.

NO-mediated signalling in epileptogenesis

NO is synthesised from L-arginine by the activity of the family of enzymes known as NO synthases (NOS). Three different forms of NOS have been identified: neural (nNOS) and endothelial NOS (eNOS), which are Ca^{2+}/calmodulin-dependent enzymes, and inducible NOS (iNOS), which shows Ca^{2+}-independent activity (62). It should be noted that all NOS isoforms have been identified in the brain (63). nNOS is found to be expressed in the hippocampus, cerebral cortex, corpus striatum and cerebellum, as well as in some cells of the autonomic nervous system (64). iNOS is reported to be expressed in the brains of humans with epilepsy. In some spontaneously epileptic mice, overexpression of iNOS is also found (65,66). Moreover, iNOS has been found to be a major contributor to the initiation/exacerbation of CNS inflammatory/degenerative conditions via the production of excessive NO (67).

Numerous studies have demonstrated the anticonvulsive activity of NO in different experimental models of epileptic activity (68-72). However, NO has been reported to play a proconvulsive role in several epilepsy models (73-77). These issues have been reviewed recently in more detail (14).

Anticonvulsive effects of NO in homocysteine thiolactone seizures: the role of NO that is produced via nNOS and iNOS

Using L-arginine and L-NAME as modulators of NO production, we investigated the role of NO in HCT-induced epileptic activity (78). We showed that the systemic administration of L-arginine significantly decreased the seizure incidence and the number of seizure episodes and prolonged the latency time to the first seizure elicited by a convulsive dose of HCT (79). In contrast, pretreatment with L-NAME increased the seizure incidence and severity and shortened the latency time to the first seizure following the injection with a subconvulsive dose of HCT. Moreover, EEG analysis showed that L-arginine decreased but L-NAME increased the median number of SWDs per rat; the duration of individual SWDs was not altered. These results showed the functional involvement of NO in HCT-induced convulsive activity.

As noted, the role of NO could depend on how it is produced. Therefore, further investigations of the roles of nNOS and iNOS in HCT-induced seizures were undertaken. Pharmacological inhibition of nNOS by 7-nitroindazole has been used to investigate the involvement of nNOS in HCT-induced seizures (79). In this study, the intraperitoneal application of 7-nitroindazole showed a tendency to increase seizure incidence, decrease the latency time to first seizure, increase the number of seizure episodes per rat and increase the severity of HCT-induced seizures.

The involvement of iNOS-derived NO in HCT-induced seizures was demonstrated using aminoguanidine, a selective iNOS inhibitor (80). Treatment with aminoguanidine increased the following behavioural seizure properties: seizure incidence, number of seizure episodes per rat and severity of HCT-induced seizures, as well as the number and duration of SWDs in the EEG. Quantitative analysis of the EEG ictal activity showed similar results. Namely, aminoguanidine increased the number and duration of SWDs induced by HCT in that study.

Numerous attempts were also made to elucidate the effects of various NOS inhibitors on the activity of different antiepileptic drugs to determine the potential therapeutic effects of NO modulation. However, the obtained results were inconsistent to some level. In particular, the results showed that NOS inhibitors could increase (76,

81), decrease (82) or not affect antiepileptic efficacy (81). Recently, it has been shown that co-administration of an iNOS inhibitor significantly decreased the beneficial effects of pioglitazone on PTZ-induced seizures in mice (83). Constitutive forms of NOS, but not iNOS, were involved in the anticonvulsant effect of lithium in PTZ-induced seizures (84). The NO modulation of NMDA receptor activity by the process of S-nitrosylation and the co-localisation of NOS and GABA to some extent suggest that the role of NO-mediated signalling involves excitability balance .

The anticonvulsive properties of NO that is produced via nNOS and iNOS in HCT-induced epileptic activity could result from several mechanisms and the interplay between NO and HCT at various levels, i.e., interaction at NMDA and GABA receptors, including the relationship of NO with the NMDA and GABA receptors, neurodegeneration, cytoprotection and oxidative stress (85-89).

CONCLUSION

The only way to develop new antiepileptic drugs and treatments is to improve the understanding of the process of epileptogenesis and the mechanisms that contribute to it. Experimental epilepsy models have been extremely useful in achieving this goal. HCT-induced seizures are particularly interesting because HCT induces two types of epileptic activity in rats. Selective REM sleep deprivation potentiates HCT-induced seizures, whereas regular physical activity beneficially affects them. The role of NO in the HCT model of epileptic activity is demonstrated to be anticonvulsive, regardless of how it is produced. Translational research on the complex interplay between the modulating factors of epileptogenesis described in this review will result in new strategies to fight epilepsy.

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THE EFFECTS OF DICLOFENAC AND IBUPROFEN ON HEART FUNCTION AND OXIDATIVE STRESS MARKERS IN THE ISOLATED RAT HEART

Maja Jevdjevic[1], Ivan Srejovic[2], Vladimir Zivkovic[2], Nevena Barudzic[2], Anica Petkovic[2], Jovana Bradic[2], Dragan Djuric[3] and Vladimir Jakovljevic[2]
[1]College of Health Studies in Cuprija, Cuprija, Serbia
[2]Department of Physiology, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia
[3]Institute of Medical Physiology "Richard Burian", School of Medicine, University of Belgrade, Belgrade, Serbia

EFEKTI DIKLOFENAKA I IBUPROFENA NA FUNKCIJU I BIOMARKERE OKSIDATIVNOG STRESA NA IZOLOVANOM SRCU PACOVA

Maja Jevđević[1], Ivan Srejović[2], Vladimir Živković[2], Nevena Barudžić[2], Anica Petković[2], Jovana Bradić[2], Dragan Đurić[3] and Vladimir Jakovljević[2]
[1]Visoka medicinska skola strukovnih studija u Cupriji, Srbija
[2]Fakultet medicinskih nauka, Univerzitet u Kragujevcu, Katedra za fiziologiju, Kragujevac, Srbija
[3]Institut za medicinsku fiziologiju "Ricard Burijan", Univerzitet u Beogradu, Beograd, Srbija

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ABSTRACT

Eicosanoids lead to the promotion of inflammation, cause fever and pain and have many other effects. NSAIDs block the action of cyclooxygenase (COX) during the process of converting arachidonic acid into inflammatory mediators, thus reducing the symptoms of inflammation. Investigations focusing on nonselective COX inhibitors, used in high doses, revealed harmful effects on myocardial function. The aim of our study was to assess the effects of two nonselective NSAIDs, diclofenac and ibuprofen, on cardiodynamic parameters, coronary flow and oxidative stress biomarkers in isolated rat hearts. The hearts of male Wistar albino rats were excised and retrogradely perfused according to the Langendorff technique at gradually increased coronary perfusion pressures (40–120 cm H_2O). The experiments were performed under controlled conditions (Krebs–Henseleit physiological solution). The hearts were perfused with 10 μmol/l diclofenac and 10 μmol/l ibuprofen. The heart function parameters, including the maximum rate of pressure development (dp/dt max), minimum rate of pressure development (dp/dt min), systolic left ventricular pressure (SLVP), diastolic left ventricular pressure (DLVP), mean perfusion pressure (MBP) and heart rate (HR), were continuously registered. Coronary flow (CF) was measured flowmetrically. Oxidative stress markers, including the index of lipid peroxidation measured as TBARS, nitric oxide measured through nitrites (NO_2^-), superoxide anion radical (O_2^-), and hydrogen peroxide (H_2O_2) in the coronary venous effluent, were assessed spectrophotometrically. Our results showed that diclofenac affected cardiodynamic parameters more significantly than did ibuprofen. Furthermore, the present data indicate that both estimated COX inhibitors do not promote the production of reactive oxygen species.

Keywords: *Diclofenac, Ibuprofen, Nonsteroidal anti-inflammatory drugs, Isolated rat heart, Oxidative stress*

SAŽETAK

Eikosanoidi dovode do zapaljenja, uzrokuju groznicu i bol, i imaju mnoge druge efekte na organizam. NSAID onemogućavaju delovanje ciklooksigenaze (COX) u procesu konvertovanja arahidonske kiseline u medijatore zapaljenja, i na taj način smanjuju simptome zapaljenja. Istraživanja koja se bave primenom neselektivnih inhibitora COX, koji se koriste u visokim dozama, pokazala su njihove štetne efekte na funkciju miokarda. Cilj našeg istraživanja je bio da ispita efekte neselektivnih NSAID, diklofenaka i ibuprofena, na kardiodinamske parametre, koronarni protok i biomarkere oksidativnog stresa izolovanog srca pacova. Srca mužijaka Wistar albino pacova su uzimana i retrogradno perfundovana prema Langedorff-ovoj tehnici sa postepenim povećanjem perfuzionog pritiska (40–120 cm H_2O). Eksperimenti su prvo izvođeni u kontrolnim uslovima (primena fiziološkog Krebs–Henseleit-ovog rastvora), nakon čega su srca perfundovana sa: 10 μmol/l dikolfenaka i 10 μmol/l ibuprofena. Parametri srčane funkcije koji su kontinuirano praćeni su: maksimalna stopa razvoja pritiska (dp/dt max), minimalna stopa razvoja pritiska (dp/dt min), sistolni pritisak u levoj komori (SLVP), dijastolni pritisak u levoj komori (DLVP), srednji perfuzioni pritisak (MBP) i frekvenca srčanog rada (HR). Koronarni protok (CF) je registrovan floumetrijski. Markeri oksidativnog stresa: indeks lipidne peroksidacije meren kao TBARS, azot-monoksid utvrđivan preko nitrata (NO_2^-), superoksid anjon radikal (O_2^-), i vodonik peroksid (H_2O_2) su mereni spektrofotometrijski u koronarnom venskom efluentu. Naši rezultati su pokazali da diklofenak ispoljava značajniji uticaj na kardiodinamske parametre u odnosu na ibuprofen. Pored toga, rezultati ove studije su pokazali da oba ispitivana inhibitora COX ne dovode po produkcije reaktivnih vrsta kiseonika.

Ključne reči: *Diklofenak, Ibuprofen, Nesteroidni-antiinflamatorni lekovi, Izolovano srce pacova, Oksidativni stres*

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Correspondence to: Prof. Vladimir Lj. Jakovljevic, MD, PhD;
Department of Physiology, Faculty of Medical Sciences, University of Kragujevac, SERBIA
Phone: +381 34 342944, Fax: +381 34 306800/ext 112, E-mail: drvladakgbg@yahoo.com

ABBREVIATIONS

CF - Coronary flow
COX - Cyclooxygenase
CPP - Coronary perfusion pressure
HRPO - Horseradish peroxidase
MBP - Mean blood pressure
NO - Nitric oxide
NADPH - Nicotinamide adenine dinucleotide phosphate
NSAID - Nonsteroidal anti-inflammatory drugs
ROS - Reactive oxygen species
TBARS - Thiobarbituric acid reactive substances
TRIS - Tris(hydroxymethyl)aminomethane

INTRODUCTION

Cyclooxygenase (COX) is an intracellular enzyme that catalyses the conversion of arachidonic acid into prostaglandin H_2, a precursor for the synthesis of prostaglandins, prostacyclin, and thromboxane, also known as eicosanoids. Eicosanoids lead to the promotion of inflammation, cause fever and pain and have many other systemic effects. There are two isoforms of COX: COX-1 and COX-2. COX-1 is the constitutive isoform of COX, and it has clear physiological effects. The inducible isoform, COX-2, is induced by pro-inflammatory stimuli in migratory cells and inflamed tissues (1). It is almost impossible to detect COX-2 in a normal heart, which indicates that the induction of COX-2 is present mostly at the site of inflammation (2).

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most common medications in the world for treating a variety of inflammatory disorders, and they are used as analgesics, anti-inflammatory drugs, and antipyretics (3). NSAIDs block the action of COX in the process of converting arachidonic acid into inflammatory mediators, thus reducing symptoms of inflammation (4). NSAIDs are classified on theas nonselective COX inhibitors and specific COX-2 inhibitors depending on the activity of each isoform (4).

Investigations focusing on nonselective COX inhibitors, used in high dosages, revealed harmful effects on myocardial function and increased mortality in patients with previous myocardial infarction (5) and as well as in patients without a prior clinical diagnosis of cardiovascular diseases (6). Recent data regarding cardiovascular risk associated with selective COX-2 inhibitors are controversial (7, 8). Over the last several years, evidence has accumulated showing that oxidative stress plays an important role in the pathogenesis of cardiovascular disease (9, 10). One of possible mechanisms by which COX inhibitors exhibit their side effects on the cardiovascular system is the induction of reactive oxygen species (ROS) (11, 12).

Diclofenac, a nonselective non-steroidal anti-inflammatory drug, has been widely used as an anti-inflammatory, analgesic, and antipyretic drug. Clinical observations have shown that long-term treatment with diclofenac correlates with the onset or aggravation of congestive heart failure, which can cause serious cardiovascular thromboembolic events, such as myocardial infarction and stroke (13). McGettigan et al claimed that diclofenac has the highest cardiovascular risk score of the nonselective NSAIDs (14). This conclusion might be because only diclofenac inhibits L-type Ca^{2+} channels and the Na^+ current in cardiomyocytes (13).

Ibuprofen is a nonselective non-steroidal anti-inflammatory drug that is often used to relieve fever and the symptoms of arthritis. This drug is well-tolerated with infrequent but well-characterised adverse effects, including heart failure (15, 16). A study on isolated guinea pig hearts showed that ibuprofen could induce cardiac arrhythmias due to the inhibition of Na^+ and Ca^{2+} channels and a decrease of the excitation propagation within the heart (17).

Based on the aforementioned discussion, the aim of the present study was to assess the effects of two nonselective NSAIDs, diclofenac and ibuprofen, on cardiodynamic parameters, coronary flow and oxidative stress biomarkers in isolated rat hearts.

MATERIALS AND METHODS

Preparation of isolated hearts

The hearts (n=24; each group 12 rats) were excised from male, eight-week old Wistar albino rats, with a body mass of 180 g to 240 g (obtained from Military Medical Academy, Belgrade, Serbia), and perfused in a Langendorff apparatus (Experimetria Ltd, 1062 Budapest, Hungary). After a short-term ether narcosis, the animals were killed by cervical dislocation (Schedule 1 of the Animals/Scientific Procedures, Act 1986, UK). After urgent thoracotomy and rapid heart arrest by superfusion with ice-cold isotonic saline, the hearts were rapidly excised, isolated and retrogradely perfused via the aorta according to Langendorff's technique at gradually increased coronary perfusion pressures (CPP) (40–120 cm H_2O). The composition of the non-recirculation Krebs–Henseleit perfusate was as follows (mmol/1): NaCl 118, KCl 4.7, $CaCl_2$ x $2H_2O$ 2.5, $MgSO_4$ x $7H_2O$ 1.7, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11, pyruvate 2, equilibrated with 95 % O_2 plus 5 % CO_2 and warmed to 37°C (pH 7.4).

Immediately after the establishment of automatic operation, the sensor was inserted (transducer BS4 73-0184, Experimetria Ltd, Budapest, Hungary) through an opening created in the left atrium of the heart and the destroyed mitral valve into the left ventricle for the continuous registration of myocardial function. Another sensor (perfusion pressure sensor, Experimetria Ltd, Hungary) was set at the same height as the end of the heart perfusion cannula to measure mean blood pressure (MBP).

Physiological assay and experimental protocol

After the heart perfusion started, a 30-min period was allowed for stabilisation of the preparation. It was performed at a basal CPP of 60 cmH_2O. To test coronary vascular reactivity, all hearts were challenged with short-term occlusions (5–30 s) and a bolus injection of 5 mmol/1 adenosine (60 µl at a flow rate of 10 ml/min to elicit maximum CF) during the stabilisation period. The hearts were discarded (approximately 25 %) if the flow did not increase by 100 % over the control value (for both tests). After an equilibration period, CPP was lowered to 50 and 40 cmH2O and then gradually increased to 70, 80, 90,100, 110, and 120 cmH2O to establish coronary autoregulation. Properly performed control experiments were included in the study (i.e., the groups of hearts in which the CPP/CF relationship was investigated twice in the absence of any drug for 120 min). It was important to confirm the stability of the preparation and the insubstantial difference between the responses to the first and second runs of changes in perfusion pressure, as described previously (18). After setting up the control experimental protocol (Krebs–Henseleit physiological solution [control group]), the hearts were perfused with 10 µmol/l diclofenac and 10 µmol/l ibuprofen.

In the control and experimental groups, after placing the sensor in the left ventricle, the following myocardial function parameters were continuously registered:

1. Maximum rate of pressure development in the left ventricle (dp/dt max),
2. Minimum rate of pressure development in the left ventricle (dp/dt min),
3. Systolic left ventricular pressure (SLVP),
4. Diastolic left ventricular pressure (DLVP),
5. Mean perfusion pressure (MBP) and
6. Heart rate (HR).

The test period started immediately after the control experiments to avoid time-dependent adverse effects. The flow was considered stable at each value of perfusion pressure when three repeated values of the CF were the same. The CF was measured flowmetrically with the following parameters:

1. Index of lipid peroxidation (measured as TBARS – thiobarbituric acid reactive substances),
2. Superoxide anion radicals (O_2^-),
3. Hydrogen peroxide (H_2O_2) and
4. Nitric oxide (NO).

The experimental protocol was approved by the Faculty of Medical Sciences Ethics Committee for the welfare of experimental animals, University of Kragujevac, Kragujevac, Serbia.

Biochemical assays

TBARS determination (index of lipid peroxidation)

The degree of lipid peroxidation in the coronary venous effluent was estimated by measuring TBARS using 1 % thiobarbituric acid in 0.05 NaOH incubated with the coronary effluent at 100°C for 15 min and measured at 530 nm. Krebs–Henseleit solution was used as a blank probe (19).

Determination of superoxide anion radical

The level of superoxide anion radical (O_2-) was measured by a nitro blue tetrazolium reaction in TRIS buffer with the coronary venous effluent at 530 nm. Krebs–Henseleit solution was used as a blank probe (20).

Determination of hydrogen peroxide

The measurement of hydrogen peroxide (H_2O_2) was based on the oxidation of phenol red by hydrogen peroxide in a reaction catalysed by horseradish peroxidase (HRPO) (21). Two hundred microlitresd of perfusate was precipitated with 800 ml of freshly prepared phenol red solution, and then 10 µl of (1:20) HRPO (made ex tempore) was added. For a blank probe (instead of coronary venous effluent), an adequate volume of Krebs–Henseleit solution was used. The level of H_2O_2 was measured at 610 nm.

Nitrite determination

Nitric oxide decomposes rapidly to form stable metabolite nitrite/nitrate products. The nitrite level (NO_2-) was measured and used as an index of nitric oxide (NO) production using Griess's reagent. A total of 0.5 ml of perfusate was precipitated with 200 µl of 30 % sulphosalicylic acid, vortexed for 30 min, and centrifuged at 3000 x g. Equal volumes of the supernatant and Griess's reagent, containing 1 % sulphanilamide in 5 % phosphoric acid/0.1 % naphthalene ethylenediamine dihydrochloride, was added and incubated for 10 min in the dark and measured at 543 nm. The nitrite levels were calculated using sodium nitrite as the standard (22).

Drugs

Both diclofenac and ibuprofen were obtained from Sigma-Aldrich Co (USA).

Statistical analysis

Values are expressed as the mean ± SE. A paired t test was used in the statistical analyses; p values less than 0.05 were considered to be statistically significant.

RESULTS

Dp/dt max

Diclofenac at a dose of 10 µmol/l induced a decrease in dp/dt max at CPP = 100 cmH_2O and 120 cmH_2O (Figure 1A), whereas ibuprofen at the same dose did not significantly affect this parameter over the entire CPP range (Figure 2A).

Dp/dt min

Similar to dp/dt max, 10 µmol/l diclofenac significantly affected dp/dt min at CPP = 120 cmH_2O (Figure 1B), and ibuprofen did not cause statistically significant changes in this parameter (Figure 2B).

SLVP

Neither diclofenac nor ibuprofen caused statistically significant changes in SLVP at any CPP (Figure 1C, 2C).

DLVP

There were no changes in the DLVP values during the administration of diclofenac or ibuprofen compared to the controls (Figure 1D, 2D) over the entire CPP range.

Mean perfusion pressure

Diclofenac induced statistically significant changes in the MBP at CPP = 120 cmH_2O (Figure 1E), whereas ibuprofen did not cause any changes in the MBP values compared to the control conditions (Figure 2E).

HR

Both diclofenac and ibuprofen induced statistically significant decreases in HR at CPP = 100 cmH_2O and 120 cmH_2O (Figure 1F, 2F) compared with the control conditions.

CF

Similar to HR, both diclofenac and ibuprofen induced statistically significant decreases in HR at CPP = 100 cmH_2O and 120 cmH_2O (Figure 1G, 2G) compared with the control conditions.

Index of lipid peroxidation (measured as TBARS)

The administration of ibuprofen induced a statistically significant increase in TBARS at CPP = 40 cmH_2O and then a statistically significant decrease at CPP = 100 cmH_2O and 120 cmH_2O (Figure 4A). Diclofenac did not cause any changes in the TBARS values over the entire CPP range (Figure 3A).

NO_2^-

Compared with the control conditions, the amount of NO_2^- released was not changed significantly during the administration of diclofenac or ibuprofen for any CPP value (Figures 3B, 4B).

H_2O_2

Diclofenac induced a significant decrease in H_2O_2 release (Figure 3C) at CPP = 120 cmH_2O, whereas ibuprofen did not significantly affect H_2O_2 release (Figure 4C).

O_2^-

There were no statistically significant changes in O_2^- release during the application of diclofenac or ibuprofen over the entire CPP range (Figures 3D, 4D).

DISCUSSION

The present study aimed to examine the effects of the acute administration of the COX inhibitors diclofenac and ibuprofen on cardiodynamic parameters, coronary autoregulation and oxidative stress biomarkers in isolated rat hearts. This work relates to earlier experiments in our laboratory (23, 12).

In the first part of our study, we focused our attention on the effects of diclofenac and ibuprofen on cardiodynamic parameters as an indicator of myocardial function. Cardiac contractility was estimated by the maximum and minimum rates of LV pressure development (dp/dt max and dp/dt min). Ibuprofen did not cause any changes in myocardial contractility, but diclofenac induced a significant decrease in contractility at higher values of CPP (Figures 1A, 1B, 2A and 2B). Our results related to ibuprofen agreere consistent with the findings of Herbretson et al (24). They showed that ibuprofen does not affect left ventricular contractility (24) in the model of porcine endotoxemia. Moreover, Beamer et al explored the possible effect of ibuprofen on hemodynamic parameters during hypovolemic shock in a canine experimental model, and they also showed that this drug had no influence on cardiac contractility (25). Our results concerning diclofenac are in agreement with Yarishkin et al (13), who concluded that diclofenac may depress cardiac excitability and contractility simultaneously.

One of the possible mechanisms by which diclofenac exhibits depressive effects on the heart is through the reversible inhibition of the Na^+ currents and irreversible inhibition the L-type Ca^{2+} channel currents in cardiac muscle cells (13, 26). Moreover, ibuprofen also exhibits a similar influence (17) on ion channels, but in our results, there was no effect on cardiac contractility.

Both drugs induced decreases in HR and CF at higher CPP values (Figures 1F, 1G, 2F, 2G). Yang et al showed that ibuprofen causes a decrease in HR (17) in the guinea pig isolated heart model. A possible mechanism for this action is decreasing the spontaneous depolarisation rate, thereby slowing the heart rate (17). Diclofenac may also affect the duration of the action potential and heart rate (26). Kristof and co-authors concluded that chronic administration of diclofenac at therapeutic concentrations does not increase the risk of arrhythmia in intact hearts (26). In our experiments, we also used intact hearts, but considering our experimental protocol and acute administration of diclofenac, this mechanism may play a role in decreasing the heart rate.

Although both diclofenac and ibuprofen are classified as nonselective NSAIDs, there are some differences in their COX selectivity; these differences could be a partial cause of their different effects. Namely, ibuprofen is a more potent inhibitor of COX-1 than diclofenac, which is a more selective COX-2 inhibitor (27) (Figure 3). In their meta-analysis, Varas-Lorenzo and co-workers found that diclofenac causes a more increased risk of vascular events compared with ibuprofen (28).

On the other hand, data regarding the influence of these drugs on ROS generation are inconsistent. Li and co-workers demonstrated that diclofenac causes decreased nitrite plasma levels (oxidation product of NO), indicating a reduction in NO bioavailability (11). In the present study, we also found decreased nitrites (Figure 3B). This may lead to vasoconstriction and consequently to a decrease in CF (Figure 1G).

Regarding these findings, we wanted to explore the possible role of oxidative stress in the appearance of side effects with these drugs. However, most of the oxidative

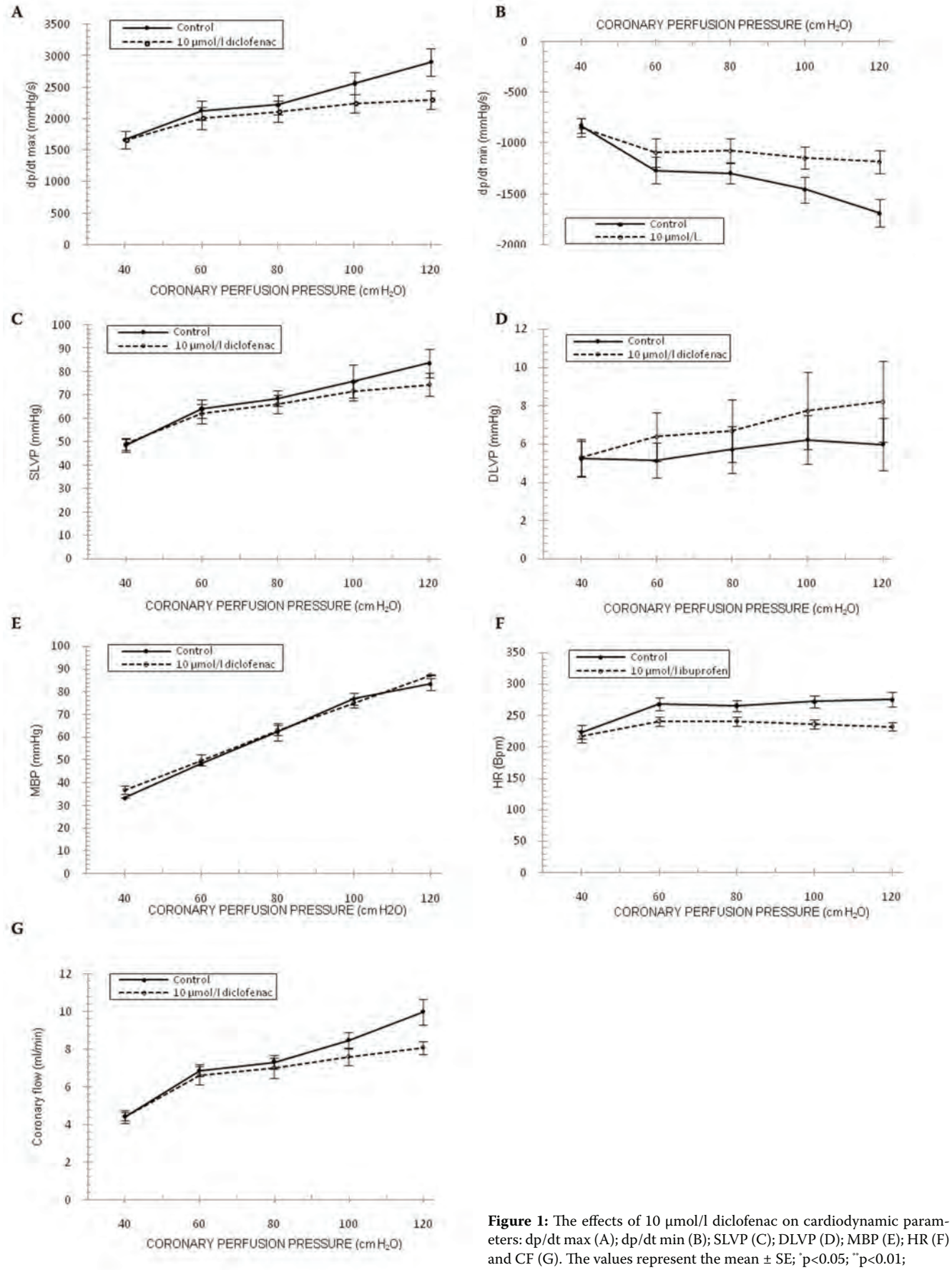

Figure 1: The effects of 10 µmol/l diclofenac on cardiodynamic parameters: dp/dt max (A); dp/dt min (B); SLVP (C); DLVP (D); MBP (E); HR (F) and CF (G). The values represent the mean ± SE; $^{*}p<0.05$; $^{**}p<0.01$;

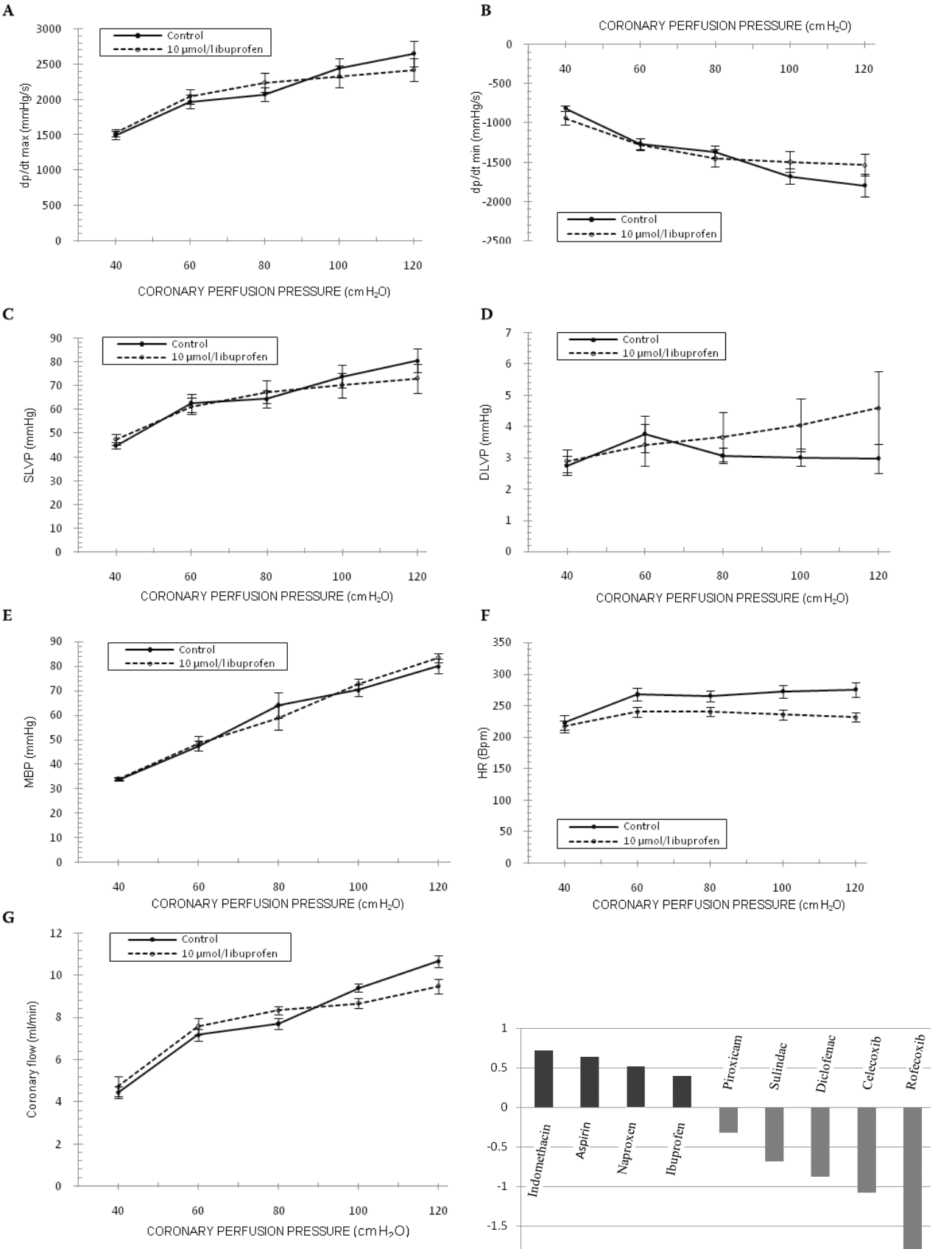

Figure 2: The effects of 10 µmol/l ibuprofen on cardiodynamic parameters: dp/dt max (A); dp/dt min (B); SLVP (C); DLVP (D); MBP (E); HR (F) and CF (G). The values represent the mean ± SE; * p<0.05; ** p<0.01;

Figure 3: COX selectivity of different NSAIDs.

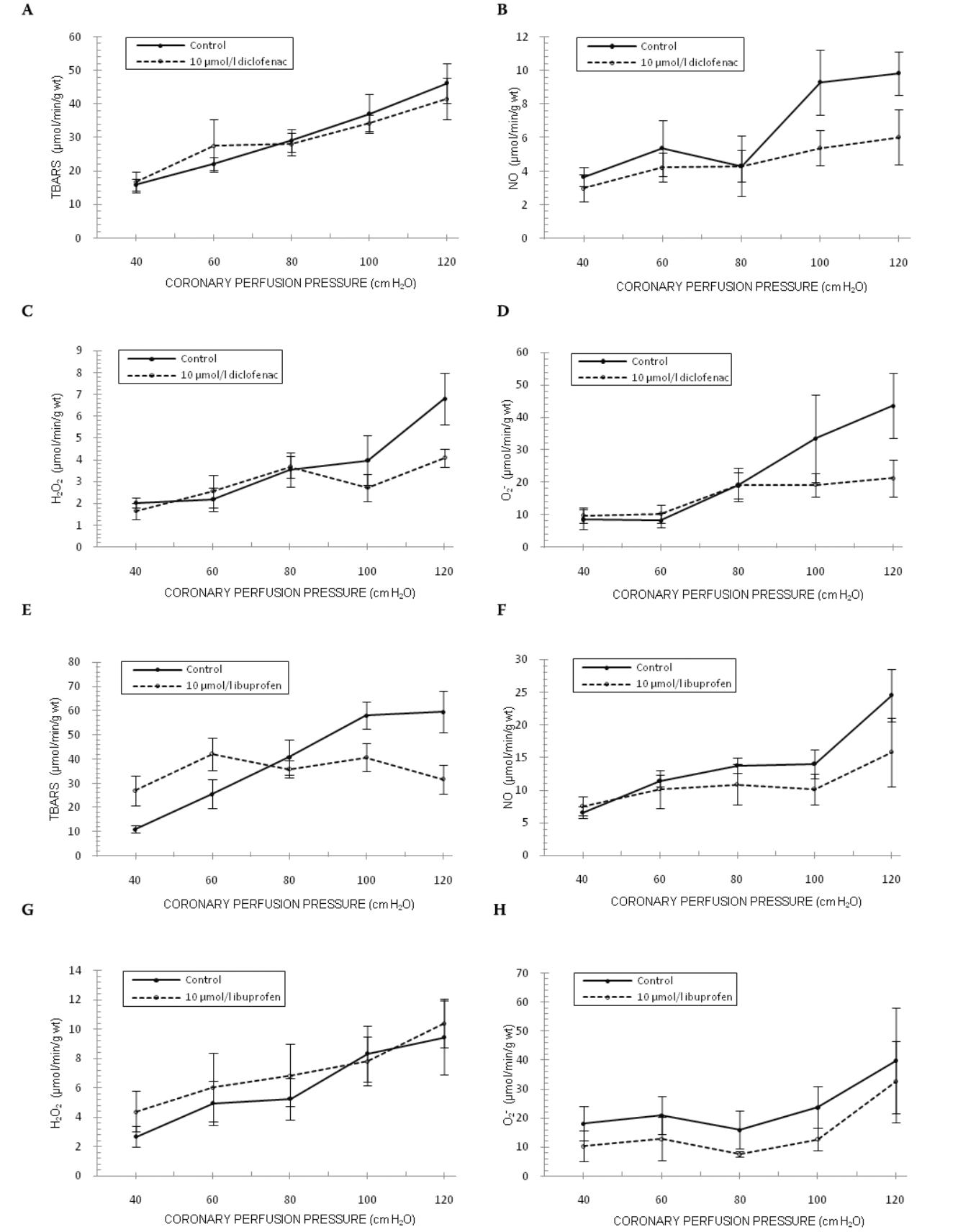

Figure 4: The effects of 10 µmol/l diclofenac and 10 µmol/l ibuprofen on oxidative stress parameters: TBARS diclofenac (A); NO diclofenac (B); H_2O_2 diclofenac (C); O_2^- diclofenac (D); TBARS ibuprofen (E); NO ibuprofen (F); H_2O_2 ibuprofen (G) and O_2^- ibuprofen (H);. The values represent the mean ± SE; $^*p<0.05$; $^{**}p<0.01$;

stress parameters did not change significantly (Figure 3). Diclofenac caused a decrease in H_2O_2 levels at CPP 40 cm H_2O (Figure 3C), and ibuprofen first induced first an increase in TBARS levels at CPP 40 and then a decrease at CPP 100 and 120 (Figure 3E). This result is in contrast with our hypothesis that oxidative stress may be one of the mechanisms by which NSAIDs exhibit their side effects. There are some results suggesting that diclofenac induces a marked increase in vascular ROS content (11). Li et al used spontaneously hypertensive rats and chronic application of NSAIDs (and among them, diclofenac). Differences between the results of our study and theirs may occur because of the different experimental models.

On the other hand, diclofenac may possess some antioxidant potential, behaving as an ROS scavenger (29). Hermann et al found that diclofenac does not improve endothelial dysfunction or affect oxidative stress parameters (30). Wilkinson and his group concluded that ibuprofen attenuates oxidative damage in the brain and has beneficial effects in Alzheimer's disease (31). The findings of these authors suggest that ibuprofen acts independently of COX inhibition to disrupt signalling cascades leading to microglial Nox2 activation, thus preventing oxidative damage. Moreover, the findings of Zhao et al suggest that ROS specifically derived from Nox2 NADPH oxidase make a substantial contribution to several key processes underlying the development of cardiac contractile dysfunction and remodelling (32). In this study, the authors examined the role of Nox2 NADPH oxidase in the development of doxorubicin-induced cardiac injury and remodelling. Considering this, we can suggest that the unchanged levels of oxidative stress biomarkers in our study could be due to the inhibition of Nox2 NADPH oxidase by ibuprofen.

Based on the present data, we can conclude that diclofenac affects cardiodynamic parameters more significantly than does ibuprofen. Furthermore, our results indicate that both estimated COX inhibitors do not promote the production of ROS; therefore, their cardiac effects do not appear to be mediated via oxidative stress, which could be important in elucidating their potential deleterious influence on cardiac muscle and coronary endothelium.

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PHENOLIC AND FLAVONOID CONTENT AND ANTIOXIDANT ACTIVITY OF *DAPHNE BLAGAYANA* GROWING IN SERBIA

Nedeljko Manojlovic[1], Miroslav Sovrlic[1], Pavle Maskovic[2], Perica Vasiljevic[3] and Marina Juskovic[3]
[1]Faculty of Medical Sciences, University of Kragujevac, 34000 Kragujevac, Serbia
[2]Faculty of Agronomy, University of Kragujevac, 32000 Cacak, Serbia
[3]Faculty of Sciences and Mathematics, University of Nis, 18000 Nis, Serbia

SADRŽAJ FENOLA I FLAVONOIDA I ANTIOKSIDATIVNA AKTIVNOST BILJKE *DAPHNE BLAGAYANA* KOJA RASTE U SRBIJI

Nedeljko Manojlović[1], Miroslav Sovrlić[1], Pavle Mašković[2], Perica Vasiljević[3] i Marina Juškoviić[3]
[1]Fakultet medicinskih nauka, Univerzitet u Kragujevcu, 34000 Kragujevac, Srbija
[2]Agronomski fakultet, Univerzitet u Kragujevcu, 32000 Čačak, Srbija
[3]Prirodno-matematički fakultet, Univerzitet u Nišu, 18000 Niš, Srbija

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ABSTRACT

The aim of this study was to examine the phenolic and flavonoid contents and antioxidant activities of methanol and chloroform extracts of leaves and twigs of Daphne blagayana. The total phenolic content in the chloroform extract of plant twigs (90.26 ± 0.69 mg GA/g) was higher than that of the other extracts (from 76.56±0.89 to 77.45±0.43 mg GA/g). In the case of flavonoids, a greater value was also obtained for the chloroform extract of twigs (35.24±0.55 mg RU/g). Several different methods were used to determine the antioxidant activity of the tested extracts, including total antioxidant capacity, metal chelating activity, hydroxyl radical scavenging activity and inhibitory activity against lipid peroxidation. Our results showed that although secondary metabolites of the plants may contribute significantly to their antioxidant activities, those antioxidant activities were not directly related to the phenolic and flavonoid amounts. The results of the present analysis demonstrated, for the first time, that Daphne blagayana leaves and twigs possess high phenolic and flavonoid contents, as well as potential antioxidant activity. This study suggests that Daphne blagayana twigs and leaves may potentially be used as an accessible source of natural antioxidants.

Key words: *Daphne blagayana, phenols, flavonoids, antioxidant activity.*

SAŽETAK

Cilj ovog rada je bio da se ispita fenolni i flavodnoidni sadržaj, kao i antioksidativna aktivnost metanolskih i hloroformskih ekstrakta lišća i grančica biljke Daphne blagayana. Ukupan sadržaj fenola u hloroformskom ekstraktu grančica (90.26±0.69 mg GA/g) bio je veći od sadržaja u ostalim ekstraktima (od 76.56±0.89 do 77.45±0.43 mg GA/g). U slučaju flavonoida, maksimalna vrednost je takođe zabeležena kod hloroformskog ekstrakta grančica (35.24±0.55 mg RU/g). Nekoliko različitih metoda su korišćeno za određivanje antioksidantne aktivnosti testiranih ekstrakata uključujući ukupan antioksidantni kapacitet, metal helacionu aktivnost, aktivnost hidroksi radikala i inhibitornu aktivnost prema lipidnoj peroksidaciji. Naši rezultati su pokazali da, iako sekundarni metabolite biljaka mogu značajno doprineti antioksidantnim aktivnostima, ove aktivnosti nisu bile uvek direktno povezane sa količinom fenola i flavonoida u ekstraktima. Rezultati ovih ispitivanja su pokazali, po prvi put, da grančice i lišće biljke Daphne blagayana poseduju visok sadržaj fenola i flavonoida i potencijalno antioksidantno delovanje. Ova studija je pokazala da se lišće i grančice ove biljke mogu potencijalno koristiti kao pristupačan izvor prirodnih antioksidanasa.

Ključne reči: *Daphne blagayana, fenoli, flavonoidi, antioksidativna aktivnost.*

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Correspondence to: Nedeljko Manojlovic,
Faculty of Medical Sciences, University of Kragujevac, Svetozara Markovica 69, 34 000 Kragujevac, Serbia
e-mail: mtnedeljko@yahoo.com

INTRODUCTION

Medicinal plants have been widely used by both ancient and modern people of all cultures for treating different illnesses and for other purposes. Plants are a good source of biologically active natural products that are all biodegradable and, more importantly, renewable. The antioxidant properties of plants could affect a range of physiological processes in the human body, thus providing protection against free radicals (1-4). Antioxidants are micronutrients that have drawn interest in recent years due to their ability to neutralise the actions of free radicals (5). Free radicals are potentially harmful products generated during a number of natural processes in the body and are associated with ageing of cells and tissues. Failure to remove active oxygen compounds over the long term can lead to cardiovascular disease, cancer, diabetes, arthritis and various neurodegenerative disorders (6).

Phenolic compounds are ubiquitous in plants. Flavonoids and other plant phenolics, such as phenolic acids, stilbenes, tannins, lignans, and lignins, are important in the plant for normal growth development and defense against infection and injury. These compounds are commonly found in plants, and they have been reported to have multiple biological effects, including antioxidant activity (7). Various investigations have implied that the concentrations of total phenolic compounds are closely related to antioxidative activity (8), with flavonoids and tannins as the major plant compounds with antioxidant activity (9).

Components of some *Daphne* species are used in natural medicine as a laxative, diuretic, anticoagulant, and in the treatment against skin diseases, toothache and malaria. Previous studies of individual species of the genus *Daphne* indicate their potential broad applications in medicine (10-13). The aim of this study was to investigate the total phenolic and flavonoid contents as well as antioxidant activity of the methanol and chloroform extracts obtained from the twigs and leaves of *D. blagayana* which have not previously been studied.

MATERIAL AND METHODS

Chemicals used

All chemicals and reagents were of analytical grade and were purchased from Sigma Chemical Co. (St Louis, MO, USA), Aldrich Chemical Co. (Steinheim, Germany) and Alfa Aesar (Karlsruhe, Germany). 1,1-Diphenyl-2-picrylhydrazyl hydrate (DPPH), Folin–Ciocalteu, ascorbic acid, butylated hydroxytoluene (BHT) and pyrocatechol were purchased from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). Hydrochloric acid, formaldehyde, anhydrous sodium carbonate, methanol and chloroform were purchased from Centrohem (Centrohem, Stara Pazova, Serbia).

Spectrophotometric measurements

Spectrophotometric measurements were performed using an MA 9524-SPEKOL 211 (ISKRA, Slovenia).

Plant material

The plant material was collected from Mt. Kopaonik, Serbia, in July, 2007. The demonstration samples are preserved in facilities of the Department of Biology and Ecology, Faculty of Science, University of Niš, Serbia (Voucher No HMN 5517).

Preparation of the plant extracts

The air-dried leaves and twigs from the plant *Daphne blagayana* (60 g) were broken into small 2– 6 mm pieces by a cylindrical crusher, and extracted separately with chloroform and methanol (500 ml) using a Soxhlet apparatus. The mixture was filtered through filter paper (Whatman, No.1) and evaporated. The residues (7.8 g for chloroform and 8.3 g for methanol) were stored in a dark glass bottle at +4°C for further processing. The extracts were used for chemical and antioxidant analysis.

Determination of total phenolic content

Total phenols were estimated according to the Folin-Ciocalteu method (15). The extracts were diluted to a concentration of 1 mg/ml, and aliquots of 0.5 ml were mixed with 2.5 ml of Folin-Ciocalteu reagent (previously diluted 10-fold with distilled water) and 2 ml of $NaHCO_3$ (7.5%). After 15 min at 45˚C, the absorbance was measured against a blank sample at 765 nm. Total phenols were determined as gallic acid equivalents (mg GA/g extract), and the values are presented as means of triplicate analyses.

Determination of flavonoid content

Total flavonoids were determined according to Brighente *et al.* (16). A total of 0.5 ml of 2% aluminium chloride ($AlCl_3$) in methanol was mixed with the same volume of methanol solution of plant extract. After 1 hour at room temperature, the absorbance was measured at 415 nm against the blank sample. Total flavonoids were determined as rutin equivalents (mg RU/g dry extract), and the values are presented as means of triplicate analyses.

Determination of total antioxidant activity

The total antioxidant activity of *D. blagayana* extracts was evaluated using the phosphomolybdenum method (17). This assay is based on the reduction of Mo (VI) to Mo (V) by antioxidant compounds and subsequent formation of a green phosphate/Mo (V) complex at acid pH. A total of 0.3 ml of sample extract was combined with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solutions were incubated at 95°C for 90 min. After cooling to room temperature, the absorbance of the solution was measured at 695 nm against the

blank sample. Methanol (0.3 ml) was used as the blank in place of an extract. Ascorbic acid was used as the standard and total antioxidant capacity was expressed as milligrams of ascorbic acid per gram of dry extract.

Determination of DPPH free radical scavenging activity

The method used by Takao *at al.* (18) was adopted with suitable modifications from Kumarasamy *et al.* (19). DPPH (8 mg) was dissolved in MeOH (100 ml) to obtain a concentration of 80 µg/ml. Serial dilutions were carried out with the stock solution (1 mg/ml) of the extract. Solutions (2 ml each) were then mixed with DPPH (2 ml) and allowed to stand for 30 min to allow any reaction to occur, and the absorbance was measured at 517 nm. Ascorbic acid (AA), gallic acid and BHT were used as reference standards and were dissolved in methanol to prepare stock solutions with the same concentrations (1 mg/ml). Control samples were prepared containing the same volume without test compounds or reference antioxidants. Ninety-five percent methanol was used as a blank. The DPPH free radical scavenging activity (%) was calculated using the following equation:

$$\% \text{ inhibition} = \frac{\text{Ac-As}}{\text{Ac}} \times 100$$

The IC_{50} value, defined as the concentration of the test material that leads to 50% reduction in the free radical concentration, was calculated using a sigmoidal dose-response curve and expressed as µg/ml.

Determination of inhibitory activity against lipid peroxidation

Antioxidant activity was determined by the thiocyanate method (20). Serial dilutions were carried out with stock solutions (1 mg/ml) of the extracts, and 0.5 ml of each solution was added to a linoleic acid emulsion (2.5 ml, 40 mM, pH 7.0). The linoleic acid emulsion was prepared by mixing 0.2804 g linoleic acid, 0.2804 g Tween-20 as emulsifier in 50 ml 40 mM phosphate buffer and the mixture was then homogenised. The final volume was adjusted to 5 ml with 40 mM phosphate buffer, pH 7.0. After incubation at 37°C in the dark for 72 h, a 0.1 ml aliquot of the reaction solution was mixed with 4.7 ml of ethanol (75%), 0.1 ml $FeCl_2$ (20 mM) and 0.1 ml ammonium thiocyanate (30%). The absorbance of the mixture was measured at 500 nm and the mixture was stirred for 3 min. Ascorbic acid, gallic acid, α-tocopherol and BHT were used as reference compounds. To eliminate the solvent effect, the control sample, which contained the same amount of solvent added to the linoleic acid emulsion in the test sample and reference compound, was used. Inhibition of linoleic acid peroxidation was calculated using the following formula:

$$\% \text{ inhibition} = \frac{\text{Ac-As}}{\text{Ac}} \times 100$$

The index of lipid peroxidation was not determined as ev . limiting factor studies.

Measurement of ferrous ion chelating ability

The ferrous ion chelating activity of the methanol extracts was measured by the decrease in absorbance at 562 nm of the iron(II)-ferrozine complex (21). One milliliter of 0.125 mM $FeSO_4$ was added to 1.0 ml of sample (with different dilutions), followed by 1.0 ml of 0.3125 mM ferrozine. The mixture was allowed to equilibrate for 10 min before measuring the absorbance. The ability of the sample to chelate ferrous ion was calculated relative to the control (consisting of iron and ferrozine only) using the formula:

$$\text{Chelating effect } (\%) = \frac{\text{Ac} - \text{As}}{\text{Ac}} \times 100$$

Determination of hydroxyl radical scavenging activity

The ability of *D. Blagayana* extracts to inhibit non site-specific hydroxyl radical-mediated peroxidation was carried out according by Hinneburg *et al.* (2006) (22). The reaction mixture contained 100 µl of extract dissolved in water, 500 µl of 5.6 mM 2-deoxy-D-ribose in KH_2PO_4–NaOH buffer (50 mM, pH 7.4), 200 µl of premixed 100 µM $FeCl_3$ and 104 mM EDTA (1:1 v/v) solution, 100 µl of 1.0 mM H_2O_2 and 100 µL of 1.0 mM aqueous ascorbic acid. Tubes were vortexed and incubated at 50°C for 30 min. Thereafter, 1 ml of 2.8% TCA and 1 ml of 1.0% TBA were added to each tube. The samples were vortexed and then heated in a water bath at 50°C for 30 min. The extent of oxidation of 2-deoxyribose was estimated from the absorbance of the solution at 532 nm. The percentage inhibition was calculated from the absorbances of the controls (Ac) and the samples (As), where the controls contained all the reaction reagents except the extract or positive control substance. The values are presented as the means of triplicate analyses.

Statistical analysis

The results are presented as mean±standard deviations of three analytical determinations. Statistical analyses were performed using Student´s t-test. IC_{50} values were calculated by nonlinear regression analysis from the sigmoidal dose-response inhibition curve. Significant differences for extracts were analysed using one way ANOVA, followed by Tukey`s HSD post hoc comparison test at $p \leq 0.05$. All computations were made using statistical software (SPSS, version 11.0).

RESULTS

The results obtained for total phenolic and flavonoid contents and total antioxidant capacity for the chloroform and methanol extracts of *D. blagayana* are presented in Table 1. The phenolic content in the chloroform extracts of twigs (90.26±0.69 mg GA/g) was higher than those in the methanol extract of leaves (77.45±0.43 mg GA/g), the chloroform extract of leaves (76.56±0.89 mg GA/g) and the methanol extract of twigs (75.88±0.54 mg GA/g). The flavonoid contents in all the tested extracts ranged from 26.79±0.34 to 35.24±0.55 mg RU/g.

Table 1. Statistical analysis of total phenols, flavonoids and total antioxidant capacity of the chloroform and methanol extracts of *D. blagayana*

Extracts of D. blagayana	Total phenolics (mg GA/g)	Flavonoids (mg RU/g)	Total antioxidant capacity (µg AA/g)
Chloroform extract of twigs	90.26±0.69	35.24±0.55	78.45±0.98
Chloroform extract of leaves	76.56±0.89	26.79±0.34	76.09±0.45
t-test	*	*	*
Methanol extract of twigs	75.88±0.54	29.95±0.39	69.50±1.00
Methanol extract of leaves	77.45±0.43	27.98±0.88	68.98±0.25
t-test	*	n.s.	n.s.
ANOVA	*	*	*

Values are the means ±SD. Data were analysed by t-test and analyses of variance (ANOVA) procedures (* $p < 0.05$; n.s. not significant)

The total antioxidant capacity was measured using the phosphomolybdenum method. The values obtained ranged from 68.98±0.25 µg ascorbic acid/g for the methanol extract of leaves to 78.45±0.98 µg ascorbic acid/g for the chloroform extract of twigs.

In this study, the antioxidant activity of the chloroform and methanol extracts were evaluated using the DPPH and hydroxy radical scavenging, lipid peroxidation and metal chelating assays. The results of the antioxidant activities were compared with control antioxidants, gallic acid, ascorbic acid, BHT and α-tocopherol.

DPPH scavenging

The DPPH assay has been widely used to determine the free radical scavenging activity of various plant extracts. The IC_{50} for DPPH scavenging activity in various extract of *D. blagayana* leaves and twigs are shown in Table 2. It can be seen that IC_{50} values of all the tested extracts of *D. blagayana* were higher than 20 µg/ml and ranged from 20.25±1.55 to 25.24±0.15 µg/ml. The chloroform extract of twigs had the highest activity (IC_{50} = 20.25±1.55 µg/ml), followed in order by the methanol extract of leaves (IC_{50} = 20.95±0.99 µg/ml), the methanol extract of twigs (IC_{50} = 21.09±0.85 µg/ml) and the chloroform extracts of leaves (IC_{50} = 25.24±0.15 µg/ml).

Inhibitory activity against lipid peroxidation

Free radical scavenging is one of the known mechanisms by which antioxidant compounds inhibit lipid oxidation. Inhibitory activity against lipid peroxidation was measured using the thiocyanate method. The results of inhibitory activity against lipid peroxidation for the tested extracts of *D.blagayana* are shown in Table 3. The chloroform extract of leaves had the highest activity (IC_{50} = 33.23±0.99 µg/ml), followed in order by the methanol extract of leaves (IC_{50} = 34.65±0.89 µg/ml), the methanol extract of twigs (IC_{50} = 35.45±0.95 µg/ml) and the chloroform extract of twigs (IC_{50} = 36.46±1.68 µg/ml).

Table 2. DPPH free radical scavenging activity of the chloroform and methanol extracts of *D. blagayana*

Extracts of *D. blagayana*	IC_{50} (µg/ml)	Tukey`s HSD test
CHL extract of twigs	20.25±1.55	CHL extract of twigs/ CHL extract of leaves*
CHL extract of leaves	25.24±0.15	CHL extract of twigs/ MET extract of twigs$^{n.s.}$
MET extract of twigs	21.09±0.85	CHL extract of twigs/ MET extract of leaves$^{n.s.}$
MET extract of leaves	20.95±0.99	CHL extract of leaves/ MET extract of twigs*
Gallic acid	3.79±0.69	CHL extract of leaves/ MET extract of leaves*
Ascorbic acid	6.05±0.34	MET extract of twigs/ MET extract of leaves$^{n.s.}$
BHT	15.61±1.26	
ANOVA	*	

IC_{50} values (means ±SD) for chloroform and methanol extracts of *D. blagayana* compared with gallic acid, ascorbic acid and BHT. Data were zanalysed by analysis of variance (ANOVA) followed by Tukey`s HSD *post hoc* comparison test. (* $p < 0.05$; n.s. not significant)

Metal chelating ability

Results of metal chelating activity of the chloroform and methanol extracts are shown in Table 4. Based on the results obtained, it can be concluded that the constituents of *D. blagayana* extracts have the ability to form complexes

Table 3. Inhibitory activity against lipid peroxidation by chloroform and methanol extracts of *D. blagayana*

Extracts of *D. blagayana*	IC_{50} (µg/ml)	Tukey`s HSD test
CHL extract of twigs	36.46±1.68	CHL extract of twigs/ CHL extract of leaves $^{n.s.}$
CHL extract of leaves	33.23±0.99	CHL extract of twigs/ MET extract of twigs$^{n.s.}$
MET extract of twigs	35.45±0.95	CHL extract of twigs/ MET extract of leaves*
MET extract of leaves	34.65±0.89	CHL extract of leaves/ MET extract of twigs*
Gallic acid	255.43±11.68	CHL extract of leaves/ MET extract of leaves*
Ascorbic acid	> 1000	MET extract of twigs/ MET extract of leaves$^{n.s}$
BHT	0.23	
α-Tocopherol	0.48±0.05	
ANOVA	*	

IC_{50} values (means ±SD) for chloroform and methanol extracts of *D. blagayana* compared with gallic acid, ascorbic acid, BHT and α-Tocopherol. Data were zanalysed by analyses of variance (ANOVA) followed by Tukey`s HSD *post hoc* comparison test. (*$p < 0.05$; n.s. not significant)

Table 4. Metal chelating activity of chloroform and methanol extracts of *D. blagayana*

Extracts of *D. blagayana*	IC_{50} (µg/ml)	Tukey`s HSD test
CHL extract of twigs	45.91±0.88	CHL extract of twigs/ CHL extract of leaves $^{n.s.}$
CHL extract of leaves	45.24±0.95	CHL extract of twigs/ MET extract of twigs*
MET extract of twigs	41.09±1.15	CHL extract of twigs/ MET extract of leaves*
MET extract of leaves	40.95±1.09	CHL extract of leaves/ MET extract of twigs*
Gallic acid	-	CHL extract of leaves/ MET extract of leaves*
Ascorbic acid	-	MET extract of twigs/ MET extract of leaves $^{n.s}$
BHT	-	
α-Tocopherol	-	
ANOVA	*	

IC_{50} values (means ±SD) for chloroform and methanol extracts of *D. blagayana* compared with gallic acid, ascorbic acid, BHT and α-Tocopherol. Data were zanalysed by analyses of variance (ANOVA) followed by Tukey`s HSD *post hoc* comparison test. (*p < 0.05; n.s. not significant)

Table 5. Hydroxyl radical scavenging activity of chloroform and methanol extracts of *D. blagayana*

Extracts of *D. blagayana*	IC_{50} (µg/ml)	Tukey`s HSD test
CHL extract of twigs	99.11±0.23	CHL extract of twigs/ CHL extract of leaves *
CHL extract of leaves	90.26±0.69	CHL extract of twigs/ MET extract of twigs$^{n.s.}$
MET extract of twigs	98.98±0.97	CHL extract of twigs/ MET extract of leaves*
MET extract of leaves	85.88±0.94	CHL extract of leaves/ MET extract of twigs*
Gallic acid	59.14±1.10	CHL extract of leaves/ MET extract of leaves*
Ascorbic acid	160.55±2.31	MET extract of twigs/ MET extract of leaves *
BHT	33.92±0.79	
ANOVA	*	

IC_{50} values (means ±SD) for chloroform and methanol extracts of *D. blagayana* compared with gallic acid, ascorbic acid and BHT. Data were zanalysed by analyses of variance (ANOVA) followed by Tukey`s HSD *post hoc* comparison test. (*p < 0.05; n.s. not significant)

with ferrous ions. As shown in Table 4, the chloroform extract of twigs had the highest value among the extracts examined (45.91±0.88 µg/ml), followed in order by the chloroform extract of leaves (45.24±0.95 µg/ml), the methanol extract of twigs (41.09±1.15 µg/ml) and the methanol extract of leaves (40.95±1.09 µg/ml).

Hydroxyl radical scavenging activity

The results of hydroxyl radical scavenging activity are shown in Table 5. For the tested extracts, the chloroform extract of twigs had the highest IC_{50} value (99.11±0.23 µg/ml), followed in order by the methanol extract of twigs (98.98±0.97 µg/ml), the chloroform extract of leaves (90.26±0.69 µg/ml) and the methanol extract of leaves (85.88±0.94 µg/ml).

DISCUSSION

In conclusion, this is the first study focused on the determination of total phenolic and flavonoid content and antioxidant activity of *D. blagayana* leaves and twigs. Our recent study investigated other similar species of the genus *Daphne* which found that methanol extracts of leaves and twigs of the plant *Daphne cneorum* have good antimicrobial and antioxidant activities (26). There are similarities between these two plants, as the methanol extract of *D. cneorum* showed a similar antioxidant activity to the methanol extract of *D. blagayana*. IC_{50} values for *D. blagayana* DPPH scavenging activity, inhibitory activity against lipid peroxidation, metal chelating activity and hydroxyl radical scavenging activity are highly comparable with the corresponding values for *D. cneorum*. Small differences between these plants (mostly less than 10%) in the examined parameters are more likely attributable to random measurement variations through repeated experiments than to genuine features of the two species. In accordance with present knowledge, it seems that phenolic and flavonoid contents play an important role in the biological activity of individual species within the *Daphne* genus. Natural phenols and flavonoids have been reported to be associated with antioxidant activity in biological systems, mainly due to their redox properties, which can play an important role in absorbing and neutralising free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (23). One of the more prominent properties of flavonoids is their excellent radical scavenging ability, which makes them valuable for therapeutic and prophylactic applications, e.g., after infection, inflammation, burns, or radiation injury (24). The activity of crude methanol extracts is due to the presence of flavonoid monomers and polymers (condensed tannins), hydrolysable tannins, and phenolics. Recently, polyphenolic compounds from plants, such as condensed and hydrolysable tannins, have been shown to be powerful antioxidants (25). The amount of total phenols varied widely in extracts and ranged from 75.88±0.54 to 90.26±0.69 mg GA/g. Among the samples tested, the chloroform extract of twigs had the highest phenolic (90.26±0.69 mg GA/g) and flavonoid (35.24±0.55 mg GA/g) contents. Analysis of variance (ANOVA) showed significant differences in the presence of total phenolics and flavonoids among the tested extracts. In order to determine statistical significance in the amount of total

phenols and flavonoids from the plant parts, Student's t-test was used. Based on the results obtained, it can be seen that there is a statistically significant difference between the total phenolic and flavonoid content of twigs and leaves, although the difference is not significant for the methanol extracts (Table 1). Variations in the total antioxidant capacity between chloroform and methanol extracts were statistically significant. Unlike chloroform extracts, methanol extracts of the twigs and leaves did not show a significant difference in the total antioxidant capacity (Table 1). In the DPPH assay, the chloroform extract of twigs showed the lowest IC_{50} value (20.25 ±1.55 μg/ml) among the samples tested, while the chloroform extract of leaves had the highest IC_{50} values. Analysis of variance of the results of DPPH activity confirmed the existence of significant differences among four tested extracts and the standards that were used. Tukey *post hoc* test for analysis of variance indicated that there are no statistically significant differences in DPPH activities between the chloroform extracts of twigs and methanol extract of twigs and leaves. For the reducing capacity of the extracts, IC_{50} values for ferrous ion chelating ability were approximately 45 μg/ml for the chloroform extracts and approximately 41 μg/ml for the methanol extracts. The metal chelating activities differ significantly, depending on the solvent. For the inhibitory activity against lipid peroxidation, IC_{50} values ranged from 33.23 ±0.99 μg/ml for the chloroform extract to 36.46 ±1.68 μg/ml for the chloroform extracts of twigs. The tested extracts showed significantly better inhibitory activity then ascorbic and gallic acids, and a lower activity then vitamin E and BHT. Tukey`s HSD *post hoc* test of the extracts showed no statistically significant difference in inhibitory activity for the parts of the plant if the same solvent is used to obtain the extract. There is a statistical significance between the chloroform and methanol extracts (Table 3). The results of hydroxyl radical scavenging activity showed higher values for the tested extracts than ascorbic acid, but lower activity than gallic acid and BHT. IC_{50} values ranged from 85.88 ±0.94 μg/ml for the methanol extract of leaves to 99.11 ±0.23 μg/ml for the chloroform extract of twigs. Statistical analysis showed that there is no statistically significant difference in activity between the chloroform and methanol extracts of twigs. The *Daphne* genus includes approximately 70 different species, and several are reported to possess significant antioxidant activity (26,27). This makes plants of the genus *Daphne* very attractive as a source of future drugs. Of course, future research should incorporate a detailed phytochemical analysis of *Daphne blagayana*, isolation of potential antioxidants and their antioxidant activity.

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SYSTEMIC MANIFESTATIONS OF PSEUDOEXFOLIATION

Tatjana S. Sarenac-Vulovic[1], Mirjana A. Janicijevic Petrovic[1], Dejan D. Vulovic[2], Sladjana M. Pavlovic[3], Sladjana Simovic[4] and Nemanja S. Zdravkovic[3]
[1]Clinic of Ophthalmology, Clinical Centre Kragujevac, Kragujevac, Serbia
[2]Centre for Plastic and Reconstructive Surgery, Clinical Centre Kragujevac, Kragujevac, Serbia
[3]Center for Molecular Medicine and Stem Cell Research, Faculty of Medical Sciences, University of Kragujevac, Serbia
[4]Health Centre ENP department Kragujevac, Kragujevac, Serbia

SISTEMSKE MANIFESTACIJE PSEUDOEKSFOLIJACIJE

Tatjana S. Šarenac-Vulović[1], Mirjana A. Janićijević Petrović[1], Dejan D. Vulović[2], Slađana M. Pavlović[3], Slađana Simović[4] and Nemanja S. Zdravković[3]
[1]Klinika za oftalmologiju, Klinicki centar Kragujevac, Kragujevac, Srbija
[2]Centar za plasticnu i rekonstruktivnu hirurgiju, Klinicki centar Kragujevac, Kragujevac, Srbija
[3]Centar za molekularnu medicinu i istrazivanje maticnih celija, Fakultet medicinskih nauka, Univerzitet u Kragujevcu, Kragujevac, Srbija
[4]Zdravstveni centar ENP odsek u Kragujevcu, Kragujevac, Srbija

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ABSTRACT

Objective: *The aim of our study was to establish a correlation between pseudoexfoliation and its systemic manifestation.*

Findings: *Pseudoexfoliation syndrome is an age-related systemic disorder that leads to the overproduction and accumulation of the pseudoexfoliated materials in the visceral organs and in the eye. Many vascular diseases are closely related with pseudoexfoliation manifestations. Our results indicated that there were no statistically significant differences (p>0.05) among patients regarding the presence of hypertension in all groups: PEX glaucoma – 45% (9 patients); PEX syndrome- 40% (8 patients); and control groups- 35% (7 patients). Ischemic heart disease was statistically significant present in the sPEX syndrome- 20% (5 patients) and PEX glaucoma- 25% (5 patients) patient groupss, in comparison with those of the control group-10%, (p<0.05). Aortic aneurism was statistically significant present in patients with PEX (syndrome-5% or glaucoma-15%), compared to those in the control group, which included no patients with aneurisms, (p<0.05). Our results indicated that a statistically significant number of patients with aneurism were in the group of patients who developed PEX glaucoma (p<0.05). Cerebrovascular diseases were detected in all groups of patients, but a significant decrease in this metric was noted in the control group- 5% (2 patients), compared with patients with PEX syndrome-15% and PEX glaucoma-25%, (p<0.05). Hearing loss, as a concomitant sign of PEX manifestation, was recorded in all patients, but in the group with PEX (syndrome-55% or glaucoma-75%), these results showed a statistically significant increase (p<0.05) in comparison with those of patients in the control group (10%). Among the patients with PEX (syndrome and glaucoma), there were no statistically significant differences in the selected categories of systemic manifestations (p<0.05). This result indicates that the main risk for systemic manifestation is the presence of PEX and that other ocular and vascular complications are, in fact, consequences of PEX.*

SAŽETAK

Cilj: *Cilj našeg ispitivanja je bio da se utvrdi korelacija između pseudoeksfolijacija i njihovih sistemskih manifestacija*

Rezultati: *Pseudoeksfolijacije su sistemski poremećaj, starijeg životnog doba, kog karakteriše povećana produkcija i nakupljanje pseudoeksfolijativnog materijala u visceralnim organima, i u oku vezane za starije. Mnoge vaskularne bolesti su u neposrednoj povezanosti sa prisustvom pseudoeksfolijacija u oku. Naši rezultati ukazuju da nije bilo statistički značjne razlike u incidenci hipertenzije kod pripadnika sve tri ispitivane grupe, (p>0.05): PEX glaukom- 45% (9 pacijenata); PEX sindrom- 40% (8 pacijenata) i kontrolna grupa- 35% (7 pacijenata). Ishemijska bolest srca je statistički značajno zastupljena u grupi bolesnika sa PEX sindromom- 20% (5 pacijenata) i PEX glaukomom-25% (5 pacijenata) u poređenju sa bolesnicima iz kontrolne grupe-10%, (p<0.05). Kod bolesnika sa PEX (sindrom-5% i glaukom-15%) uočeno je statistički značajan (p<0.05) broj bolesnika sa aneurizmom aorte u odnosu na kontrolne grupe, gde nisu zabeležene. Cerebrovaskularna oboljenja su statistički značajno umanjena, (p<0.05) u kontrolnoj grupi 5% (2 pacijenta) u poređenju sa ispitanicima iz grupe bolesnika sa PEX: sindrom- 15% i PEX glaukom-25%, (p<0.05). Gubitak sluha, koji se sreće kod bolesnika sa PEX, je zabeležen kod svih ispitanika, ali je statistički značajan broj (p<0.05) u grupi bolesnika sa PEX (sindrom-55% i glaukom-75%) u odnosu na pripadnike kontrolne grupe (10%). Naše ispitivanje ukazuje da je prisustvo PEX faktor rizika za različite vaskularne komplikacije.*

Zaključak: *Pseudoeksfolijacije su u neposrednoj povezanosti sa brojnim vaskularnim poremećajima. Bolesnici koji imaju PEX bi trebalo da budu detaljno ispitani od strane specijaliste interne medicine i neurologa. Njihova detaljna ispitivanja, naročito u ranoj fazi bolesti, bi bila od velike pomoći u prevenciji teških vaskularnih komplikacije sistemskih manifestacija PEX*

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Correspondence to: Tatjana Šarenac-Vulović, Zmaj Jovina 14/30, 34000Kragujevac
Phone number: +381 62 219915, Fax number: +381 34 37 00 73, mail: tvoja.tanja@yahoo.com

Conclusion: *Pseudoexfoliation is strongly related to systemic vascular disturbances. A detailed examination of patients with PEX by specialists in internal disease or by neurologists should be performed. Such recommended examinations can be helpful in the prevention of different vascular diseasess among patients with PEX, especially atthose in the early stages.*

Key words: *pseudoexfoliation, systemic manifestation, vascular diseases.*

Ključne reči: *pseudoeksfolijacije, sistmske manifestacije, vaskularne bolesti.*

ABBREVIATIONS

ABI-ankle brachial index
DM-diabetes mellitus
HT-hypertension
IHD-ischemic heart disease
PEX-pseudoexfoliation
PXS-pseudoexfoliation syndrome
MMPs-metalloproteinases

INTRODUCTION

Pseudoexfoliation syndrome (PXS) is an age-related systemic disorder involving the overproduction and accumulation of pseudoexfoliation materials (PEX) in the visceral organs and in the eye (1). It is characterised by an intensive production of abnormal fibres and their accumulation in the whole body and in the eye (1). PEX flakes can be detected around the blood vessels of connective tissue and have been identified using electron microscopy (2) and immunohistochemistry (3) in the lung, liver, kidney, gall bladder, and cerebral meninges (4). Some cardiovascular and cerebral diseases (angina, aortic aneurysm, dementia, etc.) have been associated with pseudoexfoliations (5). Pseudoexfoliation can be detected using a slit lamp in every part of the anterior segment of the eye, including the corneal endothelium, iridocorneal angle, pupillary margin, and iris anterior capsule of the lens. By histological examination, PEX can be detected in extra-ocular tissues, such as the conjunctiva, extra-ocular muscles, and retro-ocular tissue (1). The fibres can accumulate duringat the outflow through the trajectoriesroute of the aqueous humor, causing enhanced resistance and further increasing intraocular pressure (4). Glaucoma occurs more commonly in eyes with PEX than in those without PEX (6). Glaucoma in patients with PEX has a more serious clinical progression and worse prognosis than primary open-angle glaucoma (6).

PATIENTS AND METHODS

Our cross-sectional comparison study included 60 patients at the Clinic of Ophthalmology, Clinical Centre, Kragujevac, Serbia who were referred for cataract surgery. The study design was approved by the local ethics committee, and all enrolled patients gave their written consent at the beginning of the study.

All patients were divided in three groups according to the presence of PEX in their eyes: patients with PEX syndrome, those with PEX glaucoma and a control group (no PEX). The presence of PEX was confirmed by slit-lamp examination, measurements of intraocular pressure, and gonioscopy; to establish PEX glaucoma, fundus and visual field examination was needed. Exclusion criteria were a history of intraocular surgery, ocular trauma, uveitis, prophylactic laser photocoagulation, and myopio- or cryo-treatment. Directly before the surgical treatment, a detailed disease history was taken from every patient, including the following items: hypertension (HT), diabetes mellitus (DM), cerebrovascular stroke, ischemic heart disease (IHD), and hearing loss. Reports from an internal medicine specialist and otolaryngologist with detailed disease summary and recommended therapy were also required, and any vascular surgery was recorded. The presence of diabetes mellitus was defined as previous (detailed medical history with therapy) or newly diagnosed (no medical history). Cardiovascular disease was defined by the presence of an earlier heart attack, bypass surgery, angioplasty, cardiomyopathy or angina. Hypertension was defined by an earlier disease history with detailed therapy (systolic blood pressure of more than 160 mm/Hg or a diastolic blood pressure more than 90 mm/Hg). An otolaryngologist discovered and explained any hearing loss in our patients using standard audiometry.

The participants in the control group included patients with cataracts who were age–matched and resisted the exclusion criteria.

STATISTICAL ANALYSIS

The unpaired Student's t-test and Mann–Whitney test were performed using the SPSS 19.0 statistical software package (SPSS Inc., Chicago, IL). The results were expressed as the percentage values. All P values were 1-sided, and a P value <0.05 was considered statistically significant (significance levels as indicated in the table legends).

RESULTS

Among the participants of the study, a female majority (15, 3:1) was noted in the first group of the PXS syndrome.

Age	PEX syndrome (%)	PEX glaucoma (%)	Cataract (%)	Means±SE	P
45-60.	1(1.67)	0 (0)	11(18.33)	54.23±3.2	p=0.023 *
60-75.	10 (16.67)	6 (10)	9 (15)	70.57±5.21	p=0.078
75-90.	9 (15)	14 (23.33)	0 (0)	83.45±4.34	p=0.038 *
Mean age	73.41±6.54	77.2±3.9	63.4±4.2	72.2±7.4	p=0.029*

Table 1. Distribution of the patients according to age. Our results indicated that the older population was at a greater risk for PEX production, fibre accumulation and development of glaucoma. Our results indicated that the older population was at a greater risk for PEX production, fibre accumulation and development of glaucoma.

In the group of patients with PXS glaucoma, 14 patients were female (2.33:1), whereas in the control group, the gender ratio was approximately equal (11, 1.22:1).

The mean age of all patients was 72.2 ± 7.4 years (the youngest was 48 years; the oldest, 90 years). The group of patients with PEX glaucoma had a mean age of 77.2±3.9; the PXS group, of 73.4±6.5; and the control group, of 63.4±4.2 years, Table 1.

Our results indicated that there were no statistically significantly differences (p=0.081) among patients regarding the presence of hypertension in any of the groups: PEX glaucoma-45% (9 patients); PEX syndrome-40% (8 patients) and control groups-35% (7 patients). Ischemic heart disease was statistically significantly present in the PEX syndrome group- 20% (5 patients) and in the PEX glaucoma group-25% (5 patients), compared with controls-10%, (p=0.049). Aortal aneurisms were statistically significantly present in patients with PEX (syndrome-5% or glaucoma-15%) in comparison with patients from the control group, in which no patients had aneurisms (p=0.018). Cerebrovascular diseases were detected in all groups of patients, but in the control group- 5% (2 patients), this number was statistically significantly lower than in patients with PEX syndrome- 15% or PEX glaucoma-25% (p=0.026). Hearing loss as concomitant sign of PEX manifestation was recorded in all patients, but in the group with PXS (syndrome-55% or glaucoma-75%), those numbers were statistically significantly increased (p=0.033) in comparison with the patients in the control group (10%). Among those patients with PXS (syndrome or glaucoma), there were no statistically significantly differences among any of the selected categories of systemic manifestation (p=0.0728), Table 2.

DISCUSSION

PEX deposits can be found in many tissues in the body, especially in connective tissue or transverse organ septa (1). PEX can be presented in the eye or localised around either eye (7). Ocular PEX is associated with high rates of cataract and glaucoma (6).

PEX can be described as the abnormal production and accumulation of fibres, accompanied by the presence of fibroblasts around small blood vessels, as indicated for systemic manifestations (2). The process of this production can be activated by increased levels of different growth factors (8), a disrupted oxidative-anti-oxidative status (9), and enhanced metalloproteinase (MMP) activities (10). Homocysteine levels in the plasma are increased in patients with PEX (11). All of these parameters signify the systemic manifestations of PEX.

Many earlier studies have suggested an important relation between PEX and different systemic diseases, but their results are contradictory (12). This fact can be explained by differences among populations and selected groups. Generally, a large number of associations have been found between PXS and peripheral vascular diseases (13).

It has been established that iris vasculopathy is an obligate finding in eyes with PEX and may cause hypoxia of the anterior segment of the eye (14). Indocyanin green was used to support this finding (15). Some similar diagnostic procedures were used to establish blood flow in other tissues in the body. Cutaneous capillary perfusion was examined to demonstrate impaired vascular perfusion in the fingers of patients with PEX (16). This study indicated that the cutaneous capillary flow was significantly lower in patients with PEX than in the age-matched control group. Additionally, this study demonstrated a reduced cardiovascular regulatory function among PEX patients. Another study, using Color Doppler imaging, indicated that brachial and dorsal pedis artery circulation could be improved (12). This finding was sustained by a low ankle brachial index (ABI). Low ABI is one of the risk factors for the development of peripheral vascular disease and is strictly connected with PEX. A

	Arterial hypertension (%)	Ischemic heart disease (%)	Aortal aneurism (%)	Cerebrovascular disease (%)	Sensorineural hearing loss (%)
PEX glaucoma	9 (45)	5 (25)	3 (15)	5 (25)	15 (75)
PEX syndrome	8 (40)	4 (20)	1 (5)	3 (15)	11 (55)
Control group	7 (35)	2 (10)	0 (0)	1 (5)	2 (10)
P values	p=0.081	p=0.049*	p=0.018*	p=0.026*	p=0.033*

Table 2. Systemic manifestations of the ocular pseudoexfoliation. Patients with PEX (syndrome and glaucoma) had a greater incidence of ischemic heart disease, aortal aneurism, cerebrovascular disease and hearing loss, in comparison with patients without PEX.

biopsy of the cutaneous tissue showed pseudoexfoliation materials around the blood vessels.

Toxic agents, acoustic trauma, solicitude and other less studied causes can contribute to sensorineural hearing loss (SNHL) (17). The hair cells (inner and outer hair cells), which are placed on the basilar membrane and overlaid with the tectorial membrane, are the fragments of the compound organ of Corti. SNHL in PXF syndrome may be caused by deposits of this material in inner ear structures (organ of Corti), which can decrease the alterations in small vibrations actuated by sound analogues (18).

The present study was performed to establish a correlation between PEX patients (syndrome or glaucoma) and a control group, according to the potential systemic manifestations of PEX. Arterial hypertension was equally represented in all selected groups. Our results indicated statistically significant differences in the number of patients with ischemic cardiac disease, cerebrovascular disease, and the presence of aortal aneurism or sensorineural hearing loss between the PEX group (syndrome or glaucoma) and controls. There were no statistically significant differences between the two subgroups of the PEX group (syndrome or glaucoma). This result indicated that the main risk for systemic manifestation is the presence of PEX. Other ocular and vascular complications constitute consequences of PEX. Our results are similar to the results of recent studies, the data from which have been published (5, 7, 12). These studies suggest that ocular PEX carries a great risk for peripheral vascular disease development with sensorineural hearing loss, as was seen in the concomitant otolaryngologist's findings.

Our study showed a statistically significant association between PEX and different cardio- and cerebrovascular diseases, similar to other previous studies (5, 12, 19, 20).

Pseudoexfoliation is strongly related to systemic vascular disturbances. A detailed examination of patients with PEX by a specialist in internal diseases or a neurologist should be made. Such examinations can be helpful in the prevention of vascular disease development among patients with PEX, especially at early stages-.

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ORTHODOX CATECHISM AFFECTS GENDER DIFFERENCES IN ADOLESCENTS' NEEDS FOR AFFILIATION AND ACHIEVEMENT AND ALTERS THEIR SENSE OF PURPOSE IN LIFE

Ana Volarevic[1], Bojana Simovic Markovic[2], Nikola Jankovic[1], Sanja Bojic[1], Nebojsa Zdravkovic[1]
[1]Faculty of Medical Sciences, University of Kragujevac, Serbia
[2]Centre for Molecular Medicine and Stem Cell Research, Faculty of Medical Sciences , University of Kragujevac, Serbia

UTICAJ PRAVOSLAVNOG KATIHIZISA NA RAZLIČITO ISPOLJAVANJE SMISLA ŽIVOTA, AFILIJATIVNE MOTIVACIJE I MOTIVA POSTIGNUĆA ADOLESCENATA MUŠKOG I ŽENSKOG POLA

Ana Volarević[1], Bojana Simović Marković[2], Nikola Janković[1], Sanja Bojić[1], Nebojša Zdravković[1]
[1]Fakultet medicinskih nauka, Univerzitet u Kragujevcu, Kragujevac, Srbija
[2]Centar za molekularnu medicinu i istrazivanje maticnih celija, Fakultet medicinskih nauka, Univerzitet u Kragujevcu, Kragujevac, Srbija

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ABSTRACT

Studies conducted among Catholics and Protestants have shown higher levels of prayer and religious experience among women compared to men and have suggested that these gender differences may be a reflection of differences in personality and socialization. However, the impact of orthodox religion on adolescents' psychological and social well-being remains unknown.

The aim of this study was to investigate the influence of Orthodox religious studies (Orthodox Catechism) on gender differences in adolescents' needs for affiliation and achievement and their effect on adolescents' attitudes about their purpose in life.

This study is the first to show that Orthodox Catechism affects adolescents' needs for affiliative and achievement motivation in a gender-dependent manner. Orthodox Catechism enhances the competition motives of male adolescents and has a significant influence on the development of emotional support and goal achievement as well as providing a source of pleasure for female adolescents. Thus, the Orthodox Catechism is related to gender differences in adolescents' sense of purpose in life.

In conclusion, our findings emphasize the effects of the Orthodox Catechism on the expression of adolescents' psycho-emotional characteristics, demonstrating that the Orthodox religion has a positive influence on adolescents' needs for affiliation and achievement and affects their attitudes about their purpose in life.

Keywords: *Orthodox Catechism, gender, affiliation, achievement, motive, purpose in life*

SAŽETAK

Studije sprovedene među katolicima i protestantima su pokazali veći stepen religioznog iskustva kod žena u odnosu na muškarce, sugerišući da se ove razlike mogu odraziti i razlikama u psihološkim karakteristikama ličnosti i u njihovoj socijalizaciji i ponašanju. Međutim, uticaj pravoslavne religije na razvoj psiholoških i socijalnih karakteristika adolescenata je i dalje nepoznat.

Cilj ove studije je bio da se ispita uticaj učenja pravoslavne religije (Pravoslavni katihizis) na razlike među polovima u razvoju i ispoljavanju motiva postignuća i afilijativne motivacije adolescenata kao i da se utvrdi da li ove razlike utiču na stavove adolescenata o smislu života.

U ovom radu, po prvi put je pokazano da Pravoslavni katihizis utiče na razvoj motiva postignuća i afilijativne motivacije adolescenata različito kod muškaraca i žena. Pravoslavni katihizis pojačava takmičarski motiv muških adolescenata, ima značajan uticaj na razvoj emocionalne podrške i postizanje cilja kao izvor zadovoljstva ženskih adolescenata i utiče na stavove adolescenata o smislu života .

Rezultati predstavljeni u ovom istraživanju ukazuju da Pravoslavni katihizis utiče na ekspresiju psiho-emocionalnih karakteristika adolescenata pokazujući da pravoslavna religija ima pozitivan uticaj na razvoj motiva postignuća, afilijativne motivacije i da na pozitivan način utiče na stavove adolescenata o smislu života.

Ključne reči: *Pravoslavni katihizis, pol, afili-jativna motivacija, motiv postignuća, smisao života*

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Correspondence:
Ana Volarevic, Faculty of Medical Sciences, Svetozara Markovica 69, 34 000 Kragujevac, Serbia
volarevic_ana@yahoo.com

INTRODUCTION

For believers, complete and true devotion and obedience to God is a constant source of spiritual, emotional, and moral energy and is helpful in resisting destructive environmental attacks as well as social and mental disruption (1). For the past few decades, researchers have systematically investigated connections between religiosity and mental health and have identified a consistent relationship between them in adolescent populations (2-5).

Higher levels of religiosity and spirituality in adolescents have been associated with better mental health (6), a reduction in the negative effects of psychiatric disorders on suicidal thoughts (7-8), and protective effects for the emotional and physical well-being of adolescents exposed to traumatic events (9-10).

A general conclusion in the psychological literature is that women are more religious than men (11). Studies conducted among Catholics and Protestants have shown higher observance of prayer and religious experience among women compared to men, suggesting that these gender differences may reflect differences in personality and socialization (11).

Affiliation and achievement motives are important for the psychological and social well-being of young adults (12). Achievement motivation is an important issue for psychologists of religion because it has been correlated with personality traits and religiosity (12). Moreover, gender differences in achievement motivation have been studied widely in the context of academic achievement (13) because gender differences in motivation have been found to predict adolescents' differences in academic achievement (14).

Affiliative motivation, defined as the desire to get along with another person to create, preserve, or re-establish positive relations, is closely related to religiosity. Religion helps people to affiliate and coordinate with one another to maintain a distinctive social identity (15-16). However, this positive effect of religion on affiliative motivation may be culture-specific. Many religious traditions differentiate between the religious obligations of men and women, which also affects affiliative motivation (17).

The influence of religiousness on adolescents' needs for affiliation and achievement is carried over direct physiological pathways. Religious studies have examined gender differences in these personal traits, and a positive association has been found between intrinsic religious orientation and a sense of purpose in life among Catholics and Protestants (18-19). A greater sense of purpose in life has also been found among intrinsically religiously oriented adolescents of both genders. A sense of purpose in life is positively related to the happiness, life satisfaction, and general psychological well-being of young adults (19). In addition, religious education has a positive impact on the sense of purpose in life, and individuals who describe themselves as having strong religious faith are happier and more satisfied with their lives. This finding indicates that spirituality and religiosity are important psychological mechanisms for managing adolescents' stressful life events (6).

The consistency of the previously discussed findings across a diversity of samples, designs, methodologies, religiousness, and population characteristics serves to strengthen the inference of a positive association between religion and adolescents' emotional well-being. However, all of these studies were conducted among Catholics, Protestants, Jews, and Muslims. The impact of Orthodox religion on adolescents' needs for affiliation and achievement remains unknown.

This research examines the influence of the Orthodox religion (Orthodox Catechism) on the gender differences in adolescents' needs for affiliation and achievement that affect their attitudes about their purpose in life.

MATERIALS AND METHODS

Participants

The participants in this study were adolescents (18-21 years old) who were students of the Faculty of Medical Sciences, University of Kragujevac, Serbia (N=435). Male and female participants were divided into two groups depending on their studies during high school: Orthodox Catechism or civic education. The study was approved by the Ethical Committee of the Faculty of Medical Sciences, University of Kragujevac, Serbia.

Psychological tests

The students responded to standardized psychological tests (the purpose in life scale, the need for affiliation test, and the need for achievement test) that were previously standardized in the Serbian population .

Need for achievement test

The need for achievement test was used to measure the achievement motivation of adolescents. This test is an adaptation of the original scale constructed by David McClellan (20). It contains 55 items that assess four factors of achievement motivation: competition (example: "The biggest motivation for me is competition with others"), perseverance in achieving a goal (example: "I always finish the job that I started"), achievement of a goal as a source of pleasure (example: "Just the thought that I managed to accomplish a goal is positive for me"), and orientation towards planning (example: "I feel great satisfaction when I manage to complete my daily plan"). These four components are independent of each other, and together they form a general achievement motivation factor that is highly correlated with all four sub-factors. The participants responded on a 5-point scale ranging from 1 ("not true of myself") to 5 ("definitely true of myself"). The scale of the negatively worded items (numbers 14, 16, 18, 19, 47, 49, 52,

and 55) was reversed before the sum of all items was taken. The total score was calculated as the sum of all items.

Adolescents who scored higher on the "competition" sub-scale have a competitive spirit, a desire to be better than others, and a need to show people how successful they are. Adolescents who scored higher on the "perseverance in achieving the goal" sub-scale are willing to spend manysignificant time and effort to achieve a goal; they know what they want, and they do not give up despite the odds. Respondents who scored high on the "achievement of the goal as a source of pleasure" sub-scale feel good when they achieve their goals. Adolescents with high scores on the "orientation towards planning" sub-scale prefer to plan their activities in advance.

Need for affiliation test

The need for affiliation test is an adaptation of the Interpersonal Orientation Scale by Craig Hill (21). This scale has been used to operationalize affiliation. The original scale consists of 26 items grouped in four subscales determining four factors of affiliative motivation: emotional support (example: "Whenever something bad happens to me, it is most helpful for me to spend time with my intimate friends"), social comparison (example: "When I am not sure how good I am, it helps me to compare myself with other people"), positive stimulation (example: "The fact that I can learn something about people gives me great pleasure"), and attention (example: "I enjoy when people think that I am an important person"). Participants responded on a 5-point scale ranging from 1 ("not true of myself") to 5 ("definitely true of myself"). The total score was calculated as the sum of all items. Adolescents who had higher scores are considered to be "highly affiliated"; they more often seek social contact with others, communicate more often with colleagues, and visit friends more frequently than others. If a respondent had a high score in only a single dimension of affiliative motivation, this type of affiliative motivation is his/her main motivation for entering into social contacts.

The purpose in life scale

The purpose of life scale is used to assess adolescents' sense of purpose and meaning in life. This scale is an adaptation of the purpose in life test by James Crumbaugh (22). It consists of 23 items that assess the emotional and cognitive aspects of purpose in life (representative items: "I have discovered satisfying goals and a clear purpose in life"; "If I should die today, I would feel that my life has been worthwhile"; "My personal existence often seems meaningless and without purpose"). The participants responded on a 5-point scale ranging from 1 ("not true of myself") to 5 ("definitely true of myself"). The scale of the negatively worded items (numbers 1, 3, 5, 6, 7, 8, 12, 15, 16, and 20) was reversed before the sum of all items was taken. The total score was calculated as the sum of all items.

Procedure

The psychological tests, standardized for the Serbian population, were administered anonymously to the students in their classrooms. The students volunteered for the study after the tester briefly explained its purpose and assured them that anonymity would be maintained. If any student did not want to participant, he/she was allowed to leave the testing session . The data were collected and analyzed by a trained psychologist.

Statistical analysis

All statistical analyses were conducted using SPSS 19.0 for Windows software. The results were analyzed using Student's t-test or a Mann-Whitney test on the dependence of normal distribution determined by a Kolmogorov-Smirnov test. Correlation between the values was determined by Pearson's correlation. The data were expressed as the mean ± standard error (SEM). Values of $p < 0.05$ were considered statistically significant.

RESULTS

Orthodox Catechism enhances achievement of the goal as a source of pleasure among female adolescents

As shown in Figure 1, Orthodox religious education enhances the motivation to achieve goals as a source of pleasure among female adolescents. Female students had significantly higher scores on the "achievement of the goal as a source of pleasure" sub-scale in comparison with male students ($p<0.01$). The results from the male and female adolescents who studied civic education, who were used as the control group in this test, showed no significant difference on the "achievement of the goal as a source of pleasure" sub-scale among these participants.

The Pearson correlation test further confirmed the influence of the Orthodox Catechism on the development of motivation for the achievement of a goal as a source of pleasure among female adolescents. There was a significant correlation between gender and the score on the "achievement of the goal as a source of pleasure" sub-scale among students who studied the Orthodox Catechism ($r=0, 223$; $p<0,01$), whereas there was no significant correlation in these parameters among adolescents who studied civic education.

Orthodox Catechism enhances competition motive of male adolescents

The results obtained for the "competition" sub-scale of the need for achievement test (Figure 2) showed a significantly higher score among male adolescents who studied Orthodox Catechism compared to female students ($p <$

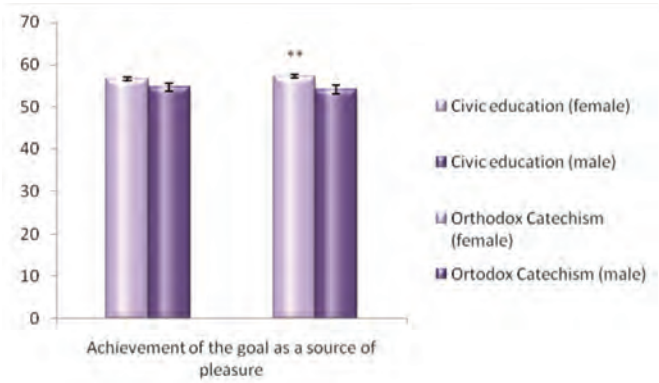

Figure 1. The results obtained on the "achievement of the goal as a source of pleasure" sub-scale of the need for achievement test

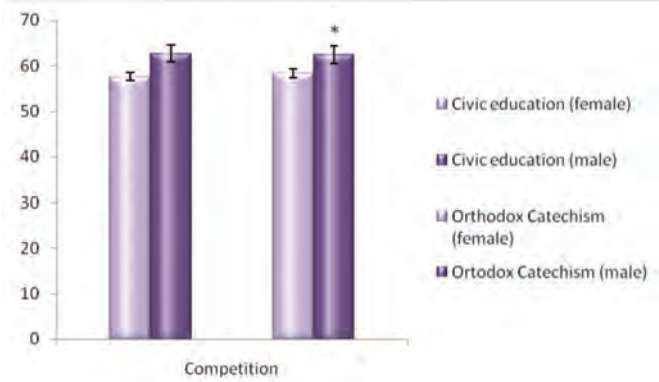

Figure 2. The results obtained on the "competition" sub-scale of the need for achievement test

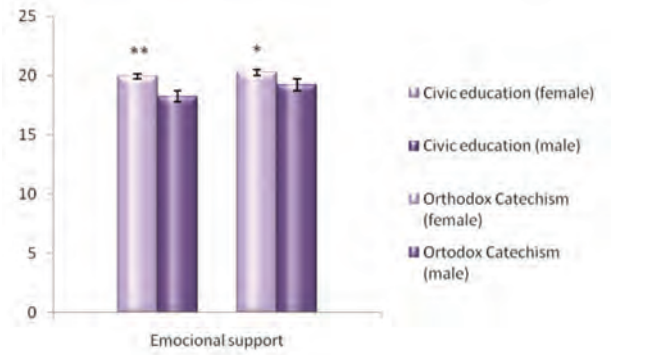

Figure 3. The results obtained on the "emotional support" sub-scale of the need for affiliation test

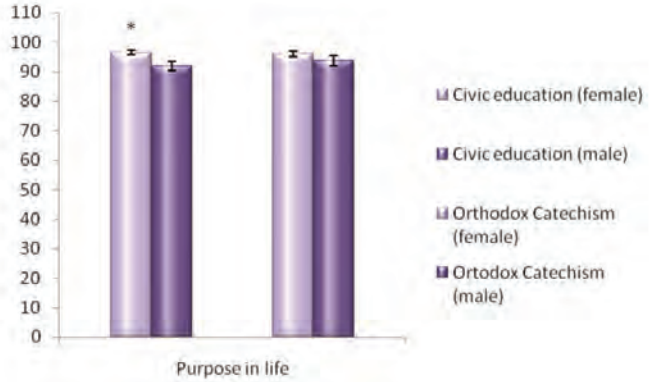

Figure 4. Results obtained on the purpose in life scale

0.05). On the contrary, there was no significant difference in the "competition" sub-scale between male and female adolescents who studied civic education, suggesting that the Orthodox Catechism enhances the competition motives of male adolescents.

As shown in Table 1, there were no significant differences for the scores on the "perseverance in achieving the goal" and "orientation toward planning" sub-scales between the examined participants, suggesting that competition motives and achievement of the goal as a source of pleasure were mainly affected by the Orthodox Catechism.

Orthodox Catechism has a significant influence on the development of emotional support as a major affiliative motive among female adolescents

The results obtained on the need for affiliation test (Figure 3) showed that emotional support is the main affiliative motive of female adolescents who studied Orthodox Catechism ($p < 0.05$) and civic education ($p < 0.01$).

The Pearson correlation test showed a significant correlation between gender and emotional support only among adolescents who studied the Orthodox Catechism ($r=0{,}145$; $p<0{,}05$), indicating that the Orthodox Catechism has a significant influence on the development of emotional support as a major affiliative motive among female adolescents.

As shown in Table 1, there were no significant differences for the scores on the sub-scales determining social comparison, positive stimulation, and attention between the examined participants, suggesting that emotional support is the main motive for female adolescents to enter into social contacts.

Orthodox Catechism affects male adolescents' sense of purpose in life

Only among adolescents who studied civic education, there was significant but negative correlation between gender and the purpose in life score ($r=-0{,}159$; $p<0{,}05$). Additionally, female adolescents who studied civic education scored significantly higher on the purpose of life scale compared to male students who studied civic education. As shown in Figure 4, Orthodox Catechism affects male adolescents' sense of purpose in life. Male adolescents who studied the Orthodox Catechism scored higher on the purpose of life scale then male students who studied civic education, with a loss of statistical significance between the genders among adolescents who studied the Orthodox Catechism.

DISCUSSION

The role of Orthodox religion in the expression of the psycho-emotional and social characteristics of adolescents is completely unknown. This study is the first to demon-

Sub-scale	Orthodox Catechism female vs. male (Mean ± SE)	civic education female vs. male (Mean ± SE)
Perseverance in achieving the goal	61.37 ± 0.56 vs. 60.02 ± 1.14	60.56 ± 0.57 vs. 58.54 ± 0.93
Orientation toward planning	26.66 ± 0.59 vs. 26.65 ± 0.99	27.29 ± 0.49 vs. 25.71 ± 1.02
Social comparison	12.69 ± 0.22 vs. 12.58 ± 0.47	12.52 ± 0.20 vs. 13.29 ± 0.34
Positive stimulation	16.76 ± 0.31 vs. 17.00 ± 0.50	16.49 ± 0.26 vs. 16.10 ± 0.46
Attention	12.17 ± 0.31 vs. 13.17 ± 0.46	12.03 ± 0.22 vs. 12.40 ± 0.43

Table 1. The mean values obtained on the need for achievement and need for affiliation tests

strate that Orthodox religious education shows gender differences in adolescents' needs for affiliation and achievement, altering their sense of purpose in life.

Numerous studies have examined the relationship between religiosity and gender differences with regard to affiliative and achievement motivation (15-16, 23-24). This study is the first to show that the Orthodox Catechism significantly enhances the achievement of a goal as a source of pleasure among female adolescents (Figure 1) and enhances the competition motive of male adolescents (Figure 2). Our data are in line with the results obtained by Allan Wigfield (25), suggesting that male students are more likely to express a competition motive due to their higher competence beliefs in sports activities, whereas female adolescents are more likely to express achievement motives that are related to social activities, satisfaction, and enjoyment. It seems that Orthodox religious education stimulates the expression and/or development of achievement motives, which are rooted in the socialization processes of male and female adolescents (23). These findings are consistent with the notion that religious education serves the relational function of establishing a shared sense of reality that helps to maintain and coordinate important social relationships (12). We are the first to show the relation between the Orthodox religion and emotional support. Our data indicate that the Orthodox Catechism has a significant influence on the development of emotional support as a major affiliative motive among female adolescents (Figure 3). Our data are in line with previous studies conducted among Protestants and Catholics, as reviewed by Ji and colleagues (26), supporting the belief that the Christian religion advances the development of emotional support among female adolescents. Importantly, these pro-social traits or emotional dispositions are typical of religiosity not only among Protestants and Catholics but also among Buddhists, Jews, and Muslims (26). According to Saroglou (27), religious people tend to attribute high importance to the value of benevolence, which is the motivational essence of emotional support. Religious people feel, think, and value things in a way that emphasizes the importance of others' interests and needs, suggesting pro-sociality as a key and universal characteristic of religious personality (27). It should be emphasized that among Protestants, Catholics, and Hindus, women are concluded to be more religiously active than men and have strongly expressed motives toward emotional support (11). In line with these findings, our data indicate that among Orthodox Christians, emotional support is the main affiliative motive and is particularly expressed among women.

A strong and an intrinsic religious orientation might be sufficient for young adults to achieve a sense of purpose in life. In turn, purpose in life is positively associated with life satisfaction and has a strong positive correlation with subjective well-being (19). Accordingly, we found that male adolescents who studied Orthodox Catechism had a higher score on the purpose of life scale then male students who studied civic education, with a loss of statistical significance between genders among adolescents who studied Orthodox Catechism. Our results are in line with data obtained by Martin Pinquart, who found small gender differences and an age-associated decline of purpose in life only in populations of older adults (28). According to Pinquart, there are no gender differences in purpose in life among young adults because most middle-aged people experience, for the first time, age-associated losses of sources of their purpose in life after they realize that some of their previous long-term goals are not realistic (28).

CONCLUSIONS

This study is the first to show that the Orthodox Catechism affects adolescents' needs for affiliative and achievement motivation in a gender-dependent manner. The Orthodox Catechism enhances the competition motives of male adolescents and has a significant influence on the development of emotional support and achievement of goals as a source of pleasure among female adolescents. Thus, the Orthodox Catechism is related to gender differences in adolescents' sense of purpose in life.

CONFLICT OF INTEREST

The authors who participated in this study declare that they do not have anything to disclose regarding funding or conflicts of interest with respect to this manuscript.

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PRESCRIBING ANTIPSYCHOTICS IN MONTENEGRO: A FOCUS GROUP ANALYSIS

Ivana Ilickovic[1], Slobodan M. Jankovic[2]
[1]Farmegra Ltd., Podgorica, Montenegro
[2]Faculty of Medical Sciences, University of Kragujevac, Serbia

PROPISIVANJE ANTIPSIHOTIKA U CRNOJ GORI: ANALIZA FOKUS GRUPE

Ivana Iličković[1], Slobodan Janković[2]
[1]Farmega Ltd, Podgorica, Crna Gora
[2]Fakultet medicinskih nauka, Univeryitet u Kragujevcu, Srbija

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ABSTRACT

Background. *Although prescribing antipsychotics to patients with schizophrenia is advised by national and/or international evidence-based practice guidelines, the implementation of the guidelines in clinical practice is still matter of concern.*

Objective. *The aim of our study was to estimate schizophrenia guideline adherence and identify eventual barriers to its implementation in Montenegro.*

Method. *This study used focus group methodology. The focus group was composed of two psychiatrists, one psychologist, one pharmacist from a community pharmacy, one pharmacist from the State reimbursement fund, one pharmacist from a drug wholesaler and the chief investigator, a clinical pharmacologist. The focus group took place in Podgorica, Montenegro, in 2013. The analysis of recordings was performed using an iterative, qualitative technique and a constant comparison method.*

Results. *The most important barriers to the implementation of evidence-based guidelines for the treatment of schizophrenia in Montenegro are non-adherence to medication, low level of psychiatrist-patient concordance, restrictive procedures for prescribing atypical antipsychotics, lack of availability of newer antipsychotics and some dosage forms, and mixing primary, secondary and tertiary care services within a tertiary care psychiatric institution.*

Conclusion. *Addressing the barriers identified by this focus group and avoiding the consequences of poor adherence would be the first steps for better mental health planning in the community.*

Key Words: *Schizophrenia, treatment guidelines, non-adherence, concordance.*

SAŽETAK

Uvod. *Mada je propisivanje antipsihotika pacijentima sa šizofrenijom regulisano nacionalnim i/ili međunarodnim smernicama dobre kliničke prakse, zasnovanim na dokazima, primena ovih smernica u praksi je daleko od željene.*

Cilj. *Cilj naše studije je bio da proceni koliko se psihijatri u Crnoj Gori pridržavaju smernica prilikom propisivanja antipsihotika, i da identifikuje eventualne prepreke za njihovu punu primenu.*

Metod. *U studiji je korišćena matodologija fokus grupe. Fokus grupu su sačinjavali dva psihijatra, jedan psiholog, jedan farmaceut iz vanbolničke apoteke, jedan farmaceut iz Fonda zdravstvenog osiguranja, jedan farmaceut predstavnik veledrogerije i glavni istraživač, klinički farmakolog. Sastanak fokus grupe je održan u Podgorici, Crna Gora, tokom 2013. godine. Analiza fonografskih zapisa sa sastanka je rađena iterativnom kvalitativnom tehnikom i metodom stalnog poređenja.*

Rezultati. *Najvažnije prepreke za punu primenu vodiča za lečenje šizofrenije su ne-pridržavanje propisanoj terapiji, nedovoljno učešće pacijenata u donošenju odluka o njihovom lečenju, komplikovana administrativna procedura za propisivanje atipičnih antipsihotika, nedostupnost novijih antipsihotika i nekih doznih formi, kao i pomešanost primarnih, sekundarnih i tercijernih zdravstvenih usluga u bolnicama namenjenim samo za tercijernu zaštitu.*

Conclusion. *Obraćanje pažnje na prepreke za primenu smernica koje je identifikovala focus grupa i popravljanje adherence pacijenata su prvi koraci ka boljem planiranju psihijatrijske zdravstvene zaštite u Crnoj Gori.*

Ključne reči: *Shizofrenija, smernice dobre kliničke prakse, slaba adherenca, učešće pacijenta.*

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Correspondence: Prof. Dr Slobodan M. Jankovic,
Faculty of Medical Sciences, University of Kragujevac,, ul. Svetozara Markovica 69, 34000 Kragujevac, Serbia
e-mail: slobodan.jankovic@medf.kg.ac.rs

INTRODUCTION

Prescribing antipsychotics to patients with schizophrenia has been advised by national and/or international evidence-based practice guidelines for decades in the majority of countries (1,2). However, implementation of the guidelines in clinical practice is still matter of concern. While there are positive examples of successful guideline implementation (3,4), there are substantial barriers in many areas, especially in developing countries (5). The nature of these barriers is diverse, ranging from psychological to cultural, managerial and financial issues. Two hundred ninety-three potential barriers to guideline implementation were described and classified in the following groups: awareness of guidelines, familiarity with the guidelines, agreement with the guidelines, self-efficiency, outcome expectations, ability to overcome the inertia of previous practice, and external barriers to conduct recommendations (6).

The identification of all relevant barriers to the implementation of guidelines is an unavoidable step in the process of designing measures to improve antipsychotic prescribing in any region. Because the barriers are culture- and country-specific (7), only research in local settings could elucidate the main obstacles for employing these guidelines (8). In Montenegro, national treatment guidelines (9) for schizophrenia were issued just 2 years ago (in 2012), and their implementation was left to practitioners. There has been no research in Montenegro up to investigate the success of this effort.

The aim of our study was to estimate schizophrenia guideline adherence and identify eventual barriers to its implementation in Montenegro, using a focus group approach.

MATERIALS AND METHODS

This study used focus group methodology. The focus group was composed of two psychiatrists and one psychologist from a mixed secondary/tertiary care health facility in Podgorica, Montenegro, one pharmacist from a community pharmacy in Podgorica, one pharmacist from the State reimbursement fund of Montenegro, one pharmacist from a drug wholesaler in Podgorica and the chief investigator, a clinical pharmacologist. The group met on one occasion (June 17th, 2013) in a physicians' room at Psychiatric Clinic, Podgorica, Montenegro. The chief investigator prepared questions for the meeting (the guide) which he used as a tool for initiating discussion. The meeting had an informal brainstorming format without any schedule limitations for expressing opinions and attitudes. The duration of the meeting was 2 hours, and it was audio-recorded.

Over the following week, the investigators analysed the recordings independently and extracted emerging themes. They then held a new meeting where they achieved a consensus about the content and conclusions. The framework approach was applied. Analysis was performed using an iterative qualitative technique and a constant comparison method. The thematic framework by which data were examined and referenced was drawn on "a priori issues" informed by the original research objectives and topics covered by the focus group guide as well as common sense categories anticipated during the process of data collection and transcribing.

As feedback validation, the investigators informed the focus group participants about the findings and conclusions, and they all agreed upon the remarks.

RESULTS

The barriers to implementation of national schizophrenia treatment guidelines that were identified by the focus group could be classified in the following theoretically informed categories: patient-related, prescriber-related, healthcare system-related and sociocultural issues.

Patient-related barriers

The participants of the focus group agreed that one of the most important patient-related barriers is low patient ***adherence*** to prescribed antipsychotic therapy. Although they said that "nobody is checking adherence of the patients to prescribed therapy", they believe that at "least 70% of the patients" were not taking their antipsychotics therapy as prescribed.

Another patient-related phenomenon could be their ***insufficient participation in decision-making process*** during prescribing. The psychiatrists said that they "...inform the patients completely, devoting a lot of manymuch time during their discharge to explaining everything about their therapy, and the patients sign documentation at the end that they are well-informed..." However, the psychiatrists also admitted that the patients with schizophrenia "...rarely take part in the prescribing decisions..."

Prescriber-related barriers

From the group discussion, it became clear that psychiatrists mostly avoid prescribing oral forms of atypical antipsychotics because "...the efficacy of risperidone and olanzapine is low in practice, although honorable professors keep saying that atypicals are very effective..." They said that atypicals "...are only helping 20-30% of patients and are suitable only for mild forms of schizophrenia..." Belief in the lack of effectiveness of risperidone and olanzapine is most likely the main reason psychiatrists avoid atypicals because because they were well-informed about and comfortable with the safety profiles. They stated, "... very frequently we observe hyperprolactinemia with risperidone, but this is easily handled by endocrinologists," and "...clozapine is effective drug, and its hematological adverse effects are rare...we check blood counts regularly..."

The discussion revealed that only two atypical antipsychotics are usually prescribed (clozapine and risperidone).

The psychiatrists had long-term experience with typical antipsychotics and their efficiency and reliability in the moderate and severe forms of schizophrenia: "...when the patient is difficult, we give him haloperidol, and we calm him safely...if we give him risperidone, he just keeps on fooling around..." Although they agreed that a combination of antipsychotics is now given rarely ("...some retired doctors gave it to everybody in the past."), they "... still have to give it in difficult patients...when one antipsychotic is not enough..." The pharmacist from a community pharmacy said that she noticed "...the patients are oversedated... probablymost likely because of co-prescribed sedatives... but maybe for some other reason, I do not know...we have to ask psychiatrists..."

Healthcare system-related barriers

The availability of atypical antipsychotics is also an important factor which may influence underprescribing these pharmaceuticals. From the focus group discussions, it was determined that there is only one available option for the treatment of acute psychosis, i.e., only one parenteral immediate-release form of antipsychotics that has marketing authorisation in Montenegro: haloperidol, a typical antipsychotic. Moreover, although psychiatrists in Montenegro make prescribing decisions autonomously, prescribing newer atypical antipsychotics is a restricted procedure which requires endorsement by a special committee composed of three psychiatrists. Finally, although atypical antipsychotics are 100% covered by the Montenegrin State Reimbursement Fund, it frequently happens that they are not available in state-owned community pharmacies, so the patients have to buy them out-of-pocket.

Special problems in prescribing antipsychotics to patients with schizophrenia are made by certain organisational issues. The psychiatrists in secondary and tertiary care health facilities are obliged by local regulations to examine and treat any patient who on the premises, regardless of whether he or she was previously evaluated by general practitioner or primary care psychiatrist; a patient or accompanying person declaring a state of emergency necessitates medical attention. This puts a huge work burden on the hospital psychiatrists, and hampers their ability to consider various treatment options, including newer atypical antipsychotics.

Sociocultural issues

There was one clear sociocultural issue that emerged from the focus groups discussion: patients with schizophrenia are unable to completely understand their situation and therapy issues, as perceived by psychiatrists, their relatives, and professionals. The pharmacist from the community pharmacy said that "...mostly relatives of patients with schizophrenia come to pharmacies for drug refills...so it is difficult to explain things to the patients..." The psychiatrists said that "...they spend more time explaining things to the patient's relatives than to the patient themselves..."

DISCUSSION

The study showed that the issue of non-adherence to prescribed antipsychotic therapy is recognised by all group members. We classified it as a patient-related barrier, however, it might be more appropriate to evaluate this issue from different perspectives; because non-adherence is adversely interrelated with both healthcare professionals and system-related issues. The identified non-adherence issue might influence physicians' prescribing choices and attitudes toward of efficacy of new treatment options. According to views expressed by focus group participants, it is difficult to determine in clinical practice whether a patient is a non-responder or non-adherent to certain antipsychotic. Prescribers may switch to another antipsychotic with false impression that the previous one (e.g., atypical) was ineffective.

Non-adherence to medications in schizophrenia patients is well-acknowledged worldwide (10, 11). The landmark Clinical Antipsychotic Trials of Intervention Effectiveness (CATIE) study revealed that 74% of patients had discontinued their medication within 18 months (11). The World Health Organization identified non-adherence as most likely "the most challenging aspect" of multidisciplinary schizophrenia treatment (10).

Although evaluating the causes of non-adherence is beyond the scope of this study, there are some features declared by participants that are already identified as predictors of poor adherence in the literature (10). As it may be concluded both from the pharmacists' and psychiatrists' input, the patients' beliefs and attitudes are discounted in decisions about the therapy. In recent literature, concordance is consistently found to be a superior concept in addressing these issues rather than the traditional paternalistic doctor- patient relationship, where the prescribing regimen is not negotiated with patients themselves. (12)

On the other hand, there are some barriers unrelated to physicians which adversely affect guideline conformance and consequently treatment outcomes. Limited access to medicines was already identified as important barrier for guideline conformance (13). Our focus group also cited a lack of availability of the whole spectrum of pharmacological treatment options for schizophrenia both in hospital (only one parenteral antipsychotic for fast tranquilisation) and in the public pharmacy settings (more complicated procedure for getting atypicals prescribed, shortages of the atypicals in public pharmacies). In guideline-conformant schizophrenia treatment services it was found that "the absence of barriers to access for pharmacological therapies likely enhances the higher conformance to these (guideline) recommendations." (13).

Although a patient-centered approach, a positive relationship with clinical staff at follow-up and guidance regarding drug use are found to be essential for improving adherence (10), there are system barriers that were identified and not addressed properly by the health authorities. Physicians emphasizised that time constraints adversely impact their capacity to provide appropriate care for their patients. The root of the problem might be traced to underuse of primary care psychiatric services and direct access to tertiary care psychiatrists for all those who think they urgently need psychiatrist.

In conclusion, the most important barriers to implementation of evidence-based guidelines for treatment of schizophrenia in Montenegro are non-adherence to medication, low level of psychiatrist-patient concordance, restrictive procedures for prescribing atypical antipsychotics, lack of availability of newer antipsychotics and some dosage forms, and mixing primary, secondary and tertiary care services within a tertiary care psychiatric institution. Addressing the barriers identified by this focus group and avoiding consequences of poor adherence would be the first steps for better mental health planning in the community.

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THE CLINICAL OUTCOME AND THERAPEUTIC TREATMENT OF A PATIENT WITH DOUBLE SERONEGATIVE MYASTHENIA GRAVIS

Ana Azanjac, Gordana Tončev, Katarina Vesić, Aleksandar Gavrilović, Tatjana Bosković Matić and Svetlana Miletić Drakulić
Clinic of neurology, Clinical center Kragujevac, Zmaj Jovina 30, 34 000 Kragujevac, Serbia

KLINIČKI ISHOD I TERAPIJA KOD BOLESNIKA SA DVOSTRUKO NEGATIVNOM MYASTHENIOM GRAVIS

Ana Azanjac, Gordana Tončev, Katarina Vesić, Aleksandar Gavrilović, Tatjana Bošković Matić i Svetlana Miletić Drakulić
Klinika za neurologiju, Klinicki Centar Kragujevac, Zmaj Jovina 30, 34 000 Kragujevac

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ABSTRACT

We reported a case of a 22-year-old male patient with swallowing difficulties and double vision. He was diagnosed on the basis of a positive pharmacological test, damage to the postsynaptic neuromuscular junction and computed tomography of the thorax. Tests for antibodies targeted to acetylcholine receptors and to muscle-specific tyrosine kinase were negative. A partial improvement in neurological findings following treatment with pyridostigmine bromide, cyclosporine and methylprednisolone was notedadministered. The patient responded favourably to plasma exchange with a withdrawal of all clinical symptoms, confirming the hypothesis that humoral factors may underlie the pathogenesis of double-negative MG .

Keywords: *seronegative Myasthenia gravis, anti-LRP4 autoantibodies*

SAŽETAK

Prikazali smo slučaj 22-godišnjeg muskarca sa kliničkom slikom otežanog gutanja i dvoslika. Bolest myasthenia gravis (MG) je dijagnostikovana na osnovu pozitivnog faramakološkog testa, pozitivnog testa neuromišićne transmisije, kompjuterizovane tomografije medijastinuma. Antitela protiv acetilholinskog receptora (AChR) i antitela protiv mišićno specifičnog receptora za tirozin kinazu (MuSK). bila su negativna. Zabeleženo je delimično poboljšanje u neurološkom nalazu na ordiniranu terapiju Piridostigminom, Ciklosporinom, kortikosteroidima. Bolesnik je imao povoljan odgovor na izmenu plazme sa povlačenjem svih kliničkih siimptoma što potvrdjuje hipotezu o učešću humoralnih faktora u patogenezi dvostruko negativne MG.

Ključne reči: *seronegativna Myasthenia gravis, anti-LRP4 antitela*

ABBREVIATIONS

AChR – acethylcholine receptor
EMNG – electromyoneurography
IgG – immunoglobulin G
MG – Myasthenia gravis
MuSK – muscle-specific tyrosine kinase
LRP4 – low density lipoprotein receptor-related protein 4
SNMG – seronegative Myasthenia gravis

INTRODUCTION

Myasthenia gravis (MG) is an autoimmune disease affecting the neuromuscular junction of skeletal muscle, and in most patients, it is caused by acetylcholine receptor (AChR)-targeted antibodies (1). These antibodies, of the IgG isotype, are detected in 80-90% of generalised MG and in 50-70% of ocular MG. The remaining 10-20% of MG patients are AChR antibody-negative (seronegative). In 40% of patients with seronegative MG, IgG antibodies targeted to the muscle-specific kinase (MuSK) are found, which do not occur in patients with seropositive MG (2). However, approximately 10% of patients with generalised myasthenia gravis show no antibodies to AChR or MuSK, findings that may be revealed using routine methods, and these patients are described as having double seronegative MG (3).

In this case study, we reported a patient with double seronegative m yasthenia gravis (SNMG), who was negative for anti-AChR and anti-MuSK antibodies.

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Correspondence:
Svetlana Miletić Drakulić Zmaj Jovina 30, 34000 Kragujevac. 063 8127637, Email: mileticdrakulic@gmail.com

CASE REPORT

In September 2011, a 22-year-old male patient was admitted to the Department of Neurology in Kragujevac, who had difficulty in swallowing and double vision. These symptoms had occurred on the day prior to admission. Neurological examination upon admission showed *diplopiae* when looking up and *dysphagia* with nasal regurgitation of liquids together with slurred speech. A fatigue test was positive. Motor power to the neck and proximal muscles of the upper and lower limbs was intact.

As part of the diagnostic procedures, a pyridostigmine test was performed, which was positive. Antibodies to acetylcholine receptors and to muscle-specific tyrosine kinase were not detected (AChR antibodies less than 0.1 nmol/l and anti-MuSK antibodies less than 0.01). Computed tomography of the thorax showed inhomogeneous density with increased fat tissue in smaller areas of the thymus. A test of neuromuscular transmission (TNT) in the n. axillaris-m. deltoideus l. dex. and n. facialis-m. nasalis l. dex. system performed upon admission was normal.

After three weeks, a second test of neuromuscular transmission in the n. axillaris -m. deltoideus l. dex system was performed. A rapid nerve conduction protocol was used at a, frequency of 1-3 Hz. That study indicated a marked decrease in amplitude of action potentials (39%-54%) at all stimulations and demonstrated accumulated damage to the postsynaptic neuromuscular junction level. Three days after admission, the patient developed proximal weakness of the neck anteflexors and proximal weakness of the musculature of the lower limbs. On admission, the following therapy was administered: tbl. Pyridostigmine bromide at a dose of 60 mg 1 to 6 hours, sol. Cyclosporine 2x1, 5 ml, amp. Methylprednisolone at a dose of 80 mg/day, three times a day infusions, amp. Prostigmin with 1.5 mg, amp. Atropine 0.5 mg and a partial improvement in neurological findings was observed. *Plasmapheresis* was then administered, and six therapeutic plasma exchanges were performed on the second day after admission. The patient responded favourably to plasma exchange, and all clinical symptoms withdrew. After a few days of plasma exchange, the symptoms reappeared and progressed in intensity with a worsening response to therapy observed. On the ninth day of hospitalisation, the patient developed respiratory insufficiency. The patient then received intravenous immunoglobulin (Intratect) at 0.4 g/kg daily for 5 days, and a partial response to therapy was observed. Control EMNG on the 21st day after symptom onset showed damage to the postsynaptic neuromuscular junction.

A thymectomy was performed during the fourth month of disease. A histological examination of thymic tissue indicated hyperplasia of the thymus.

DISCUSSION

Myasthenia gravis (MG) is an autoimmune disease characterised by a defect in synaptic transmission at the neuromuscular junction that leads to fluctuating muscle weakness (3).The term seronegative myasthenia gravis (SNMG) refers to a generalised disease without detectable anti-acetylcholine receptor (anti AChR) antibodies (4). However, approximately 10% of patients with generalised myasthenia gravis show no antibodies to AChR or MuSK and are described as having double seronegative MG (5).

Up to 66% of these patients (double seronegative patients) show a low titre of low affinity antibodies targeted to AChRs. After 12 months, 15,2% of initially seronegative patients had become seropositive, yielding a seronegativity rate of 8,2%. Of seronegative patients not receiving immunosuppressants, 38% were MuSK antibody-positive and 43% were seropositive for non-muscle autoantibodies (6). In the Serbian population, anti-AChR antibodies were detected in sera of 84,1% of patients, whereas 15,9% of patients did not show these antibodies (AChR antibody-negative patients). Of these AChR antibody-negative patients, anti-MuSK antibodies were detected in 36,4% of patients (7).

In our case, we report upon a male patient with double seronegative myasthenia gravis, who showed no detectable anti-AChR or anti-MuSK antibodies. This profile follows the distribution of gender in SNMG with anti-MuSK antibodies, where a higher prevalence is observed in females (3). Seronegative myasthenia gravis is more similar to acetylcholine receptor antibody-positive myasthenia gravis than MuSK antibody-positive myasthenia gravis with respect to clinical features, immunosuppressive treatments and thymic pathology (8); however, subtle differences exist in age at onset, maximum severity and regional distribution of myasthenic weakness (1).

In MuSK-negative patients, the clinical profile is more heterogeneous. The majority of patients show mild disease. Limb muscles are more commonly affected, and bulbar signs are both less frequent and less severe than in MuSK-positive subjects (9). Involvement of the bulbar musculature was observed in 60,1% of MuSK-positive patients, 35,2% of AChR-positive and 23,8% of double negative patients at onset. The number of patients who showed bulbar symptoms at the final observation was 29,1% in the MuSK-positive group vs. only 6,7% in the double negative group (10). In our patient, his disease began with acute bulbar musculature weakness with ptosis and diplopia. Additionally, in seronegative MuSK patients, respiratory crises are rare, whereas our patient developed respiratory failure on the ninth day of disease.

We noted a good response of our patient to pharmacological tests and a positive pyridostigmine test for neuromuscular junction. The single fibre EMG test is particularly useful in complete seronegative MG cases in which the RNS test is negative; however, this test is not used in our institution (11). A recent report indicated that more than 60% of AChR and MuSK antibody-negative MG patients show low-affinity antibodies to AChRs. These data indicate that at least some double seronegative MG patients have antibodies directed against AChR that are not detected by routine immunoprecipitation assays. These findings strongly imply that the SNMG antibodies are directed towards AChRs but that they

bind appreciably only when the AChRs are packed densely in relatively immobile clusters. Some evidence has suggested that co-expression with MuSK and Dok-7 increases further the sensitivity of the test, leaving open the possibility that intracellular modifications of either AChRs or MuSKs or changing the packing geometry of the clusters may influence the binding of these low-affinity antibodies (12).

Identification of LRP 4 as the MuSK-binding agrin receptor in skeletal muscle tissue suggested the possibility that autoantibodies for this membrane protein may underlie myasthenia gravis (13). Consistent with this hypothesis, antibodies targeted against the extracellular portion of LRP4 have recently been detected in 9 out 300 AChR and MuSK seronegative MG patients. LRP4 is a member of the low density lipoprotein receptor-related protein family of transmembrane proteins and has important functions during development and morphogenesis of limbs and the ectodermal organs, lungs and kidneys (14, 15). In adult skeletal muscle, LRP4 is specifically expressed at the neuromuscular junction. In particular, this result indicates that autoantibodies are targeted against LRP4 in approximately 50% of double seronegative MG causes, which is considerably higher than the 3% previously reported (3). All patients improved following treatment with a combination of AChE inhibitors and two or more forms of immunotherapy, and all achieved stable clinical and pharmacological remission.

Studies investigating therapeutic responses in double-negative MG support the hypothesis of humoral factors underlying the pathogenesis of this form of MG. Double seronegative groups show a higher percentage good outcomes and lower maintenance prednisolone doses than AChR- or MuSK-positive groups. The good outcome in the SN patients was unrelated to the composition of the group because even the most severely involved patients showed good outcomes (16). A classification as being seronegative MG should be reserved for non-immunosuppressed patients with generalised MG who lack muscle AChR binding, AChR modulating or MuSK antibodies at presentation and after a follow-up of at least 12 months (6). Here, in our case study, we have presented a patient with partial improvement of neurological findings upon administration of administered pyridostigmine bromide, cyclosporine and methylprednisolone. The patient responded favourably to plasma exchange, with the withdrawal of all clinical symptoms, confirming the hypothesis that humoral factors are involved in the pathogenesis of double negative MG.

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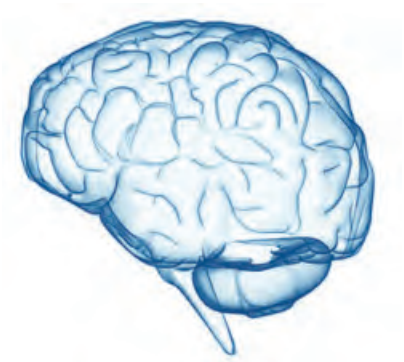

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FACULTY OF MEDICAL SCIENCES
Svetozara Markovica 69, 34000 Kragujevac, SERBIA
P.O. Box 124
Tel. +381 (0)34 30 68 00 • Tfx. +381 (0)34 30 68 00 ext. 112
e-mail: sjecr@medf.kg.ac.rs

www.medf.kg.ac.rs