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ROLE OF DEPRESSION IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE PATIENTS

Aishwarya Gowda MB[1], Sayoojya R Nair[1], Shefali Deo[1], Norah H Vanlalhriatmawii[1], Mohammed Numanuddin[2] and Khayati Moudgil*
[1] *PharmD Intern, Department of Pharmacy Practice, JSS College of Pharmacy Ooty, TamilNadu- 643 001, JSS Academy of Higher Education And Research, Mysuru, INDIA*
[2] *Clinical Pharmacologist, Breach Candy Hospital, Mumbai, INDIA*

ULOGA DEPRESIJE KOD PACIJENATA SA HRONIČNOM OPSTRUKTIVNOM BOLESTI PLUĆA

Aishvaria Govda MB[1], Saioojia R Nair[1], Shefali Deo[1], Norah H Vanlalhriatmavii[1], Mohammed Numanuddin[2] i Khaiati Moudgil*
[1]*Odeljenje za farmaceutsku praksu, JSS Farmaceutski koledž Ooti, TamilNadu- 643 001, JSS Akademija za visoko obrazovanje i istraživanje, Misuru, INDIJA*
[2]*Breach Candi Hospital, Mumbai, INDIJA*

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ABSTRACT

Chronic obstructive pulmonary disease is a progressive lung disease characterized by chronic obstruction of the lung airflow that interferes with normal bleeding and is not fully reversible. Chronic smoking is the most common risk factor for COPD causing severe cough, wheezing, labored breathing and reduced functionality. COPD not only exerts pulmonary symptoms but also has a spill over the extra pulmonary effects. Owing to the impact of the disease, it may lead to conditions like osteoporosis, cardiovascular complications as well as to psychological effects such as depression and anxiety. Such comorbidities are hidden and are not effectively treated. Depression, one of the most common hidden comorbidities is known to be present but never diagnosed. Various scales like HAM-D and Bode Index can be used to diagnose the extent of depression. Our review mainly focuses on the various studies conducted worldwide and comparing the results of the same. Based on the worldwide analysis, depression is known to affect a COPD patient at later stages and requires immediate diagnosis and appropriate treatment.

***Keywords**: COPD, Cough, Depression, HAM-D, Bode Index.*

SAŽETAK

Hronična opstruktivna bolest pluća je progresivna bolest pluća koju karakteriše hronična opstrukcija protoka vazduha u plućima koja remeti normalno krvarenje i nije u potpunosti reverzibilna. Hronično pušenje je najuobičajeniji faktor rizika za HOBP i prouzrokuje jak kašalj, zviždanje u grudima, otežano disanje i smanjenu funkcionalnost. HOBP ne samo da ispoljava simptome u plućima već dovodi do određenih posledica izvan pluća. Ova bolest može dovesti do stanja poput osteoporoze, kardiovaskularnih komplikacija kao i do psiholoških efekata kao što su depresija i anksioznost. Takvi komorbiditeti su prikriveni i ne leče se efikasno. Za depresiju, jednu od najuobičajenijih prikrivenih komorbiditeta se zna da je prisutna ali nikada dijagnostikovana. Različite skale poput HAM-D i Bod indeksa se mogu koristiti kako bi se dijagnostikovao stepen depresije. Naš rad se uglavnom fokusira na različite studije koje su sprovedene širom sveta i poredjenje rezulata. Zasnovana na svestkoj analizi, za depresiju se zna da utiče na pacijenta sa HOBP u kasnijim stadijumima i zahteva neposrednu dijagnozu i odgovarajuće lečenje.

***Ključne reči:** HOBP, kašalj, depresija, HAM-D i Bode indeks.*

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***Address for Correspondence**
Clinical Resident,
Department of Pharmacy Practice, JSS College of Pharmacy Ooty, TamilNadu- 643 001, JSS Academy of Higher Education And Research, Mysuru, INDIA
E-mail: khayatimoudgil@jssuni.edu.in
Mob-no: +91-9489238815

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a progressive lung disease, which when left untreated can lead to fatal outcomes. COPD commonly manifests as persistent coughing, wheezing, breathlessness and it reduces functional ability. It is estimated that by 2030, COPD will be the third leading cause of death worldwide [1]. COPD mainly affects the bronchus and alveoli due to excessive smoking. The epithelial cells are irreversibly damaged due to nicotine dependence. Chronic COPD initially affects only the lungs but if not treated, it can exhibit extra pulmonary effects [2]. The predominant ones are cardiovascular abnormalities like Cor Pulmonale, diabetes, weight loss and osteoporosis. Apart from these, there are certain hidden comorbidities which are underdiagnosed and left untreated such as anxiety and depression. There are various scales to diagnose these hidden comorbidities such as HAM-D scale, GDS scale and Bode Index etc. This review mainly focuses on the worldwide studies conducted to assess depression among COPD patients and also attempts to urge the diagnosis and treatment of this comorbidity. Treating depression will not cure COPD, however it will help to cope with the progression of the disease course.

How common is COPD worldwide?

COPD might not be as common as diabetes mellitus, hypertension or cancer, but as per studies, it is the third leading cause of death and by 2030, it is estimated to affect 6 million people worldwide. Almost every year, various studies are conducted to estimate the burden of the disease. In 2013, one such study estimated that 300 million people were victims of this disease worldwide [3].

As per the Indian epidemiological details as of March 2018, COPD is the second notable cause of death ranging between 2% to 22% among men and 1.2% to 19% amongst women. COPD ranks 7th among the north eastern states like– Manipur, Mizoram, Arunachal Pradesh, Nagaland, Meghalaya, Tripura and Sikkim and 4th among the remaining states of India [4]. As per a study conducted by the Indian State-level disease initiative collaborators, the disease transition from 1990 to 2016 was compared. Based on this study, the population of COPD in 1990 and 2016 were compared. COPD crude prevalence had a 29.2% increase over the years Table 1 which depicts the progressive incidence of the disease. Disease Adjusted Life Years (DALY) along with the total death percentages due to COPD were also estimated and reflected in Table 2.

Chronic Obstructive Pulmonary Disease can affect any adult age group, however, it predominantly occurs over 40 years of age. The 2011-2012 epidemiological data in Europe claimthat the prevalence of COPD in adult population over 40 years falls in the range of 15%-20% [5]. The same study also reveals the prevalence to be higher in men than in women, reason being, a high amount of smoking among men. From the past few years, the number of women with COPD is rising quickly than in men as smoking has grown progressively in women too [6]. Studies claim a faster decrease in FEV1 values in women with just a few cigarettes than in men. According to the study conducted in a large population, females were observed to have more severe COPD with an early onset (<60yr) and a greater susceptibility to COPD with lower tobacco exposure [7]. The prevalence of COPD is considerably higher in smokers than in the non-smoker group, owing to the etiology of the disease. On account of the study conducted in Israel, one fifth of the smokers of the age above 45 years developed COPD [8].

How common is depression in COPD?

Depression has always been an integral comorbidity of COPD [9]. As per a review of various studies, it demonstrates a prevalence of 6% - 80% of COPD patients [10,11,12]. COPD patients with an underlying comorbid cardiac disease, the chance of depression is between 15% - 23% [13,14] and with cancer, it ranges from 13% - 38% [15].

Etiology of depression in COPD

It is commonly believed that the relation is uni-directional i.e. COPD progressively leads to depression. However, it is now postulated that early depression at a young age, urges people towards nicotine dependence, leading to COPD at a later age. The various factors which can lead to depression due to COPD are mainly-

a. Nicotine dependence:

The mood disorders associated with COPD are largely due to the nicotine dependence [16]. The evidence shows that the inflammatory mediators are major contributors in depressive symptoms. A recent study shows that in elderly population the inflammatory biomarkers like interleukin-6 and C-reactive protein play a major role in depressive symptoms and pulmonary obstruction [17,18]. Another important mechanism of depression and suicidal thoughts is due to the nicotine dependence that shows serotonin and its metabolite levels in the cerebrospinal fluid are lesser than that of a non-smoker. The prolactin levels in smokers are also seen lesser in response to the serotonin inhibition [19].

b. Genetics:

A study on blood relatives gives an idea that the first degree relatives of population with major depression are at a higher risk if exposed to smoking. The chances of depression in smokers with the first degree relatives of major depression are 2 to 3% higher than the non-blood related group. In addition to this statement, the studies on twin and adopted groups prove that the risk of depression in monozygotic twins is high up to of 50% whereas dizygotic twins are 10 to 25% [20].

c. Chronic illness:

The major contribution of depression in patients with a chronic illness like COPD is due to loss of functionality. The loss of functionality has an attributable risk of 34% in such patients. The major reasons that lead to depression may be reduced by mobility, inability to perform day to day works and occupational activities [21],that reduces the ability to participate in family and recreational activities that were previously performed. The psychological changes because of these insults may cause depression in the population [22]. Studies have shown that patients with chronic illness have less chance for depression when they receive a good social support. Mood disorders, anxiety and depression in patients with chronic illness have less chance to develop if they get a better social support [23].

d. Effects of COPD on CNS:

COPD has a direct relationship on cerebrovascular diseases due to which COPD patients can be a vulnerable population for depression [24]. This type of depression is termed as "Vascular Depression". Studies using the Magnetic Resonance Imaging (MRI) show that the development of subcortical hyper intensities (SH) contributes a major role [25]. In conclusion, it is mentioned that the patients with hypertension or any coronary artery disease due to COPD are at a higher risk.

Are all COPD patients at a risk of depression?

A few articles were collected and analyzed regarding this topic. We found out that only one third of COPD patients suffered from depression. All analyzed data have been revealed in Table 3.

Based on all the above studies conducted to assess depression in COPD, it was commonly observed that depression is significantly present as a hidden comorbidity among COPD patients, and frequently used scales are needed to assess depression.

How to diagnose depression in COPD patients?

Primary health care professionals play an important part in screening and early detection of depression among patients with COPD [26]. However, this remains challenging for several reasons: these include the lack of a standardized approach in diagnosis, inadequate knowledge or confidence in assessing the psychological status, as well as the time constraints such as competing agendas, high patient load which leads to less time contact with the patient and the physician. In addition, the system-based barriers such as scarcity of medical records as well as the communication gap between primary health care and mental health care [27]. Furthermore, patients are hesitant to reveal the symptoms, owing to the stigma of psychiatric illness. Though there are Biomarker panel available for diagnosing depression, the usage of Structured Interview Method (SDI) questionnaire is standard due to the convenience in administering and quicker result[28]. An efficient way to detect the psychological distress is to use reliable, valid and standardized measures of the disease impact, such as questionnaires. The Diagnostic and Statistical Manual (DSM 5) requires at least five or more symptoms to diagnose the patient mainly, anhedonia or depressed mood. Apart from these, somatic and non-somatic symptoms can be considered. The DSM 5 can only classify the patient as depressed or not, however questionnaires like the Hamilton Depression Scale (HAM-D) can classify the severity of depression too[29]. The commonly and widely used questionnaire scales include:

- Beck Depression Inventory (BDI)
- Hamilton Depression Scale (HAM-D)
- Hospital Anxiety and Depression scale (HAD)

Hospital Anxiety and Depression Scale (HADS):[30]

The HADS was constructed in 1983 by Zigmond and Snaith. It is a self-administered questionnaire which is used to measure the symptoms of depression and general anxiety in patients of non-psychiatric hospital clinics. 10-15 minutes are required to administer the questionnaire and there are no items regarding somatic symptoms to prevent the overlapping symptoms between the somatic illness and mood disorder. Both inpatient and outpatient can benefit from this scale. Both anxiety (HAD-A) and depression (HAD-D) can be evaluated through 7 items each.

The scores obtained are categorized for both depression and anxiety as follows:

0-7 = Normal
8-10 = Borderline abnormal (borderline case)
11-21 = Abnormal (case)

Beck Depression Inventory (BDI):

The BDI is 21 items self-administered questionnaire for measuring the severity of depression in normal and psychiatric patients. 10 minutes are required to complete the questionnaire. The highest possible score is 63 [31].

Based on the score obtained, the score of depression can be interpreted as follows:

1-10= Normal
11-16= Mild mood disturbance
17-20= Borderline clinical depression
21-30= Moderate depression
31-40= Severe Depression
Over 40= Extreme depression

Hamilton Depression Rating Scale (HDRS):

The HDRS also known as the HAM-D scale is a widely used scale for assessment of the severity and change in depression symptoms of depression in elderly patients. There are 17 items ($HDRS_{17}$) in the original version relating to the

symptoms of depression experienced over the past week. About 20-30min are required to administer the questionnaire to the subject. The scale emphasizes melancholic and physical symptoms of depression and it is used more commonly in a hospital inpatient. The drawback of the HDRS is that atypical symptoms of depression are not assessed (e.g., hyperphagia, hypersomnia) [32].

The scores can be categorized and interpreted on the following basis:

0-7= Normal
8-13= Mild Depression
14-18= Moderate Depression
19-22= Severe Depression

In general, there are different types of scales available to measure the level of depression. But, when it comes to clinical practice, the selection of the scale depends upon the clinician's level of comfort with the scale and availability of time. Some clinicians choose to use the self-rating scales, while other clinicians prefer to ask directly the patients about the symptoms during the visit. Therefore, clinicians play a major role in the selection of the scale depending upon the limitation and strength of a few commonly used scales [33].

MANAGEMENT

There are no specific treatment guidelines for COPD with comorbid depression. Antidepressants were found to be effective with better quality of life. The treatment depends on the patients who fall in this category of mild, moderate or severe from the results of the scales. The patients are treated accordingly with pharmacological or non-pharmacological treatment.

Pharmacological

Antidepressants: Antidepressants are commonly used for the treatment of anxiety and depression. According to the study done by AM Yohannes and GS Alexopoulos, these are the drugs used in their study [34].

a) Tricyclic antidepressants (TCA):

i. **Nortriptyline:** This drug shows effectiveness in depression, anxiety and other respiratory diseases when compared with placebo. The study used 0.25mg/kg of body weight and the weight increased weekly up to 1mg/kg.
ii. **Desipramine:** This drug was initiated with a dose of 25mg and increased weekly to a targeted dose of 100mg/kg. There was no significant improvement between the placebo and the control group.
iii. **Doxepin:** This drug was initiated with a dose of 25mg/kg till maximum 105mg/kg for 6 weeks. But, there was no significant improvement between the two groups.
iv. **Protriptyline:** This drug shows anticholinergic side effects like dryness of mouth; it does not show any clinical improvement given as a dose of 10mg/day for 12 weeks between the two groups.

b) Selective Serotonin Reuptake inhibitors (SSRIs):

i. **Fluoxetine:** It was given as a dose of 20mg daily for 8 weeks. When compared with the placebo and the control group, 67% had a response to the depressions score scales. But, there was no significant difference between the two groups.
ii. **Sertraline:** There were no significant improvements in depression patients as well as in COPD patients. A dose of 12.5mg/kg was initiated till maximum of 100mg/kg for two weeks. But, a retrospective done with a dose of 25mg to 100mg in 7 patients with chronic respiratory disease having depression shows an improvement clinically and physically.
iii. **Paroxetine:** A dose of 20mg/kg daily over 12 weeks to the end stage COPD with comorbid depression. Hence, there was a clinical improvement in the controlled when compared with the placebo.

Non-pharmacological

i. **Cognitive-behavioral therapy (CBT):** It is a form of psychotherapy used for intervening patients in such a way that will boost their mood, emotional thoughts, resolve problems by encouraging them to challenge distorted cognitions and change destructive patterns of behavior [35]. Anja Fritzsche et al study shows that self-help educational program revealed significant decreases in the Psychosocial and Total Sickness Impact Profile (SIP) scores which indicate improvements in the disease's impact on everyday life and their health status [36].
ii. **Pulmonary rehabilitation:** According to Kurt B Stage et al, pulmonary rehabilitation has been used for COPD patients for co-morbid depression and anxiety by exercising the patients' breathing related problems and psychological education to help them improve the symptoms and better quality of life.
iii. **Singing therapy:** A randomized community-based study done by Hua Liu et al, in China concluded that group singing decreases depression, puts them into a happy mood and it was found to be effective in all patients improving their quality of life [37].

Table 1: Change in prevalence of COPD from 1990 to 2016 in Indian population

Year	1990	2016	Percentage change 1990-2016
India (1316 million)	3254 (3124 to 3385)	4204 (4032 to 4378)	29·2% (27·9 to 30·4)

Table 2: Percentage of death and DALY due to COPD in India as of 2016

COPD	Male	Female	Both sexes
Percentage of total deaths in India 2016 (95% UI)	8.7%	8.6%	8.7%
Percentage of total DALY in India, 2016 (95% UI)	5.2%	4.4%	4.8%

Table 3: Analyzed data from various studies

AUTHOR	SAMPLE SIZE (patients)	ASSESSMENT TOOLS	OUTCOME/RESULTS
Richard W. Light et al,1985(10)	45 COPD	Beck depression inventory (BDI)	19 had significant depression
Mark E. Kunik et al, 2005(20)	557 COPD	Beck Depression Inventory (BDI)	Minimal: 156 patients Mild: 121 patients Moderate: 147 patients Severe: 133 patients
Sajal De, 2010(21)	100 COPD	Patient Health Questionnaire (PHQ-9)	17 patients with severe depression
Niresh Thapa et al, 2017(22)	93 COPD	Beck Depression Inventory (BDI)	Minimal: 4 patients Mild: 14 patients Moderate: 41 patients Severe: 34 patients
Debabani Biswas et al, 2017(23)	75 COPD	Hamilton Depression Scale (HAM-D)	37 patients of COPD with depression Mild to Moderate: 37 patients Severe: 4 patients
Ruchi Dua et al, 2018(2)	128 COPD	Hospital Anxiety and Depression scale (HAD)	29 patients with depression
Abhishek Agarwal et al, 2018(24)	150 COPD	Hamilton Depression Scale (HAM-D)	46 patients of COPD with depression Mild: 18 patients Moderate: 26 patients Severe: 2 patients
Sujeer Khan, 2017(25)	120 COPD	Beck's Depression scale	Mild: 6 patients, Moderate: 18 patients Severe: 42 patients
Kang Xu, 2018(26)	53 COPD	HAM-D/Hamilton Depression Rating Scale (HDRS)	40 patients with symptoms of depression
Marc Miravitlles et al, 2018(27)	684 COPD	Beck Depression Inventory (BDI)	104 patients with severe depression
Josep Montserrat - Capdevila et al, 2018(28)	512 COPD	Hospital Anxiety and Depression scale (HAD)	64 patients with depression
Tian Xiao et al, 2018(29)	275 COPD	Hospital Anxiety and Depression Score - Depression (HAD- D)	13.1% depression

CONCLUSION

This study clearly shows that there exists a link between COPD and depression. So, we have to focus on the comorbidities and their management prior to their severity. More studies should be conducted and the appropriate therapy should be implemented in the future.

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CONFLICT OF INTEREST

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A STUDY TO COMPARE HYPOLIPIDEMIC EFFECTS OF ALLIUM SATIVUM (GARLIC) ALONE AND IN COMBINATION WITH ATORVASTATIN OR EZETIMIBE IN EXPERIMENTAL MODEL

Sudipta Sil[1],Uttam Kumar Roy[2], Supreeti Biswas[3], Purnendu Mandal[4], Keya Pal5
[1]*North Bengal Medical College and Hospital, Department of Pharmacology, India*
[2]*Raiganj Govt. Medical College and Hospital, Department of Pharmacology, India*
[3]*Burdwan Medical College and Hospital, Department of Pharmacology, India*
[4] *Raiganj Govt. Medical College and Hospital, Department of Pharmacology, India*
[5]*Burdwan Medical College & Hospital, Department Of Biochemistry, India*

STUDIJA ZA UPOREĐIVANJE HIPOLIPIDEMIJSKIH EFEKATA ALLIUM SATIVUM (BELI LUK) I U KOMBINACIJI SA ATORVASTATINOM ILI EZETIMIBOM U EKSPERIMENTALNOM MODELU

Sudipta Sil[1],Uttam Kumar Roy[2], Supreeti Biswas[3], Purnendu Mandal[4], Keya Pal5
[1] *Medicinski koledž i bolnica Severnog Bengala, Odeljenje za farmakologiju, Indija*
[2] *Vlada Raiganj. Medicinski fakultet i bolnica, Odeljenje za farmakologiju, Indija*
[3] *Medicinski fakultet i bolnica Burdvan, Odeljenje za farmakologiju, Indija*
[4] *Vlada Raiganj. Medicinski fakultet i bolnica, Odeljenje za farmakologiju, Indija*
[5] *Medicinski fakultet i bolnica Burdvan, Odeljenje za biohemiju, Indija*

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ABSTRACT

Background: Dyslipidemia is a major cause of atherosclerosis and atherosclerosis induced conditions. Atorvastatin is an effective drug for dyslipidemia and reduce the risk of cardiovascular morbidity and mortality. Ezetimibe is used as an adjunct to statins hypercholesterolemia. Garlic is known for the hypolipidemic effect in traditional medicine. There are very limited studies comparing the additive effects of Allium sativum on atorvastatin and ezetimibe. Aims: To compare the additive hypolipidemic effects of Allium sativum with atorvastatin and ezetimibe. Setting and Design: The experimental study was done in Department of Pharmacology and Biochemistry, Burdwan Medical College, Burdwan from February 2014 - October 2015. Material and Methods: Dyslipidemia rat by (induced by atherogenic diet) were randomized into five groups of six rats in each and each cage was labelled for identification of different groups and treated with drugs (atorvastatin, ezetimibe, garlic homogenate, atorvastatin + garlic homogenate, ezetimibe + garlic homogenate) for twelve weeks and assessment of lipid profiles were done. Change of parameters checked for any significant difference by appropriate statistical tests. Results: Significant TC (Total Cholesterol) & TG (Triglyceride) concentrations reduction were maximum among ezetimibe group (51% and 47%) respectively. LDL (Low Density Lipoprotein) & VLDL (Very Low-Density Lipoprotein) concentrations reduction were maximum (62% and 26%) among combination of atorvastatin and garlic group when compared to other treatment groups. HDL (High Density Lipoprotein) concentration was maximally increased (31%) among combination ezetimibe and garlic group which was also statistically significant. Conclusion: Garlic have significant hypolipidemic effect when used in combination with atorvastatin and ezetimibe.

Keywords: *atorvastatin, dyslipidemia, experimental, ezetimibe, garlic.*

SAŽETAK

Dislipidemija je glavni uzrok ateroskleroze i indukovane ateroskleroze uslovi. Atorvastatin je efikasan lek za dislipidemiju i smanjuje rizik od kardiovaskularnog morbiditeta i mortaliteta. Ezetimib se koristi kao dodatak leku statini hiperholesterolemija. Beli luk je poznat po hipolipidemijskom dejstvu u tradicionalnoj ishrani lek. Postoje vrlo ograničene studije u kojima se upoređuju aditivni efekti Alliuma sativum na atorvastatinu i ezetimibu. Ciljevi: Uporediti aditivne hipolipidemijske efekte Allium sativum sa atorvastatin i ezetimib. Eksperimentalna studija rađena je u Odeljenju za Farmakologija i biohemija, Medicinski koledž Burdvan, Burdvan od februara 2014. do oktobra 2015. godine. Materijal i metode: Dislipidemija pacova (indukovana aterogenom ishranom) randomizirana je u pet grupa od po šest pacova u svakom kavezu, koji su označeni za identifikaciju različitih grupa i lečeni lekovima (atorvastatin, ezetimib, homogenat belog luka, atorvastatin + homogenat belog luka, ezetimib + beli luk homogenat) tokom dvanaest nedelja pri čemu je urađena procena lipidnih profila. Promena parametara proverena na svim značajnim razlikama odgovarajućim statističkim testovima. Rezultati: Značajno smanjenje koncentracije TC (ukupnog holesterola) i TG (triglicerida) bilo je maksimalno među grupama ezetimiba (51% i 47%). Smanjenje koncentracije LDL (lipoproteina male gustine) i VLDL (lipoproteina vrlo male gustine) bilo je maksimalno (62% i 26%) u kombinaciji kombinacije atorvastatina i belog luka u poređenju sa drugim lečenim grupama. Koncentracija HDL (lipoprotein visoke gustine) je maksimalno povećana (31%) među kombinacijama ezetimiba i belog luka, što je takođe bilo statistički značajno. Zaključak: Beli luk ima značajan hipolipidemijski efekat kada se koristi u kombinaciji sa atorvastatinom i ezetimibom.

Ključne reči: *atorvastatin, dislipidemija, eksperimentalni, ezetimibe, beli luk.*

Corresponding author:
Dr.Uttam Kumar Roy
Address- Dilip Das Sarani, Hill View Main, PaschimBardhaman,
Pin-713304, West Bengal, India
Phone numbers: 9434163225
E-mail address: uroy951@gmail.com

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INTRODUCTION

Dyslipidemia is a major cause of atherosclerosis and atherosclerosis-induced conditions. Both genetic disorders and lifestyle (sedentary behaviour and diets high in calories, saturated fat, and cholesterol) contribute to the dyslipidemias seen in countries around the world [1].

Atorvastatin competitively inhibits HMG-CoA reductase and reduces conversion of HMG-CoA to mevalonate in cholesterol biosynthesis. Ezetimibe used as an adjunct to statins in inhibits absorption of cholesterol (and of plant stanols) from the duodenum.

With the growing interest in screening plants for medicinal activity, reports of hypolipidemic effects of various plants are increasingly encountered in literature. The usage of herbal therapies along with prescription and over the counter medications is increasing day by day. In both animal and human studies, Allium sativum has been reported to lower cholesterol, triglycerides, and change blood lipoproteins.[2-12]

There are gaps in existing knowledge comparing the additive effects of Allium sativum on atorvastatin and ezetimibe in dyslipidemia. Keeping the above facts in view, the present work was undertaken to compare the hypolipidemic effect of Allium sativum alone and in combination with atorvastatin[13] or ezetimibe in dyslipidemia albino rats induced by atherogenic diet.[14-16]

AIMS

To establish the hypolipidemic effect of Allium sativum, Atorvastatin and Ezetimibe when used as single agent as well as in combination.

SUBJECTS AND METHODS

Subjects: Albino rats of both sexes (100-160g) procured from institutional animal house for study.

Drugs and chemicals:

1. Tablet Atorvastatin -Stator-10, Batch no: STA4009 of atorvastatin (Abbott Healthcare Pvt Ltd)
2. Tablet Ezetimibe- Ezentia and Batch no: SKN 0060 of ezetimibe (Sun Pharma Laboratories Ltd)
3. Carboxy methyl cellulose (CMC) sodium salt, medium viscosity (25-350 cps), LOT: S36561201, CAS: 9004-32-4, S: 22-24/25, F: 3, LobaChemie Private Limited)
4. Garlic homogenate
5. Cholesterol powder (Thermo Fisher Scientific India Pvt Ltd), CAS no: 57-88-5, molar weight: 386.66, Prod no: 12312, LOT no: 63557005-1
6. Distilled water.

All chemicals used for biochemical analysis of blood parameters were available in Department of Biochemistry.

Study setting**:** Planning of the protocol and animal experiment was carried (from February 2014 - October 2015) in the Department of Pharmacology, Burdwan Medical College, Burdwan and biochemical part in the Department of Biochemistry.

Ethical considerations: Study was started only after approval from IAEC

[BMC/AEC/10(2) /2013, DATED 16/12/2013]. The guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), Animal Welfare Division, Ministry of Environment, Govt. of India for the care and use of the laboratory animals were strictly followed throughout the experimental procedure. Albino rats of either sex with weight of 100 – 160 grams were included in our study. Rampant rats and dietary pattern out of control were excluded from our study.

In accordance with principles of 3R's[17], Replacement was not possible, Reduction with the use of minimum number of experimental animals and Refinement by provision of species appropriate housing and climate conditions (CPCSEA guidelines) , minimum invasive procedures, no animals were sacrificed. Care was taken for Rehabilitation.

Preparations and dose calculation

All drugs administered by gavage feeding. Atherogenic diet/ high fat diet (HFD) was prepared from cholesterol powder.[18] Cholesterol requirements of each albino rat was calculated at a dose of 125mg/kg body weight/day by extrapolation from rabbit dose (0.5g/kg body weight daily) [19] and total requirement made up in coconut oil. Then1%(w/v) cholesterol solution was prepared by mixing 5 g dry powder in 500 ml coconut oil and dose came to 1.25 mL/100 g/day. Atorvastatin dose calculated by extrapolation from maximum human dose (80 mg/day)[20]came to 7.2 mg/ kg body weight/day. [19] So, when one atorvastatin (80 mg) was mixed with 100 ml 2% CMC suspension (w/v), the dose of atorvastatin for a 100g rat was 0.9 ml/day.

Ezetimibe dose calculated by extrapolation from maximum human dose (10 mg/day)[21] came to 0.9 mg/ kg body weight/day. So, when one ezetimibe tablet (10 mg) was mixed with 100 ml 2% CMC suspension (w/v), the dose of ezetimibe for a 100g rat was 0.9 ml/day.

Preparation of garlic homogenate -The fresh bulbs of Allium sativum were identified and authenticated(Office Of Scientist-'D', Central National Herbarium, Ministry of Environment And Forests, Botanical Survey of India, P.O.-Botanic Garden, Howrah-711103, Government of India, No: BSI/CNH/SD/TECH./2014, dated 19.03.2014).

Garlic 50gm was homogenized in 100 ml of cold distilled water. After centrifugation and filtration, concentration of this garlic preparation was 500 mg/ml, based on the weight

of the starting material (50g/100 ml)[22.] Garlic homogenate was administered as a dose of 500mg/kg[23], which came to 0.1 ml for a 100grat.

Experimental design:

Albino rats of both sexes (100-160g) procured from institutional animal house were acclimatized for 3 weeks [24] with free access to food and water (ad libitum). The rooms were equipped with lighting, conditioning, moisture and heat control. After acclimatization, rats were kept on normal diet for 6 weeks. After that period all the rats were kept on overnight fasting with water ad libitum. The fasting rats on the next day properly weighed. The standard cholesterol diet with normal diet and water was administered for 6 weeks to induce dyslipidemia. At the end of the 6 weeks blood was withdrawn (all the rats were kept on overnight fasting with water ad libitum before blood withdrawal) from the tail vein to analyze [25] lipid profiles to confirm the induction of dyslipidemia.

Now the dyslipidemia rats were randomized (computer generated in Excel) into five groups of six rats in each and treated with drugs orally (along with HFD) accordingly for 12 weeks.[26] Allocated groups were labeled as Group-I: atorvastatin, Group-II: ezetimibe, Group-III : garlic homogenate,Group-IV : combination of atorvastatin and garlic homogenate and Group-V : combination of ezetimibe and garlic homogenate. At the end of 12 weeks, blood was withdrawn in the same manner from the tail vein to analyze lipid profiles to observe the hypolipidemic effects of these drugs. Assessment of lipid profiles (TC, TG, LDL, HDL, and VLDL) done (Table 1,2,3).

Measurement of parameters:

TC, HDL cholesterol and TG concentrations were measured by enzymatic methods. Calculation of LDL cholesterol concentration

LDL cholesterol = TC— [HDL cholesterol + (TG/ 5)]

The VLDL is estimated by dividing the plasma TG by 5. This formula is reasonably accurate if the fasting TG level does not exceed 200 mg/dL, it cannot be used if the TG level is >400 mg/dL. Oral feeding and blood collection procedure from lateral tail vein of rat were done properly.[27] Data analysis was done using Microsoft Excel and IBM SPSS version 17 software.

RESULTS

In our study, there were 5 groups which were fed high fat diet and were treated with atorvastatin, ezetimibe, garlic, combination of atorvastatin and garlic and combination of ezetimibe & garlic. As there were different groups and concentrations of TC, TG, HDL, LDL, VLDL were measured after treatment, we analyzed the data with mixed analysis of variance (ANOVA), as the primary purpose of mixed ANOVA is to understand if there is any interaction between two independent variables on the dependent variable, that is to help us to determine whether there are differences among groups over time. Here one between-subject factor is intervention (5 groups), one within subject factor (time) and six dependent variables those are continuous.

In summary we wished to know whether the concentrations differ significantly over time to different interventions (between groups). There was a statistically significant interaction between the intervention and time on lipid profiles. As we had a statistically significant interaction, reporting the main effects could be misleading and we wanted to determine the difference between groups at each level of time and within groups (known as simple main effects).

Simple main effect of group (between groups)

Testing for the simple main effects for group means testing for differences in lipid profiles between groups at each level of time.

Simple main effect of time (within group)

It means testing for differences in lipid profiles within a group at two different time points.

TOTAL CHOLESTEROL (TC)

There was a statistically significant interaction between the intervention and time on Total cholesterol concentration, $F(4, 25) = 48.097$, $p < .05$, partial $n2 = .885$. So, simple main effects were done.

Simple main effect of group (between groups)

There was a statistically significant difference in total cholesterol concentration between different groups after drug treatment, $F(4, 25) = 836.714$, $p<.05$, partial $n2=.993$.

Total cholesterol concentration was increased from ezetimibe group (124.17 ± 2.93) to atorvastatin + garlic group (134.17 ± 1.84), atorvastatin group (146.33 ± 6.59), ezetimibe + garlic group (207 ± 1.27) and garlic group (212 ± 2.28) in that order . Games Howell post-hoc analysis (as there was no homogeneity of variances) revealed that this increase was statistically significant for all groups.

Simple main effect of time (within group)

There was a statistically significant effect of time on total cholesterol concentration for all the groups and for all the group's total cholesterol concentration was significantly reduced after treatment.(Table 1)

TRIGLYCERIDE (TG)

There was a statistically significant interaction between the intervention and time on Triglyceride concentration, $F(4, 25) = 87.256$, $p < .05$, partial $n2 = .933$. So, simple main effects were done. There was a statistically significant difference in Triglyceride concentration between different groups after drug treatment, $F(4, 25) = 318.944$, $p<.05$, partial $n2=$

.981.Triglyceride concentration was increased from ezetimibe group (117.33 ± 1.21) to atorvastatin + garlic group (117.50 ± 1.52), atorvastatin group (136.33 ± 5.68), ezetimibe + garlic group (157.83 ± 1.47) and garlic group (171.33 ± 4.08), in that order .Games Howell post-hoc analysis (as there was no homogeneity of variances) revealed that this increase was statistically significant among all groups except among ezetimibe and atorvastatin plus garlic group.

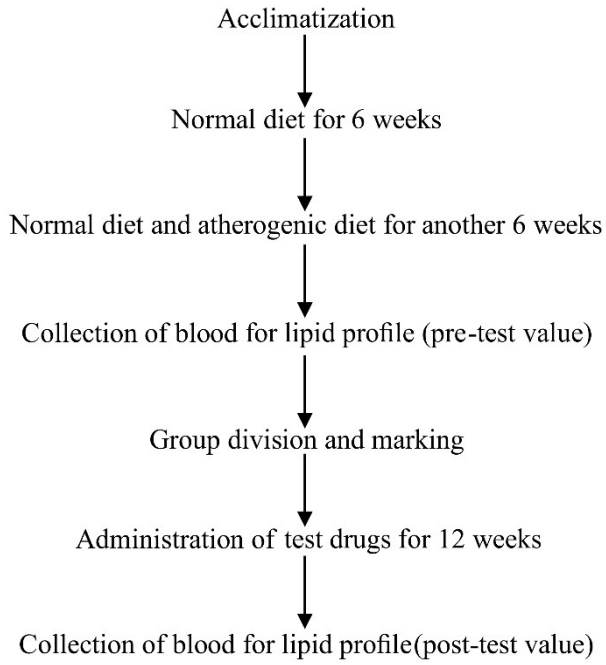
Simple main effect of time (within group)

There was a statistically significant effect of time on triglyceride concentration for all the groups and for all the groups, triglyceride concentration was significantly reduced after treatment. (Table 1, Figure 2)

Table 1. Change in Cholesterol and Triglyceride (mg/dl)

	CHOLESTEROL		Percentage reduction	TRIGLYCERIDE		Percentage reduction
	PRE	POST		PRE	POST	
Atorvastatin	255.17±19.09	146.33±6.59 P=0.016	43	226.83±7.17	136.33±5.68 P=0.015	40
Ezetimibe	251.67±13.47	124.17±2.93 P=0.001	51	220.33±1.86	117.33±1.21 P=0.001	47
Garlic	252.50±13.11	212±2.28 P=0.032	16	222.17±3.97	171.33±4.08 P=0.048	23
Atorvastatin+Garlic	264.33±20.38	134.17±1.84 P=0.001	49	221.50±2.43	117.50±1.52 P=0.001	47
Ezetimibe +Garlic	259±12.81	207±1.27 P=0.031	20	224.17±6.68	157.83±1.47 P=0.042	30
P value Between group	0.141	<0.001		0.201	<.001	

Figure 1. Study flow chart



HIGH DENSITY LIPOPROTEIN (HDL)

There was a statistically significant interaction between the intervention and time on HDL concentration, F (4, 25) = 34.308, p < .05, partial n2= .469. So, simple main effects were done.

Simple main effect between groups group

There was a statistically significant difference in HDL concentration between different groups after drug treatment, F (4, 25) = 12.33, p<.05, partial n2= .664.

HDL concentration was decreased from ezetimibe + garlic group (52 ± 1.41) to atorvastatin + garlic group (49.67 ± 1.37), garlic group (49 ± 1.67), ezetimibe group (48 ± 1.41), and atorvastatin group (46.17 ± 1.60) in that order.

Tukey HSD post-hoc analysis (as there was homogeneity of variances) revealed that this decrease was statistically significant among

1) atorvastatin group and garlic group,
2) atorvastatin group and atorvastatin plus garlic group,
3) atorvastatin group and ezetimibe plus garlic group,
4) ezetimibe group and ezetimibe plus garlic group and
5) garlic group and ezetimibe plus garlic group.

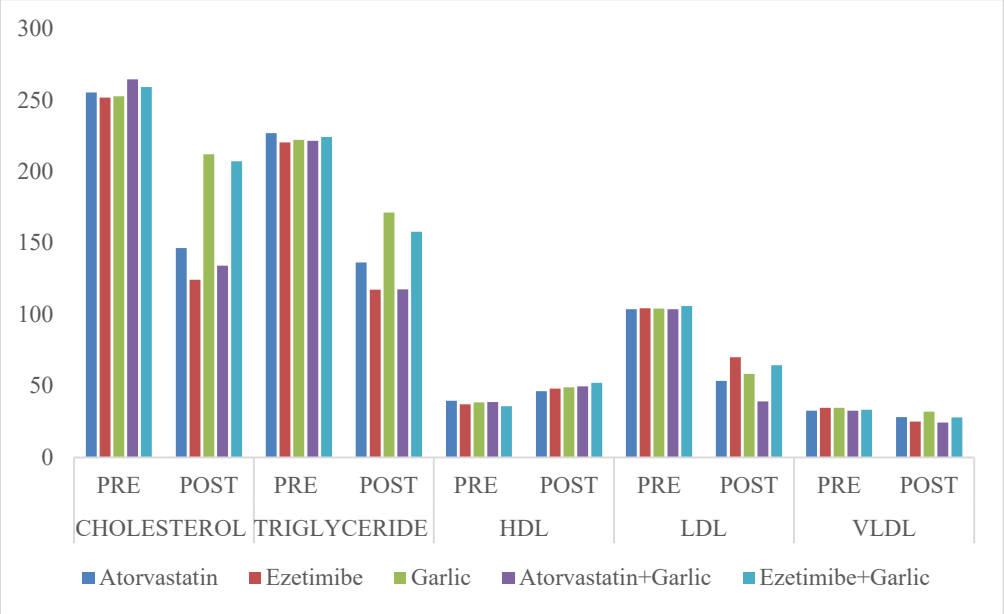
Simple main effect of time

There was a statistically significant effect of time on HDL concentration for all the groups and for all the groups HDL concentration was significantly increased after treatment.

Table 2. Change in HDL(mg/dl)

	HDL		Percentage change
	PRE	POST	
Atorvastatin	39.50±3.08	46.17±1.6 P=0.049	14
Ezetimibe	37.17±2.48	48±1.41 P=0.04	23
Garlic	38.50±3.27	49±1.67 P=0.040	21
Atorvastatin +Garlic	38.67±3.01	49.67±1.37 P=0.04	22
Ezetimibe +Garlic	35.83±2.23	52±1.41 P=0.003	31
P value Between group	0.220	<0.001	

Figure 2. Change in Biochemical Parameters



LOW DENSITY LIPOPROTEIN (LDL)

There was a statistically significant interaction between the intervention and time on LDL concentration, F (4, 25) = 44.721, p < .05, partial n2= .877. So, simple main effects were done.

Simple main effect between groups

There was a statistically significant difference in LDL concentration between different groups after drug treatment, F (4, 25) = 56.051, p<.05, partial n2= .90.

LDL concentration was increased from atorvastatin + garlic group (39 ± 2.53) to atorvastatin group (53.33 ± 6.56),

garlic group (58.33 ± 1.21), ezetimibe + garlic group (64.33 ± 1.37) and ezetimibe group (70 ± 4.73) in that order.

Games Howell post-hoc analysis (as there was no homogeneity of variances) revealed that this increase was statistically significant among all groups except among 1) atorvastatin group and garlic group, 2) ezetimibe group and ezetimibe plus garlic group.

Simple main effect of time

There was a statistically significant effect of time on LDL concentration for all the groups and for all the groups LDL concentration was significantly reduced after treatment. (Table 3, Figure 2)

Table 3. Change in LDL and VLDL(mg/dl)

	LDL		Percentage reduction	VLDL		Percentage reduction
	PRE	POST		PRE	POST	
Atorvastatin	103.50±2.43	53.33±6.56 P=0.001	48	32.67±0.81	28.17±2.79	14
Ezetimibe	104.17±2.14	70±4.73 P=0.014	33	34.67±2.16	25±0.89 P=0.041	28
Garlic	104.±1.41	58.33±1.21 P=0.001	44	34.67±2.16	31.83±1.17 P=0.053	8
Atorvastatin +Garlic	103.50±2.43	39±2.53 P=0.001	62	32.67±0.82	24.33±2.25 P=0.041	26
Ezetimibe +Garlic	105.83±1.3	64.33±1.37 P=0.015	39	33.17±0.75	27.83±1.47 P=0.048	16
P value Between groups	0.271	<0.001		0.634	<0.001	

VERY LOW-DENSITY LIPOPROTEIN (VLDL)

There was a statistically significant interaction between the intervention and time on VLDL concentration, F (4, 25) = 7.659, p < .05, partial n2= .551. So, simple main effects were done.

Simple main effect of group

There was a statistically significant difference in VLDL concentration between different groups after drug treatment, F (4, 25) = 15.549, p<.05, partial n2= .713.

VLDL concentration was increased from atorvastatin + garlic group (24.33 ± 2.25) to ezetimibe group (25 ± .89), ezetimibe + garlic group (27.83 ± 1.47), atorvastatin group (28.17 ± 2.79) and garlic group (31.83 ± 1.17) in that order.

Games Howell post-hoc analysis (as there was no homogeneity of variances) revealed that this increase was statistically significant among 1) ezetimibe group and garlic group, 2) ezetimibe group and ezetimibe plus garlic group, 3) garlic group and atorvastatin plus garlic group.

Simple main effect of time

There was a statistically significant effect of time on VLDL concentration for all the groups and for all the groups VLDL concentration was significantly reduced after treatment. (Table 3, Figure 2).

DISCUSSION

Garlic is well known in traditional and folklore medicine for its hypolipidemic effect. Many in vitro and in vivo studies revealed organosulphur compound of Garlic cause inhibition of the hepatic activities of lipogenic and cholesterogenic enzymes that are thought to be the genesis for dyslipidemias, increased excretion of cholesterol and suppression of LDL-oxidation.[28-30]

There are very limited studies [14,15,16] comparing the additive effects of Allium sativum on these 2 drugs (atorvastatin and ezetimibe). The present work was undertaken to compare the hypolipidemic effect of Allium sativum alone and in combination with atorvastatin or ezetimibe in albino dyslipidemia rats (induced by atherogenic diet). After treating with different drugs all parameters (TC, TG, HDL, LDL and VLDL) were recorded and compared with revised baseline values obtained after giving high fat diet. Here we found that, these values were significantly reduced compared to revised baseline values (HDL was increased) and there was significant difference among all the group.

Atorvastatin (7.2 mg/kg body weight/day) was able to significantly reduce TC (43%), TG (40%), LDL (48%), VLDL (14%) and increase HDL (14%) after 12 weeks of drug treatment in animal models. Our result was supported by various previous studies. Kumar DS et al had showed that atorvastatin (1.2 mg/kg body weight) had significantly reduced TC, TG, LDL, VLDL and increased HDL level after 9

weeks of treatment in animal models. The same type of result was also shown by Krause BR and Newton RS in another study. Rajyalakshmi G et al had showed that plasma TC, TG and LDL levels were significantly reduced, and HDL levels were increased after treatment with atorvastatin in animal models [31-33]. Significant reduction in the level of serum cholesterol, triglyceride, LDL, VLDL and increase in HDL level by HFD+ standard atorvastatin drug was shown by Rajendran Rand Krishnakumar E. The same result was also shown in few another studies where atorvastatin was used 1mg/kg body weight and 10mg/kg body weight [34-37].

In our study we had found that, ezetimibe (0.9 mg/kg body weight/day) was able to significantly reduce TC (51%), TG (47%), LDL (33%), VLDL (28%) and increase HDL (23%) after 12 weeks of drug treatment in animal models . Mohammadi A et al had shown in a study that chow + 0.005% (w/w) Ezetimibe Compared with hypercholesterolemic control rats significantly decreased LDL ($P<0.05$) and TC ($P<0.05$) after one-month treatment.[13] The results of many experiments have shown that ezetimibe decline LDL by about 20% when administered alone.[22] Studies have shown that ezetimibe decrease the levels of serum triglyceride by 1.7 to 9.4%, but this reduction was not noticeable always.[15]

Garlic homogenate was able to significantly reduce TC (16%), TG (23%), LDL (44%), VLDL (8%) and increase HDL (21%) after 12 weeks of drug treatment in animal models in our study. This is also supported by many other studies. Yeh YY et al had showed that hypocholesterolemia effect of water-soluble sulfur compounds of garlic is due to the inhibition of cholesterol synthesis pathway, while the inhibition by lipid-soluble extracts of garlic results from the strong toxic properties of this lipid-extract. They also proved that water extract inhibited cholesterol synthesis more than methanol and petroleum extractable fraction .[38] Elmahdi B et al had showed in a study that adding 8% raw garlic to rat atherogenic diet (diet containing 2% cholesterol), declined serum total cholesterol and LDL levels and increased HDL .[39]Aouadi et al. also, showed that addition of 10% fresh garlic to atherogenic diet (diet containing 2% cholesterol) led to significant reduction in LDL levels, and raised HDL levels in rat model . [40] In a study, HDL significantly increased in garlic extract-treated animals when compared with normal chow diet. A study [38] was conducted to evaluate the effectiveness of aged black garlic (ABG) extract in alleviating obesity and hyperlipidemia, and regulating antioxidant properties in rats fed high-fat diet by Kim I et al and they had shown that administration of ABG extract had improved the body weight gain and dyslipidemia through the suppression of body lipid profiles and antioxidant defense system.[4 1]

Atorvastatin plus garlic group were able to significantly reduce TC (49%), TG (47%), LDL (62%), VLDL (26%) and increase HDL (22%) after 12 weeks of drug treatment in animal models in our study. In a study it was shown that after one-month treatment significant reduction in total cholesterol levels in rats treated with either Garlic Extract or AT (80 mg/kg) when compared to control group. Similarly, Concurrent administration of atorvastatin with garlic extract significantly decreased total cholesterol level compared to control group. Moreover, combined administration of atorvastatin and garlic extract produced a synergistic effect on the reduction of total cholesterol levels. This synergistic effect was observed at lower dose level of AT (20 mg/kg) while it is lost at higher dose level of AT (80 mg/kg) which had contradicted our study result. However, no significant change in triglyceride levels was observed in garlic extract-treated rats (which had also contradicted our study) whereas, administration of AT (20 and 80 mg/kg) either alone or in combination with GE produced significant decrease in triglycerides levels compared to control group, which had supported our study result. [42]

Ezetimibe plus garlic group were able to significantly reduce TC (20%), TG (30%), LDL (39%), VLDL (16%) and increase HDL (31%) after 12 weeks of drug treatment in animal models in our study. Maheswari G et al had shown in an animal study that the combination of ezetimibe (30 µg/kg) with garlic extract (10 mg/kg) resulted in lowering of serum total body cholesterol (12%), triglycerides (30%), LDL (33%), VLDL (21%) along with increase in serum HDL (46%) after treatment which indicated that garlic extract augmented hypolipidemic effect of ezetimibe .[14] This result was similar to our result but augmented hypolipidemic effect was not properly found in our study.

Mohammadi A et al had shown in a study that serum levels of LDL and TC significantly decreased in ezetimibe ($p<0.05$), garlic ($p<0.05$), and much more in combination of garlic and ezetimibe groups ($p<0.001$). TG and VLDL markedly decreasedingarlicandcombinationofgarlicandezetimibegroups($p<0.05$) while change of TG and VLDL in ezetimibe-treated animals were not significant. In their study they had used 4% (w/w) garlic powder and 0.005% (w/w) ezetimibe .[15]

In our study we had found that, after drug treatment given, significant TC & TG concentrations reduction were maximum among ezetimibe group, significant LDL & VLDL concentrations reduction were maximum among combination of atorvastatin and garlic group when compared to other treatment groups. HDL concentration was maximally increased among combination ezetimibe and garlic group which was also statistically significant.

Our study had certain limitations- Blood samples were drawn only at one point of time after the treatment, short study duration.

CONCLUSION

Biochemical parameters (TC, TG, LDL, and VLDL) could be effectively reduced and HDL could be increased after treating the animals with atorvastatin, ezetimibe, garlic homogenate, combination of atorvastatin and garlic homogenate, combination of ezetimibe and garlic homogenate. The

maximum effectivity in such reduction with ezetimibe for TC and TG. For LDL & VLDL, combination of atorvastatin and garlic homogenate was most effective. The maximum effectivity in increase of HDL was noted with combination of ezetimibe and garlic homogenate. In summary, Garlic extract augmented hypolipidemic effect of ezetimibe and atorvastatin. Moreover, there is maximum effect with ezetimibe on **HDL and with atorvastatin on LDL &VLDL, though study with various dose ranges is required**

There remains a scope for future studies with larger sample size and longer duration. It is recommended for future extrapolation in human after toxicity study.

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GALECTIN-3 IN CRITICALLY ILL PATIENTS WITH SEPSIS AND/OR TRAUMA: A GOOD PREDICTOR OF OUTCOME OR NOT?

Jasna Jevdjic[1], Maja Surbatovic[2], Snezana Milosavljevic[3], Goran Rondovic[2], Ivan Stanojevic[4], Stevan Eric[1], Nenad Zornic[1]
[1]Department of Surgery, , Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia
[2]Clinic of Anesthesiology and Intensive Therapy, Military Medical Academy, Faculty of Medicine of the Military Medical Academy, University of Defence, Belgrade, Serbia
[3]Department of Anesthesiology, Clinical Center Kosovska Mitrovica, Kosovska Mitrovica
[4]Institute for Medical Research, Military Medical Academy, Faculty of Medicine of the Military Medical Academy, University of Defence, Belgrade, Serbia

GALEKTIN - 3 KOD KRITIČNO OBOLELIH PACIJENATA SA SEPSOM I/ILI TRAUMOM: DOBAR PREDIKTOR ISHODA ILI NE?

Jasna Jevdic[1], Maja Šurbatović[2], Snežana Milosavljević[3], Goran Rondović[2], Ivan Stanojević[4], Stevan Erić1, Nenad Zornić[1]
[1]Katedra za hirurgiju, Fakultet medicinskih nauka, Univerzitet u Kragujevcu, Kragujevac, Srbija
[2]Klinika za anesteziologiju i intenzivno lečenje, Vojnomedicinska akademija, Medicinski fakultet Vojnomedicinske akademije, Univerzitet odbrane, Beograd, Srbija
[3]Služba anestezije, Klinički centar Kosovska Mitrovica, Kosovska Mitrovica
[4]Institut za medicinska istraživanja, Vojnomedicinska akademija, Medicinski fakultet Vojnomedicinske akademije, Univerzitet odbrane, Beograd, Srbija

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ABSTRACT

Severe sepsis and/or trauma complicated with multiple organ dysfunction syndrome are leading causes of death in critically ill patients. The aim of this prospective, observational, single centre study was to assess the prognostic value of galectin-3 regarding outcome in critically ill patients with severe trauma and/or severe sepsis. The outcome measure was hospital mortality.

In total, 75 critically ill patients who were admitted to the intensive care unit of the tertiary university hospital were enrolled in a prospective observational study. Blood samples were collected upon fulfilling Sepsis-3 criteria and for a traumatized Injury Severity Score > 25 points.

Levels of galectin-3 were significantly higher in nonsurvivors on the day of enrolment – Day 1 ($p<0.05$). On Day 1, the area under the curve (AUC) for the galectin-3 for lethal outcome was 0.602. At a cut-off level of 262.82 ng/mL, the sensitivity was 53%, and the specificity was 69.7%, which was objectively determined by a Youden index of 0.20.

The discriminative power of galectin-3 in predicting outcome was statistically significant. Galectin-3 on Day 1 is a fairly good predictor of lethal outcome.

Keywords: *galectin-3; critical care; outcome; hospital mortality*

SAŽETAK

Teška sepsa i/ili trauma kod koje se kao komplikacija javlja sindrom multiple organske disfunkcije je vodeći uzrok smrti kod kritično obolelih. Cilj ove prospektivne, observacione studije je bio da se proceni prognostička vrednost galektina – 3 u smislu ishoda kod kritično obolelih sa teškom traumom i/ili teškom sepsom. Mera ishoda je bio hospitalni mortalitet.

75 kritično obolelih pacijenta, primljenih u jedinicu intenzivne terapije tercijarne univerzitetske bolnice, obuhvaćeno je prospektivnom, observacionom studijom. Uzorci krvi su sakupljani na dan ispunjavanja SEPSIS-3 kriterijuma, a kod traumatizovanih ISS >25 bodova.

Vrednosti galektina – 3 su bile statistički značajno veće kod umrlih na dan uključenja u studiju – Dan 1 ($p<0.05$). Vrednost AUC/ROC za galektin – 3 prvog dana u smislu predikcije ishoda je bila 0.602. Pri cut-off vrednosti od 262.82 ng/mL senzitivnost je bila 53% a specifičnost 69.7%, što je objektivno utvrđeno korišćenjem Youden indeksa čija vrednost je bila 0.20.

Vrednost galektina – 3 prvog dana je dobar prediktor letalnog ishoda.

Ključne reči: *galektin - 3; kritično oboleli; ishod; hospitalni mortalitet*

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Corresponding author:
Nenad Zornic, MD, PhD
Clinical Center Kragujevac,
Zmaj Jovina 30, Kragujevac 34000, Serbia,
Faculty of Medical Sciences University of Kragujevac,
Svetozara Markovica 69, Kragujevac 34000, Serbia,
Email: nenadzornic@gmail.com

INTRODUCTION

Critical illness is defined by the presence of altered organ function in acutely ill patients such that homeostasis cannot be maintained without medical intervention in intensive care units, such as mechanical ventilation, vasoactive support for haemodynamicsa, and renal replacement therapy. It usually involves two or more organ systems. Immune dysfunction is common in critically ill patients, and it may modulate the immune response and affect patient morbidity and mortality, particularly in severe trauma and/or sepsis. Immune cells and mediators, in the critical care setting, are understudied and do represent a challenging area. Inflammatory mediators can be predictive biomarkers of organ dysfunction and outcome in critically ill patients, so they are of interest for both researchers and clinicians (1). One of the most interesting mediators, galectin-3, belongs to a family of conserved proteins with carbohydrate-recognition domains (CRDs). CRDs bind beta-galactosidase, and they consist of approximately 130 amino acids. Galectin-3 is unique in this family of proteins because it is a chimaera-type with two terminals: a C-terminal CRD and an N-terminal that is a protein-binding domain (2, 3, 4).

It is difficult to find adequate biomarkers of the immune response in critical illness, regardless of its cause, with good predictive value regarding outcome because there is a wide and complex array of immune-related mediators. Many of them were explored in this clinical setting (5, 6, 7). Recently, in a few studies, galectin-3 has been investigated as a novel potential biomarker regarding its accuracy and clinical usefulness.

The aim of our prospective observational study was to assess the prognostic value of galectin-3 regarding outcome in a cohort of critically ill patients with secondary sepsis and/or trauma. The outcome measure was hospital mortality.

PATIENTS AND METHODS

Ethical Approval

Approval in concordance with the Declaration of Helsinki was obtained from the local ethics committee, and informed consent was obtained from a patient or first-degree relative.

Patients and study design

A total of 75 critically ill and injured patients, admitted to the surgical intensive care unit (SICU) were enrolled in a prospective study conducted in a tertiary university hospital (Military Medical Academy, Belgrade, Serbia). Patients with secondary sepsis (underlying conditions were peritonitis, pancreatitis and trauma) were enrolled if they had fulfilled current Sepsis-3 diagnostic criteria for sepsis (formerly severe sepsis) and/or septic shock (acute change in total SOFA score ^{3} 2 points and vasopressors required to maintain mean arterial pressure ^{3} 65 mmHg and serum lactate level > 2 mmol/L despite adequate volume resuscitation) (8). The diagnostic criteria encompass any of the following variables thought to be a result of the infection: sepsis-induced hypotension, lactate levels greater than 2 mmol/L, urine output less than 0.5 mL/kg/hr for more than two hours despite adequate fluid resuscitation, acute lung injury with PaO_2/FiO_2 less than 250, creatinine greater than 2.0 mg/dL (176.8 micromol/L), bilirubin greater than 2.0 mg/dL (34.2 micromol/L), platelet count less than 100,000 and coagulopathy (international normalised ratio – INR) greater than 1.5. Additionally, critically ill patients with severe trauma [Injury Severity Score – ISS (determined using Abbreviated Injury Scale – AIS) > 25 points] were enrolled. Only adult patients, at least 18 years of age, were recruited. The exclusion criteria were as follows: (1) secondary sepsis and/or septic shock with an underlying condition other than severe peritonitis, pancreatitis or trauma; (2) malignant disease of any origin; (3) long-term SICU stay before criteria fulfilment; (4) pre-existing immunodeficiency. The Sequential Organ Failure Assessment (SOFA) score, the Simplified Acute Physiology Score (SAPS) II and the Acute Physiology and Chronic Health Evaluation (APACHE) II score were calculated and recorded within the first 24 h after admission to the SICU.

Sampling and analysis

Patient's venous blood was drawn by trained, qualified phlebotomists on the first day of enrolment in the study. The concentration of galectin-3 was determined with the Quantikine Human Galectin-3 Immunoassay ELISA test (R&D Systems Europe Ltd, UK). This assay employs the quantitative sandwich enzyme immunoassay technique. Briefly, a solution of a monoclonal antibody specific for human galectin-3 was prepared according to the manufacturer guidelines. Polystyrene microplates (96 wells; 12 strips of 8 wells) were coated with a prepared solution of monoclonal antibody specific for human galectin-3 (100 µl/well, overnight, + 4 °C). After the washing and blocking procedure, the wells were filled with 100 µl assay diluent and subsequently with 50 µl of standards, controls and samples per appropriate well. Plates were covered and incubated for 2 hours at RT. After another washing procedure, the wells were filled with 200 µl of prepared human galectin-3 conjugate and were covered and incubated for an additional 2 h at RT. After the final washing procedure, 200 µl of substrate solution was placed in each well, and the plate was incubated for 30 min at RT, protected from light. Development of the colour reaction was terminated with stop solution (50 µl/well), and the optical density of each well was determined at 450 nm in a microplate reader (BioTek Synergy HT, Winooski, Vermont, USA)T. The concentrations of the tested samples were obtained by

Table 1. Demographic and clinical data

Total no. of patients	75
Age (average, range)	59.2 (from 18 to 85 yrs)
Sex, n (%) male female	 45 (60.0%) 30 (40.0%)
Simplified Acute Physiology Score II – SAPS II score, mean ± SD	55.23 ± 8.25
Acute Physiology and Chronic Health Evaluation II – APACHE II score, mean ± SD	24.25 ± 4.23
Sequential Organ Failure Assessment – SOFA score, mean ± SD	6.78 ± 2.42
Overall hospital mortality	45.3%

plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis, from a best fit curve through the points on a log/log graph.

Statistical analysis

A complete statistical analysis of the data was performed with the statistical software package, SPSS Statistics 18. Variables were presented as the mean value ± standard deviation (SD), median, minimal and maximal values. The Kolmogorov-Smirnov test was used for evaluation of the distribution of the data. Statistical significance between groups was tested by Kruskal-Wallis or Mann-Whitney tests. ROC curves were constructed to determine the sensitivity and specificity of mediators for the prediction of outcome. The Youden's index (J), the difference between the true positive rate and the false positive rate, was used. Maximizing this index allows one to find, from the ROC curve, an optimal cut-off point independently from the prevalence. All the analyses were estimated at $p<0.05$ level of statistical significance.

RESULTS

Demographic and clinical data of 75 patients is shown in Table 1.

Baseline characteristics of the patient population regarding galectin-3 according to outcome (hospital mortality) are shown in Table 2.

We compared levels of galectin-3 between survivors and nonsurvivors on Day 1. Levels of galectin-3 were significantly higher in nonsurvivors (Mann-Whitney U Z = -1.972; $p<0.05$). Data are shown in Figure 1.

The clinical accuracy of galectin-3 in predicting outcome was investigated. The discriminative power of this mediator in predicting lethal outcome was statistically significant. A receiver operator curve was generated to determine the cut-off values for optimal sensitivity and specificity for the galectin-3 levels on Day 1. The results are shown in Table 3.

Table 2. Baseline characteristics of the patient population regarding galectin-3 according to outcome

Galectin-3 (ng/mL)	Survivors	Nonsurvivors
N	41	34
Mean	211.20	425.33
Standard Deviation	110.45	715.18
Median	190.25	282.57
Minimum	53	97
Maximum	742	7945

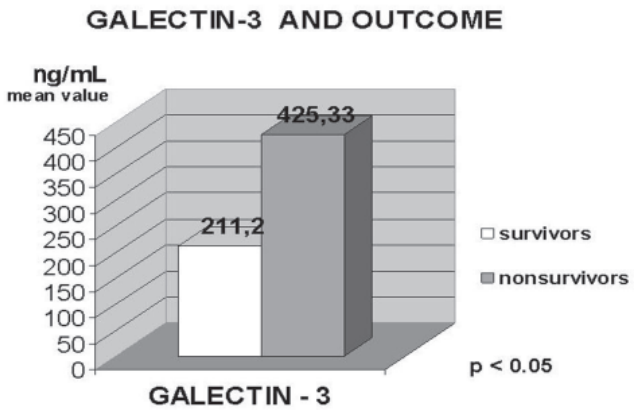

Figure 1.

Table 3. Clinical accuracy of galectin-3 in predicting lethal outcome on Day 1

Parameter	AUC ROC	*p* value	95% Confidence Interval		Cut-off value	Sensitivity (%)	Specificity (%)	Youden index
			Lower Bound	Upper Bound				
Galectin – 3 on Day 1	0.602	p< 0.05	0.547	0.850	262.82	53.0	69.7	0.20

A galectin-3 level that is higher than the cut-off value on Day 1 is a good predictor of lethal outcome in this patient population.

DISCUSSION

Galectin-3 can be found in a variety of tissues and is highly expressed on myeloid cells (like monocytes, macrophages and neutrophils) as well as on epithelial and endothelial cells. After various stimuli, these cells release this mediator, so galectin-3 can be found extracellularly. In our cohort of 75 critically ill and injured patients, we compared levels of galectin-3 between survivors and nonsurvivors. The analysis was performed on the first day of enrolment in the study. The outcome measure was hospital mortality. That is, that these patients were followed for a rather long time (greater than one year) until either hospital discharge or death. Levels of galectin-3 were significantly higher in nonsurvivors. The clinical accuracy of galectin-3 in predicting outcome was investigated. The discriminative power of this mediator in predicting lethal outcome was statistically significant with an AUC/ROC of 0.602. A galectin-3 level higher than the cut-off value of 262.82 pg/mL on Day 1 is a good predictor of lethal outcome in this patient population with a moderate sensitivity of 53% and a rather good specificity of almost 70%.

Free extracellular galectin-3 is involved in immunity against pathogens, in various stages of inflammation with complex roles (9, 10). Recently, it has been shown that galectin-3 can recognize microbial structures (PAMPs – pathogen-associated molecular patterns); also it can be released by damaged tissues, so it serves as a DAMP - damage-associated molecular pattern (11). Galectin-3 is a proinflammatory mediator, so one of its properties is to enhance infiltration of the site of infection with neutrophils and other immunocompetent cells. Galectin-3 is synthesized and stored in the cytoplasm, and it can be either secreted by activated cells or passively released from dying cells. In addition to being a potential DAMP, galectin-3 can act as a PRR - pattern-recognition receptor, so it can modulate innate immunity (12).

Various pathogens are inducers of galectin-3 synthesis and release. Therefore, this glycan binding protein secretion is upregulated by bacteria and fungi. Additionally, it has been shown that this lectin can bind to pathogens. In their study, Quattroni and co-authors, investigated the interaction between galectin-3 and *Neisseria meningitidis*, an important extracellular human pathogen that is a leading cause of meningitis, which can also be complicated by onset of meningococcal sepsis (13). The authors demonstrated by immunohistochemical analysis that galectin-3 is expressed during meningococcal disease and colocalizes with bacterial colonies in infected tissues from patients. They also found that galectin-3 binds to *Neisseria meningitidis*. In animal studies, they used galectin-3 deficient (Gal-3(-/-)) mice to evaluate the contribution of galectin-3 to meningococcal bacteraemia and found that Gal-3(-/-) mice had significantly lower levels of bacteraemia compared with wild-type mice after challenge with live bacteria, indicating that galectin-3 confers an advantage to *Naisseria meningitidis* during systemic infection. It has been shown that expression of galectin-3 influences the course of infection of *Mycobacterium leprae*. Among other things, it diminished the ability of monocytes to differentiate into dendritic cells in response to granulocyte-macrophage colony-stimulating factor – GM-CSF. Additionally, the ability of dendritic cells, which are derived from monocytes, to stimulate T-cell proliferation in response to mycobacterial antigens was also hampered (14). Therefore, in intracellular infection with Mycobacterium, activation of T lymphocytes was affected by high expression of galectin-3. In that scenario, the cell-mediated adaptive immune response, which is necessary to control infection, is inadequate.

As mentioned before, a variety of lectins serve as pattern-recognition receptors during innate immune responses against pathogens, including ficolins and collectins which are soluble molecules (15), as well as C-type lectin family members, which are membrane receptors. Researchers demonstrated that galectin-3 serves as a pattern recognition receptor for bacteria (binds LPS – lipopolysaccharide-endotoxin from *Escherichia coli* and *Pseudomonas aeruginosa*, for instance), virus, fungi and parasites (16). Interestingly, elevated release of galectin-3 can exacerbate tissue damage through activated leukocyte infiltration but can also bind lipopolysaccharide, thus, acting as a negative regulator of endotoxic shock induced by lipopolysaccharide.

Galectin-3 is synthesized and released by a myriad of immune cells acting such as sentinels. This interesting mediator affects immunocompetent cells in an autocrine and/or paracrine fashion, i.e., if it is extracellular, galectin-3 binds to a membrane receptor, and if it is intracellular, galectin-3 modulates intracellular proteins activity. One study showed that injection of exogenous galectin-3 induced migration of neutrophils to the injection site, although, in vitro, galectin-3 is not a neutrophil chemoattractant (17). This suggests that, after being released from cells, galectin-3 does indirectly induce migration of neutrophils acting as a DAMP. It has to be emphasized that galectin-3 does not only enhance neutrophil infiltration of the inflammation site, but it also plays a role in terminating the inflammatory response by being a part of the neutrophil removal process. It has been demonstrated recently, in an animal model of self-resolving peritonitis, that galectin-3-deficient mice exhibited reduced apoptosis and efferocytosis of neutrophils (18). In cell biology, efferocytosis (from efferre, Latin for 'to take to the grave', 'to bury') is the process by which dying/dead cells (e.g., apoptotic or necrotic) are removed by phagocytic cells. It can be regarded as the 'burying of dead cells'. theTherefore, the authors of this study showed the existence of impaired neutrophil clearance without galectin-3 being present.

One of the many roles of galectin-3, the only chimeric galectin, is to act as an alarmin. That was investigated by Mishra and co-authors in animal model of pulmonary infection with *Francisella novicida* (19). As extensive cell death is pivotal in severe infection, the authors focused on host endogenous molecules called alarmins released from dead or dying host cells leading to a proinflammatory response. The authors demonstrated an upregulated expression and extracellular release of galectin-3 in the lungs of mice undergoing lethal pulmonary infection with a virulent strain of *F. novicida* but not in those infected with a non-lethal, attenuated strain of the bacteria. In comparison with their wild-type counterparts, *Francisella novicida*-infected galectin-3-deficient (galectin-3(-/-)) mice demonstrated significantly reduced leukocyte infiltration, particularly neutrophils in their lungs. They also exhibited a marked decrease in inflammatory cytokines, vascular injury markers, and neutrophil-associated inflammatory mediators. *Francisella novicida*-infected galectin-3(-/-) mice exhibited improved lung architecture with reduced cell death and improved survival over wild-type mice, despite similar bacterial burden. The authors concluded that galectin-3 acts as an alarmin by augmenting the inflammatory response.

The effect of galectin-3 on immune cells is, as described above, versatile. As far as mononuclear phagocytes are concerned, one study demonstrated that in microglia (mononuclear phagocytes of central nervous system), galectin-3 functions as an endogenous paracrine ligand for Toll-like receptor (TLR)-4, and so, it induces important TLR-4-mediated activation of the immune cascade (20). Galectin-3-TLR-4 interaction was further confirmed in a murine neuroinflammatory model (intranigral lipopolysaccharide-LPS injection). Dendritic cells are a crucial link between innate and adaptive immunity as they play a pivotal role in determining the Th1/Th2/Th17 polarization of the adaptive immune response. Intracellular galectin-3 predominantly modulates cytokine release by dendritic cells. It has been shown that neutralizing antibodies against this chimeric galectin did not reverse its effects; the evidence is, therefore, pointing to crucial participation of intracellular galectin-3 in the regulation of cytokine release by dendritic cells (21).

Surprisingly, there are only a few studies and a paucity of clinical data regarding galectin-3, a complex multifaceted molecule, as a predictor of infection and/or outcome in critically ill and injured patients. This mediator has dual roles as both a circulating DAMP and a cell membrane-associated PRR. ten Oever and co-workers focused their investigation on assessing the potential of circulating galectin-3 for discriminating between infections and non-infectious inflammatory disorders on the one hand, and between fungal and bacterial infections on the other (22). Galectin-3 was measured in the plasma of 127 patients with either non-infectious inflammatory disorders (gout, autoinflammatory syndrome or pancreatitis) or an infection (viral lower respiratory tract infection, bacterial sepsis or candidaemia). Circulating galectin-3 concentrations were increased in patients with infections when compared with healthy volunteers or patients with non-infectious inflammatory diseases. The clinical accuracy of predicting infection was also assessed. Galectin-3 was a good predictor of infection with AUC/ROC of 0.73. At cut-off value of 20.6 ng/ml specificity was 95%, and sensitivity was 43%. Galectin-3 concentrations were similar in patients with bacterial and Candida sepsis, while being lower in viral respiratory infections. So, galectin-3 could not discriminate between bacterial and fungal sepsis. Another study investigated galectin-3 as a diagnostic marker with opposite results. Mueller and co-workers evaluated to which extent plasma concentrations of galectin-3, among other biomarkers, is increased in heart failure compared with diverse non-cardiac conditions such as infectious disease or chronic kidney disease (23). Compared to healthy controls, the median galectin-3 concentration was a ~1.5-fold increase in patients with heart failure; a ~1.4-fold in pneumonia; a ~2.4-fold in heart failure with pneumonia; a ~2.5-fold in renal disease, and a ~2.7-fold in sepsis ($p<0.001$ for all compared to controls). Galectin-3 was not significantly increased in chronic obstructive pulmonary disease. The authors concluded that, because increased plasma concentrations of galectin-3 are not specific for a distinct disease group, this biomarker is not useful for diagnostic purposes.

Only rather recently, focus regarding galectin-3 has changed towards its outcome predictive value in critical care medicine, i.e., in a patient population similar to ours. Dieplinger and co-workers conducted a study aimed to assess prognostic value, for the prediction of 90-day all-cause mortality, of several biomarkers, including galectin-3, in an unselected cohort of critically ill patients (24). In univariate analyses, increased galectin-3 plasma concentrations at baseline were strong prognostic markers. Last year, Kim and co-workers investigated whether a biomarker could be objective and reliable tool to predict mortality in sepsis and explored the prognostic utility of emerging biomarker galectin-3 regarding the prediction of mortality in patients with sepsis (25). In this retrospective study, 157 septic patients were included. Procalcitonin (PCT), presepsin, galectin-3, and soluble suppression of tumourigenicity 2 (sST2) concentrations were analysed in relation to the 30-day all-cause mortality. Median values of galectin-3 were significantly higher in nonsurvivors (58.6 vs. 24.5 respectively). Our findings are in line with both of these studies (Deplinger et al.; Kim et al.). The trend is the same; nonsurvivors had higher values of galectin-3 than survivors. Levels of galectin-3, in both survivors and nonsurvivors in these two studies, were lower than in our study. That can be explained by the fact that our patients had more severe critical illness, which is reflected by a higher SAPS II score in our patient population when compared to results published by Deplinger et al. Additionally, our patients were obviously sicker, with a hospital mortality rate which was almost doubled compared to the results demonstrated by Kim and co-workers (45.3% vs. 25.5%, respectively). The clinical accuracy of galectin-3

in predicting a lethal outcome on Day 1 was statistically significant in both of these studies, as it was as in ours, with a slightly higher AUC/ROC of 0.7 compared to our AUC/ROC of 0.6. As far as the study published by Kim et al. is concerned, we have to bear in mind that we calculated the clinical accuracy of this mediator in predicting hospital mortality, so the outcome measure was different (in their study it was 30-day mortality).

The complexity of the myriad of galectin-3 activities also makes this mediator very interesting as a biomarker in a variety of fields, such as heart failure (26), kidney diseases (27, 28) and malignant diseases (29, 30).

CONCLUSION

We demonstrate that galectin-3 is an emerging prognostic biomarker in critically ill patients. The levels of galectin-3 were significantly higher in nonsurvivors. The clinical accuracy of galectin-3 in predicting a lethal outcome in critically ill and injured patients was assessed. The discriminative power of this mediator in predicting a lethal outcome, with the outcome measure being hospital mortality, was statistically significant. Trends and patterns in the investigated biomarker that we found should be validated in a larger patient population, so further studies are warranted.

CONFLICT OF INTEREST

No conflicts of interest, financial or otherwise, are declared by the authors.

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REDOX STATUS IN WOMEN WITH RHEUMATHOID ARTHRITIS

Aleksandra Vranic[1], Aleksandra Antovic[2], Nevena Draginic[3], Marijana Andjic[1], Marko Ravic[1], Vladimir Jakovljevic[3,4] and Mirjana Veselinovic[5]
[1]Department of Pharmacy, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia
[2]Karolinska Institutet, Department of Medicine, Solna, Rheumatology Unit
and Academic Specialist Center, Center for Rheumatology, Stockholm Health Services, Stockholm, Sweden
[3]Department of Physiology, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia
[4]Department of Human Pathology, 1st Moscow State Medical University IM Sechenov, Moscow, Russia
[5]Department of Internal medicine, Faculty of Medical Sciences, University of Kragujevac, Serbia

REDOKS STATUS KOD ŽENA SA REUMATOIDNIM ARTRITISOM

Aleksandra Vranic[1], Aleksandra Antovic[2], Nevena Draginic[3], Marijana Andjic[1], Marko Ravic[1], Vladimir Jakovljevic[3,4] i Mirjana Veselinovic[5]
[1]Katedra za farmaciju, Fakultet medicinskih nauka, Univerzitet u Kragujevcu, Kragujevac, Srbija
[2]Karolinska Institut, Odsek medicina, Solna, Odeljenje za reumatologiju
i Akademski specijalisticki centar, Centar za reumatologiju, Medicinski centar Stokholm, Stokholm, Svedska
[3]Katedra za fiziologiju, Fakultet medicinskih nauka, Univerzitet u Kragujevcu, Kragujevac, Srbija
[4]Odsek za humanu patologiju, Prvi Moskovski državni medicinski univerzitet I.M. Sechenov, Moskva, Rusija
[5]Katedra za internu medicine, Fakultet medicinskih nauka, Univerzitet u Kragujevcu, Kragujevac, Srbija

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ABSTRACT

The aim of this study was to assess oxidative status and to set baseline characteristics for female population with established rheumatoid arthritis. Total of 42 patients with rheumatoid arthritis and 48 age- and sex-matched controls were included in the study. Clinical examination was performed and assessed disease activity. Peripheral blood samples were used for all the assays. The markers of oxidative stress were assessed, including plasma levels of index of lipid peroxidation – thiobarbituric acid reactive substances, hydrogen peroxide, superoxide anion radical, nitrites and activity of superoxide dismutase, catalase and reduced glutathione levels as antioxidant parameters. In the patients group, levels of hydrogen peroxide and index of lipid peroxidation were higher than in controls. Patients with rheumatoid arthritis had decreased superoxide dismutase and catalase activity compared to healthy subjects. Interestingly, controls had higher levels of nitrites compared to patients. Patients showed a marked increase in reactive oxygen species formation and lipid peroxidation as well as decrease in the activity of antioxidant defense system leading to oxidative stress which may contribute to tissue and cartilage damage and hence to the chronicity of the disease.

Keywords: *rheumatoid arthritis, oxidative stress, reactive oxygen species, women*

SAŽETAK

Studija ima za cilj da proceni oksidacioni status i utvrdi osnovne karakteristike ženske populacije sa dijagnozom reumato. U istraživanje je uključeno 42 pacijenta sa reumatoidnim artritisom i 48 zdrava ispitanika iste starosti i pola. Urađen je klinički pregled i procenjena je aktivnost bolesti. Uzorci periferne krvi su korišćeni za sve testove. Određivani su markeri oksidacionog stresa, uključujući plazma koncentracije indeksa lipidne peroksidacije, vodonik peroksida, superoksid anjon radikala, nitrita kao i aktivnost superoksid dismutaze, katalaze i nivo redukovanog glutationa kao antioksidacionih parametara. Kod pacijenata, nivoi vodonik peroksida i indeksa lipidne peroksidacije bili su veći nego u kontrolnoj grupi. Pacijenti sa reumatoidnim artritisom imali su smanjenu aktivnost superoksid dismutaze i katalaze u poređenju sa zdravim osobama. Interesantno, kontrole su imale veće vrednosti nitrita u poređenju sa pacijentima. Pacijenti sa reumatoidnim artritisom pokazali su značajno povećanje koncentracije reaktivnih kiseonicnih vrsta, kao i smanjenje aktivnosti antioksidacionog sistema, što dovodi do nastanka oksidacionog stresa koji može doprineti oštećenju tkiva i hrskavice, a time i hroničnom toku bolesti.

Ključne reči: *reumatoidni artritis, oksidacioni stres, reaktivne kiseonične vrste, žene*

ABBREVIATIONS

Anti-CCP – Anticyclic citrullinated polypeptide
aPTT – activated partial thromboplastin time
BMI – Body mass index
CRP – C-reactive protein
DAS28 – Disease activity score
ESR – Erythrocyte Sedimentation Rate
INR – International Normalised Ratio
LPO – lipid peroxidation
PT – prothrombin time
RA – Rheumatoid arthritis
RF – Rheumatoid factor
ROS – Reactive oxygen species

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Corresponding author:
Mirjana Veselinovic,
Faculty of Medical Sciences, University of Kragujevac,
Kragujevac, SERBIA,
Email:veselinovic.m@sbb.rs

INTRODUCTION

Rheumatoid arthritis (RA) is systemic autoimmune disease manifested as erosive polyarthritis with chronic manner, intermittent acute inflammatory episodes and extra-articular manifestation (1). It consequently leads to progressive cartilage and joints damage as well as to disability (2) and represents a significant burden both for the individual and for society (3). RA affects almost 1% of the total population whereby it is characterized by a strong systemic inflammation with a shortened lifetime and increased mortality (4). Data suggests that the risk to develop this disease in 3:1 in favor of the female (5) with a significant higher disease activity in this population (6).

Oxidative stress and decreased antioxidant status are the hallmarks in patients of RA as observed in recent years. It is a dynamic phenomenon involved in the disease pathogenesis in a complex way. Oxidative stress represents an imbalance between pro-oxidants and antioxidants in the body in favor of reactive oxygen species (ROS) (7).

As a response to inflammatory conditions in RA an ROS overproduction is usually generated from endogenous source such as mitochondrial electron transport chain by the process of oxidative phosphorylation. Generally, oxygen species in RA have different origin and it can be produced by activated macrophages in the synovial membrane, by chondrocytes, and also through activated neutrophils in the synovial cavity (8). Reaction of ROS production is followed by upregulation of different enzymes such as NADPH oxidase, nitric oxide synthase as well xanthine oxidase (9). Thereafter, NADPH oxidase complex catalyses oxygen reduction to superoxide anion radicals. Unless the concentration of this ROS is not neutralized by superoxide dismutase it consequently leads to deadly combination with NO, synthesis of toxic peroxynitrite. Components of cartilage and extracellular matrix are damaged either directly or indirectly by superoxide anion along with other oxygen and nitrogen radicals by reducing the synthesis of matrix components such as collagen and proteoglycans (10). In addition, the H_2O_2-dependent inhibition of proteoglycan synthesis contributes to cartilage destruction in RA through interfering with the repairing mechanisms of the proteolytic and oxidative damages (11).

Increased values od ROS are potentially harmful for different macromolecules and it may result in impairment of physiological processes and damage cell membranes, lipids, proteins and nucleic acids. This leads to fast protonation, - SH group's depletion, deamination od DNA bases and lipid peroxidation (12). Afterwards, lipid peroxidation has been implicated in pathogenesis of many diseases such as cancer, degenerative disease as well inflammatory disease. It can be measured as thiobarbituric acis reactive substance (TBARS) and it may play an important role (13). Increased lipid peroxidation has been reported in various biological samples of RA patients (14).

Body has developed an antioxidant defense system in order to prevent damaging effects of the pro-oxidants and to protect the cells from oxidative damage. Some common enzymes involved in the neutralization or elimination of ROS are superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) as well as glutathione reductase (GR), glutathione-S-transferase (GST), thioredoxin reductase (TR) and heme oxygenase (10). Catalase and reduced glutathione have affinity to H_2O_2. Affinity of CAT increases due to higher values of H_2O_2 (15) while SOD has mail effect in superoxide anion radical neutralization (10).

RA is a condition with strong influence of both genetic and environmental factors. Accordingly, data available about this disease and the biochemical aspects in our population are very minimal, limited and non-compliant. Regarding, the aim of this study was to assess oxidative status and to set baseline characteristics for female population with established rheumatoid arthritis.

PATIENTS AND METHODS

Patients

Forty two women with diagnosed rheumatoid arthritis referred to the outpatient of Internal Clinic, Department of Rheumatology were included in the study (mean age 54.8±9.1). The mean disease duration was 12.8±8.0 years and the mean value of DAS28 was 3.8±1.1 at the moment of blood sampling. All patients were treated with the standard treatment protocol methotrexate (15-25mg per week) and prednisolone (≤10mg per day). The diagnose of rheumatoid arthritis was established according to Classification criteria for RA American College of Rheumatology (ACR)/ European League Against Rheumatism (EULAR) 2010. Patients included in this study did not have history of diabetes, malignancy, inflammatory disease except rheumatoid arthritis, liver- or renal insufficiency, previous hospitalization due to cerebrovascular, cardiovascular disorders and venous thromboembolism. The patients treated with high doses of steroids (≥10 mg/day, including parenteral administration) and those treated with biologic therapy were excluded from the study. The number of swollen and tender joints of the selected patients was recorded with the help of a rheumatologist. Disease activity score (DAS28) was calculated using a universally accepted formula. Additionally, at the time of blood sampling patients were free of any medication known to influence oxidative balance.

Healthy controls

Forty eight age- and sex-matched subjects (mean age 54.1±6.2) were included in the study as healthy controls and had a medical investigation to ensure absence of a history of diabetes, malignancy, inflammatory disease including rheumatoid arthritis, liver- or renal insufficiency. As well as in patients group, controls were free of any medication known to influence oxidative balance.

Written informed consent was obtained from all participants, and the study protocol was approved by the Ethics Committee of the Clinical Center Kragujevac prior to the onset of the study. The investigation was conducted in accordance with the principles outlined in the Declaration of Helsinki and principles of Good Clinical Practice (GCP).

Laboratory analysis

During blood sampling, blood was taken to perform laboratory analyzes. Biochemical and hematological analyzes (RF, anti-CCP, ESR, CRP, lipid status, leukocytes, leukocyte formula, fibrinogen, aPTT, PT, INR) for all research participants were obtained in the Central Laboratory of the Clinical Center Kragujevac.

Biochemical assays

Blood sampling was obtained in the same manner for all patients at the Internal Clinic, Department of Rheumatology, Clinical Center Kragujevac, Serbia. The examinations were performed in a quiet, air-conditioned, temperature-controlled room (22–24°C). Venous blood from all participants was collected between 8–10 a.m at least a 10-h fast in both patients and controls. Blood was collected in Vacutainer tubes containing 0.129 M sodium citrate (BD Vacutainer Blood Collection System) using 21-gauge polyethylene catheter for taking blood samples (BD Vacutainer needles). Blood was centrifuged to separate plasma and red blood cells (RBCs).

Redox status

Redox status was evaluated spectrophotometrically by measuring the levels of hydrogen peroxide, superoxide anion radical, nitrites and index of lipid peroxidation in plasma. Activities of corresponding antioxidative enzymes superoxide dismutase, catalase, and reduced glutathione were measured in erythrocytes in the same manner.

Index of lipid peroxidation (thiobarbituric acid reactive substances)

The degree of lipid peroxidation in plasma was estimated by measuring of thiobarbituric acid reactive substances (TBARS) using 0.4 ml 1% thiobarbituric acid (TBA) in 0.05 NaOH mixed with 0.8 ml of plasma, incubated at 100 °C for 15 min and measured at 530 nm. Distilled water was used as a blank probe. TBA extract was obtained by combining 0.8 ml plasma and 0.4 ml TCA (trichloroacetic acid). Thereafter, samples were put on ice for 10 min, and centrifuged for 15 min at 6000 rpm (16).

Nitrite determination

Nitric oxide (NO) decomposes rapidly to form stable metabolite nitrite/nitrate products. The method for detection of the plasma nitrite levels is based on the Griess reaction. Nitrites (NO_2^-) were determined as an index of NO production with Griess reagent (forms purple diazocomplex) (17). 0.1 ml 3 N PCA (perchloric acid), 0.4 ml 20 mM EDTA (ethylenediaminetetraacetic acid), and 0.2 ml plasma were put on ice for 15 min, then centrifuged 15 min at 6000 rpm. After pouring off the supernatant, 220 µl K_2CO_3 was added. Nitrites were measured at 550 nm. Distilled water was used as a blank probe.

Superoxide anion determination

The level of superoxide anion radical (O_2^-) was measured using Nitro Blue Tetrazolium (NBT) reaction in TRIS-buffer with plasma and read at 550 nm. Distilled water was used as a blank probe (18).

Hydrogen peroxide determination

Determination of hydrogen peroxide (H_2O_2) concentration is based on oxidation of phenol red using hydrogen peroxide, in reaction catalyzed by enzyme peroxidase from horse radish (POD) (19). 200 µl sample with 800 µl PRS (phenol red solution) and 10 µl POD were combined (1:20) and measured at 610 nm.

Determination of catalase, superoxide dismutase, and reduced glutathion

Isolated RBCs were washed three times with 3 volumes ice-cold 0.9 mmol/l NaCl and hemolysates containing about 50 g Hb/l, prepared according to McCord and Fridovich (20), were used for the determination of catalase (CAT) activity. Determination of CAT activity was determined according to Beutler (21). Lysates were diluted with distilled water (1:7 v/v) and treated with chloroform–ethanol (0.6:1 v/v) to remove hemoglobin. Then 50 µl CAT buffer, 100 µl sample, and 1 ml 10 mM H_2O_2 were added to the samples. Detection was performed at 360 nm. Distilled water was used as a blank probe. Determination of superoxide dismutase (SOD) activity is based on epinephrine method of Misra and Fridovich (22). A 100 µl lysate and 1 ml carbonate buffer were mixed, and then epinephrine in a volume of 100 µl was added. Detection was performed at 470 nm. This method belongs to'negative' type group of methods, since it monitors decrease of autoxidation speed in alkaline medium, which is dependent of $O_2^{-\cdot}$. The level of reduced glutathione (GSH) concentration was determined based on GSH oxidation with 5.5-dithiobis-6.2-nitrobenzoic acid, using Beutler method (23). Measurement of the absorbance is carried out at a wavelength of maximum absorption of 420 nm.

Statistical analysis

The statistical analysis was performed using the statistical package SPSS 20.0 for Windows. The results are expressed as means ± standard deviation of the mean (SD). The differences between parameters in different time measurements were assessed by analysis of variance test with repeated measures and independent samples t-test as post hoc. The alpha level for significance was set to $p < 0.05$.

Table 1. Baseline characteristics of healthy subjects (controls) and patients with rheumatoid arthritis

Features of the participants	Controls	RA patients
Number of patients	48	42
Age (years)	54.15 (6.29)	54.81 (9.05)
Weight (kg)	71.75 (12.04)	72.02 (13.37)
Height (cm)	166 (6)	166 (6)
BMI (kg/m^2)	26.13 (3.90)	26.16 (4.87)
Disease duration (years)	/	12,81 (7.99)
Number of tender joints	/	5 (5)
Number of swollen joints	/	2 (2)
DAS28 - ESR	/	3.75 (1.09)
RF (IU/ml)	/	91.50 (71.43)
RF, n (%)	/	36 (85.71)
Anti-CCP antibodies (U/ml)	/	173.05 (128.68)
Anti-CCP antibodies, n (%)	/	40 (95.24)
Menopause (Yes/No)	34/14	31/11
Smokers, n (%) Current smokers Non-smokers Past smokers	 20 (41.7) 20 (41.7) 8 (16.6)	 17 (40.5) 19 (45.24) 6 (14.26)

The values are expressed as means ± SD. **BMI** – Body Mass Index; **DAS28** – Disease Activity Score 28; **ESR** – Erythrocyte Sedimentation Rate; **RF** – Rheumatoid factor; **anti-CCP** – Anticyclic citrullinated polypeptide.

Table 2. Comparison of values of biochemical, hematological and hemostatic analysis between healthy subjects (controls) and patients with rheumatoid arthritis

Parameter	Controls	RA patients
ESR (mm/h)	13,33 (8,83)**	22,95 (13,50)
CRP (mg/L)	2,54 (2,40)**	11,55 (26,42)
Cholesterol (mmol/L)	6.50 (1.23)	6.06 (1.01)
Triglycerides (mmol/L)	1.5 (0.65)	1.49 (0.62)
HDL (mmol/L)	1.60 (0.30)	1.49 (0.29)
LDL (mmol/L)	4.21 (1.12)	3.81 (0.94)
Leukocytes (10^9/L)	6,51 (1,98)	7,58 (3,02)
Neutrophils (10^9/L)	3.77 (1.62)*	4.76 (2.69)
Neutrophils (%)	56,95 (6,40)**	61,92 (9,28)
Lymphocytes (%)	32,71 (5,68)**	27,87 (7,79)
Fibrinogen	3.26 (0.52)*	3.55 (0.69)
aPTT	28.16 (3.17)	28.35 (3.12)
PT	11.15 (0.67)**	11.89 (1.95)
INR	1.01 (0.06)**	1.08 (0.2)

The values are expressed as means ± SD. Statistical significance between control and experimental group presented as* ($p < 0.05$); **($p < 0.01$). **ESR** – Erythrocyte Sedimentation Rate; **CRP** – C-reactive protein; **HDL** – High-Density Lipoprotein; **LDL** – Low-density lipoprotein; **aPTT** – activated partial thromboplastin time; **PT** – prothrombin time; **INR** – International Normalised Ratio.

RESULTS

Subjects characteristics

Demographic and clinical characteristics of the study population as well as results regarding the presence of traditional CV-risk factors are presented in Table 1. Thirty six out of 42 patients were RF seropositive and 40 of 42 had anti-CCP antibodies. There wasn't any statistical difference between healthy controls and patients regarding age, BMI and smoking.

Parameters of laboratory analysis

Patients with RA had higher ESR, CRP, fibrinogen levels as well as values of PT and INR. Furthermore, leukocyte formula showed higher percent of neutrophils and lower percent of lymphocytes in RA patients compared to healthy controls. Also, higher value of neutrophils was noticed in patients. These results can indicate increased inflammation and changes in hemostatic balance in RA patients. Results for biochemical, hematological and hemostatic analysis are presents in Table 2.

Redox status parameters

The dynamics of oxidative stress parameters (H_2O_2, O_2^-, NO_2^- and TBARS) in healthy control and patients with rheumatoid arthritis are shown in Figure 1, while activities of the antioxidative enzymes (SOD, CAT, GSH) are shown in Figure 2.

Oxidative parameters

Values of hydrogen peroxyde were lower in healthy subjects compared to patients suferring of RA (0.82 ± 0.33 vs. 1.05 ± 0.41 nmol/min/g wt, respectively; $p<0.01$) (Fig 1a). When it comes to O_2^- values, there was no statistically significant difference between controls compared to patients (26.92 ± 5.68 vs. 23.05 ± 5.64 nmol/min/g wt, respectively) (Fig 1b). Values of nitrites were higher in control group relative to patients with RA (4.98 ± 0.9 vs. 3.83 ± 0.52 nmol/min/g wt, respectively; $p<0.01$) (Fig 1c). Futhermore, the same trend as for the H_2O_2 was noticed for TBARS. The values were lower in controls relative to RA patients (1.04 ± 0.11 vs. 0.94 ± 0.13 μmol/min/g wt, respectively; $p<0.01$) (Fig 1d).

Activity of antioxidant parameters

Activity of CAT was statistically increased in control group compared to patients (3.39 ± 0.58 vs. 3.10 ± 0.51 U/gHbx10^3, respectively; $p<0.05$) (Fig 2a). The same trend was noticed in SOD activity with statistically higher values in controls relative to RA group (23.57 ± 5.27 vs. 13.95 ± 5.80 U/gHbx10^3, respectively; $p<0.05$) (Fig 2b). Values of GSH were not statistically different between controls and patients (75071.02 ± 8616.41 vs. 69814.68 ± 8503.38 nmol/ml RBCs, respectively) (Fig 2c).

Correlation

There was no correlation between the measurements of the serum oxidative stress markers and antioxidant capacity and diseases duration, DAS28, CRP, ESR, fibrinogen, the values of RF, anti- CCP antibodies.

DISCUSSION

In addition, it is well known that a pro-inflammatory mediator such as cytokines and prostaglandins plays an important role in the RA pathogenesis. However there is strong evidence that oxidative stress has additional impact through the overproduction of ROS at inflammation sites. Oxidative stress occurs either due to the overproduction

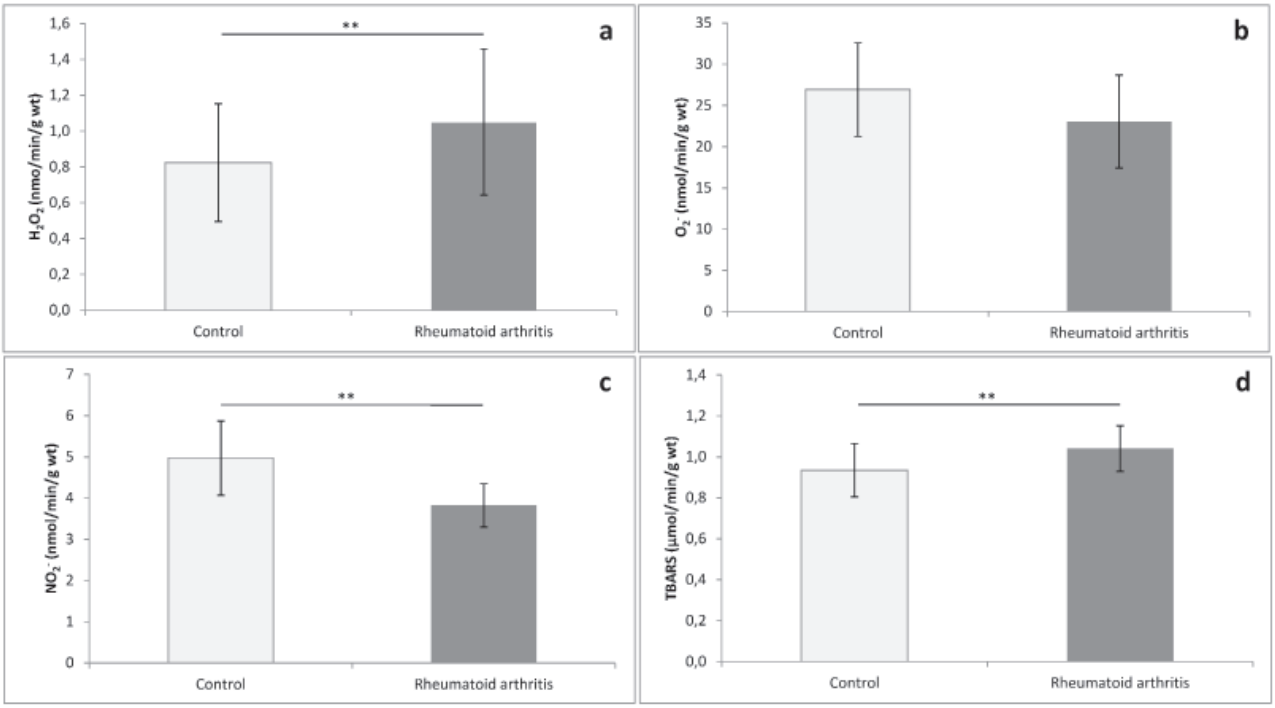

Figure 1. Oxidative stress parameters in control group compared to patients with rheumatoid arthritis. Data are presented as following: **a** – Level of hydrogen peroxide (H_2O_2); **b** – Level of superoxide anion radical (O_2^-); **c** – Level of nitrites (NO_2^-); **d** – Level of index of lipid peroxidation measured as thiobarbituric acid reactive substances (TBARS). Data are expressed as mean ± SD (*p<0.05; **p<0.01).

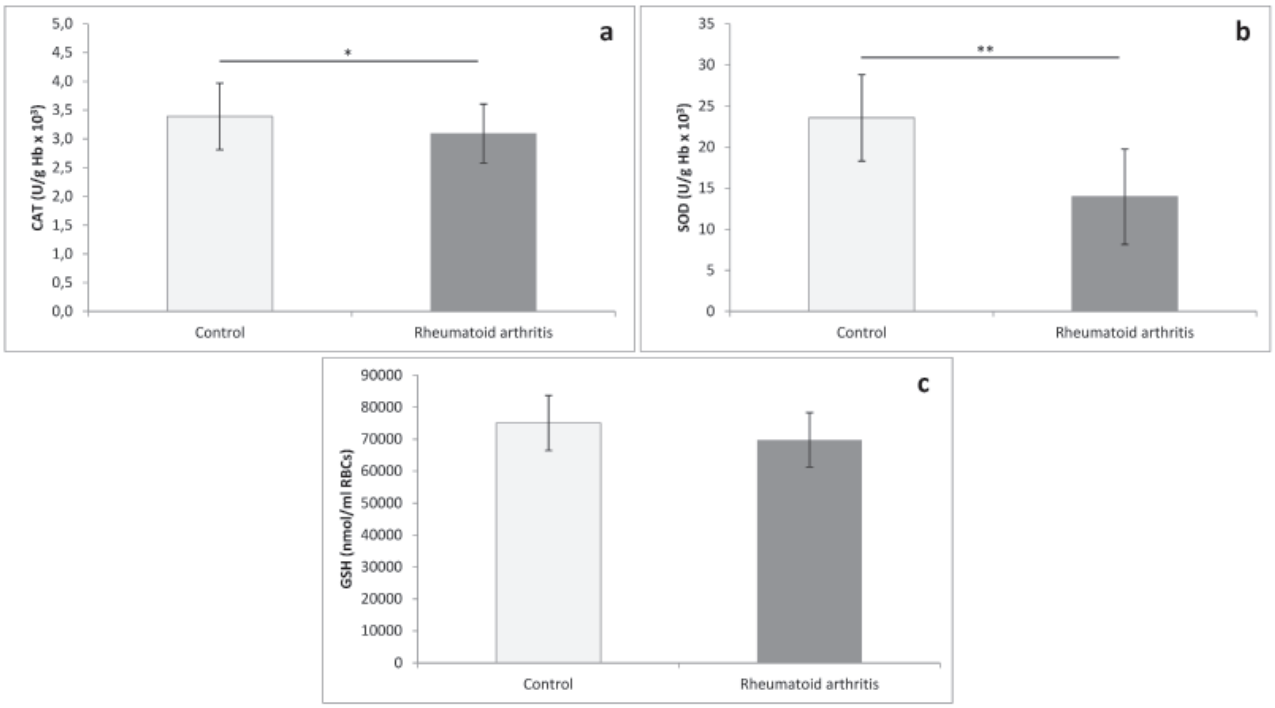

Figure 2. Activity of antioxidant parameters in control group compared to patients with rheumatoid arthritis. Data are presented as following: **a** – Catalase activity (CAT); **b** – Superoxide dismutase activity (SOD); **c** –Level of reduced glutathione (GSH). Data are expressed as mean ± SD (*p<0.05; **p<0.01).

of oxidants or their elimination weakness by antioxidant defense system. In this study, balance of pro-oxidative and oxidative parameters was assayed to gain insight into the oxidative stress status in RA.

Previously, it has been shown that NO values were statistically higher in patients with RA compared with healthy controls (1) (8) NO is a reactive nitrogen species produced mainly by macrophages and it is an important component of the oxidative burst during inflammation and autoimmunity. Interestingly, in our study, statistically higher values of nitrites were noticed in healthy control compared to patients suffering of RA (Fig 1c). Some of possible explanation for these results is in the fact that combined NO and superoxide anion radical can induce deadly peroxynitrite formation. Nitric oxide ($NO^{\cdot}$) is produced by the deamination of L-arginine from nitric oxide synthase. This oxide reacts with superoxide radical in favor of peroxynitrite production (24). In our study we noticed lower values of O_2^- in patients compared to control group without statistical significance (Fig 1b). This can be result of the previously mentioned peroxynitrite synthesis due to primary higher values of NO and O_2^- in RA patients that induced reaction between themselves and reduce O_2^- and NO levels. O_2^- has a half-life of 10^{-6} of a second, and NO molecules last for few seconds, both of which support this possibility (25). Furthermore, it is quite possible that the highest values of NO is in inflammation is *in situ* on the original place of inflammation origin, in joints. It has been found that one more important source of NO were articular chondrocytes and synovial fibroblasts. During chronic inflammation in affected joints, different type of cells such as neutrophils, lymphocytes, mast cells and macrophages could result with increased production of NO (1). Kundu et al showed higher concentration of NO in different biological fluids. The levels of intracellular NO were 1.56 times higher in isolated monocytes from the blood of RA patients relative to healthy as well as in monocytes of the synovial infiltrate (12). Increased levels of the marker molecule for peroxynitrite, nitrotyrosine, were found in synovial fluid samples from rheumatoid patients (24). During long-term of disease duration and high disease activity measured as DAS28 it is possible that in the place of ROS origin in inflammated joint there is strong and fast reaction between NO and O_2^- and production of peroxynitrite. But, that remains unknown considering that in our study we didn't measure values of this radical.

The primary targets of ROS are double bonds in polyunsaturated fatty acids in the cell membrane, which increase lipid peroxidation (LPO) and result in more oxidative damage. Deleterious by-product is generated by oxidative damage mediated by ROS as well as a thiobarbituric acid reactive substance (TBARS), malondialdehyde (MDA) which leads to synthesis of various adducts with protein inducing pathogenic antibodies and tissue damage in patients with RA. MDA has been reported as a primary biomarker of free radical induced lipid damage and oxidative stress (26). Increased LPO has been shown in previous studies confirming the presence of oxidative stress in rheumatoid arthritis. Vasanthi et al showed statistically higher values of MDA in patients compared to control group (1). Desai and coworkers pointed out that mean level of blood malondialdehyde (in nmol/ml) in controls was lower compared to cases (27). Large number of previous studies indicated increased levels of MDA in RA patients relative to healthy subjects in different biological fluids (14, 28, 29). Pasupathi et al showed that level of plasma and erythrocyte thiobarbituric acid reactive substances (TBARS) was markedly increased in the rheumatoid arthritis patients when compared to control subjects (30) as well as Veselinovic et al (31) who correlate with our study.

In our study values of H_2O_2 were statistically increased in patients compared to controls (Fig 1a). That follows decreased CAT activity in RA patients due to detoxified concentration of H_2O_2 by CAT. Several studies in patients with RA documented evidence of increased values of H_2O_2 levels compared to healthy control. Veselinovic and coworkers noticed higher serum concentrations in RA patients compared to healthy subjects that correlate with our results (31). The higher values of H_2O_2 were noticed in previous study by Khojah et al (8).

In our study, statistically higher SOD and CAT activity as antioxidant defense system enzymes was noticed in controls as well as higher values of GSH without statistic significance in the same group (Fig 2a-c). These enzymes have an important function in antioxidant defense system. Catalase has a different role in physiological and pathological conditions. Under physiological condition, catalase has low affinity for H_2O_2 (compared to glutathione peroxidase) but CAT affinity increases due to increased concentration of H_2O_2 (15). Glutathione peroxidase uses reduced glutathione as hydrogen donor for metabolizing H_2O_2 to water. In addition, previous findings promoted glutathione (GSH), the main thiol antioxidant, as an important anti-inflammatory mediator (32). Two enzymes of antioxidative protection, gluthathione peroxidase and catalase are very important in metabolism regulation of hydrogen peroxide which in excess can cause damage to DNA, RNA and lipids. The role of SOD is in superoxide anion neutralization one of the mail ROS involved in inflammation (10).

Futhermore, SOD and CAT activity was higher in healthy subjects relative to patients (Fig 2 a-b). The same trend was noticed in Feijóo and coworkers research. In control group there were higher SOD and CAT activity compared to RA patients (7). Likewise, higher activity of SOD and CAT followed by decreased level of pro-oxidative parameters in healthy control compared to RA patients were noticed in other studies (14, 26, 27) that correlate to our results. In previous studies values of GSH were higher in control group compared to RA patients (7, 14, 26, 27). But, our results showed correlation with Cimen and coworkers study. They showed unchanged values of GSH in controls compared to RA patients (33).

Our results are in agreement with other recent studies that indicate that oxidative stress generated within an inflamed joint can produce connective tissue destruction leading to joint and periarticular deformities in RA.

CONCLUSION

This study confirmed that redox status in RA patients is changed and that antioxidant defense system is compromised in these patients. There is a shift in the oxidant/antioxidant balance in favor of lipid peroxidation, A marked increase in ROS formation and lipid peroxidation as well as decrease in the activity of antioxidant defense system can lead to oxidative stress which may contribute to the tissue and cartilage damage observed in RA and hence to the chronicity of the disease. These results may suggest a potential benefit from exogenous antioxidant supplements in women with RA.

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MORTALITY CHARACTERISTICS IN SUMADIJA DISTRICT FROM 2010 – 2017

Natasa Mihailovic [1], Nebojsa Zdravkovic [2], Biljana Milicic [3], Sanja Kocic [1,4], Vesna Milicic [5] and Olivera Rakic [6]
[1] Institute of Public Health Kragujevac, Kragujevac, Serbia, Department of Biostatistics and Informatics
[2] University of Kragujevac, Kragujevac, Serbia, Faculty of Medical Sciences, Department of Medical statistics and informatics
[3] University of Belgrade, Belgrade, Serbia, Faculty of Dental Medicine, Department of Medical Statistics and Informatics
[4] University of Kragujevac, Kragujevac, Serbia, Faculty of Medical Sciences, Department of Social medicine
[5] University of Kragujevac, Kragujevac, Serbia, Faculty of Medical Sciences, Department of Dermatovenerology
[6] Faculty of Medicial Science, University of Priština - Kosovska Mitrovica

KARAKTERISTIKE MORTALITETA U ŠUMADIJSKOM OKRUGU 2010-2017. GODINA

Nataša Mihailović[1], Nebojša Zdravković [2], Biljana Miličić[3], Sanja Kocić[1,4], Vesna Miličić[5] i Olivera Rakić[6]
[1] Centar za biostatistiku i informatiku, Institut za javno zdravlje Kragujevac, Kragujevac, Srbija
[2] Katedra za medicinsku statistiku i informatiku, Fakultet medicinskih nauka, Univerzitet u Kragujevcu, Kragujevac, Srbija
[3] Katedra za biostatistiku i informatiku, Stomatološki fakultet, Univerzitet u Beogradu, Beograd, Srbija
[4] Katedra za socijalnu medicinu, Fakultet medicinskih nauka, Univerzitet u Kragujevcu, Kragujevac, Srbija
[5] Katedra za dermatovenerologiju, Fakultet medicinskih nauka, Univerzitet u Kragujevcu, Kragujevac, Srbija
[6] Medicinski fakultet Priština, Univerzitet u Prištini sa privremenim sedištem u Kosovskoj Mitrovici, Kosovska Mitrovica, Srbija

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ABSTRACT

Mortality rate represents a number of deaths on a particular territory per time unit. There are general and specific mortalities.

The aims at analysing the characteristics of mortality in Sumadija District for the time period ranging from 2010 – 2017.

The study includes all death cases in the District of Sumadija in period 2010-2017, 34681 cases. The data are described and analysed with χ^2 test and linear trend.

The trend analysis does not indicate any significant variations during the given time span. The gender analysis reveals that there is a significantly higher number of deceased persons among male population (52.2%, 47.8%, p<0.05). The average age of the deceased females (76.2±13.4) was higher than the average age of males (73.8±14.1) (p<0.05). The cause-specific analysis shows that natural causes of death dominate absolutely (96.5%) over violent deaths (2.7%) and undetermined causes (0.8%). The distribution of death causes according to ICD 10 shows that the most frequent causes of death are heart and blood vessel diseases, respiratory and neoplasm diseases.

The life expectancy of the inhabitants of Sumadija District is increasing over time. There was a slight decrease in the mortality rates during the observed time period. The highest number of the deceased people is 65 or more years old. Men have higher mortality rates throughout their lives. Natural death and non-communicable diseases are dominant. The most common causes of death are heart and blood vessels diseases, in women, and respiratory and neoplasm diseases, in men.

Keywords: *mortality, death causes, gender-specific mortality, Sumadija District*

SAŽETAK

Mortalitet predstavlja broj umrlih na određenoj teritoriji u određenom vremenskom periodu. Mortalitet može biti opšti i specifični.

Cilj istraživanja je analiza karakteristika smrtnosti u Šumadijskom okrugu u periodu od 2010 do 2017. Godine.

U studiji su uključene sve osobe preminule na teritoriji Šumadijskog okruga u periodu 2010-2017. Godina, njih 34681. Podaci su opisani deskriptivnim metodama i analizirani χ^2 testom i linearnim trendom.

Analiza trenda ne ukazuje na značajne varijacije broja umrlih u posmatranom periodu.

Analiza po polu ukazuje na statistički značajno veći broj preminulih osoba muškog pola (52.2% naspram 47.8%, r<0.05). Prosečna starost preminulih žena veća je u celom periodu analize (76.2±13.4) u poređenju sa preminulim osobama muškog pola (73.8±14.1) (p<0.05).

Analiza vrsta smrti pokazuje apsolutnu domninaciju prirodne smrti (96.5%) naspram nasilne (2.7%) i neodređenu (0.8%). Distribucija uzroka smrti prema MKB10 grupama bolesti pokazuje da su najčešći uzroci smrti: Bolesti srca i krvnih sudova, Bolesti respiratornog sistema i Neoplazme.

Životni vek stanovnika Šumadijskog okruga raste iz godine u godinu. Istovremeno, beleži se blago smanjenje stope mortaliteta u posmatranom periodu. Najveći broj preminulih čine osobe starosne dobi 65 i više godina. Muškarci imaju veću stopu smrtnosti tokom čitavog života. Prirodna smrt je dominantna vrsta smrti, a najčešći uzroci smrti su nezarazne bolesti. Najčešći uzrok smrti kod žena su bolesti srca i krvnih sudova, a kod muškaraca bolesti respiratornog sistema i neoplazme.

Ključne reči: *mortalitet, uzroci smrti, polno-specifični mortalitet, Šumadijski okrug .*

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Corresponding author:
Natasa Mihailovic, MD,
Institute of Public Health,
Nikole Pasica 1, 34000 Kragujevac, Serbia,
Tel: +381 34 504 533, Fax: + 381 34 331 344,
E-mail: natalimihailovic@gmail.com.

INTRODUCTION

Mortality rate represents a number of deaths on a particular territory per time unit (1, 2). In favourable circumstances, the curve which represents mortality rates has the shape of the letter "J" which indicates a low mortality during the initial years of life. This follows from biological factors so the curve has the similar shape for both sexes and all populations. However, a curve level is determined by a mortality rate of a given population which depends on external (exogenous) factors. Some of the most important external factors include life standard, health care development level, cultural characteristics, etc (3).

There are general and specific mortalities. General mortality is a demographic indicator of vital statistics which is, together with birth rate, used in the analysis of natural population growth. General mortality does not only depend on the intensity of dying, but also on age structures of populations. Populations with higher proportions of the elderly have a higher general mortality (4). Specific mortality represents mortality levels of a population relative to individual characteristics, such as sex, age, cause of death, etc.

Populations generally differ in terms of their age structures and consequently there are differences in general mortality rates. A method for comparing two or more populations is called standardization (5). Standardization can be direct and indirect. Direct standardization is used when morality rates of different age groups within observed populations are known. Global, i.e. European, population is used as standard population in such comparisons (2). Indirect standardization is used when mortality rates for age groups are not known for one population. In this case, the standardization is performed by using standardized mortality ratio.

The leading cause of death is an illness or injury that initiated a sequence of conditions that directly contributed to death or an accident or act of violence with deadly outcome. The International Classification of Diseases (10^{th} revision) is used in the collection and processing of the data about death causes (6).

AIMS

This paper aims at analysing the characteristics of mortality in Sumadija District for the time period ranging from 2010 – 2017.

MATERIAL AND METHODS

The research is designed as a retrospective cohort study. The study includes all death cases in the District of Sumadija occurring from January the 1^{st}, 2010 to December the 31^{st}, 2017. The total number of deaths during the given time period equals 34681 cases.

As a data source, the study uses Death Certificates issued on the territory of Sumadija District. Death Certificates are filled in by an appointed doctor when death occurs in a medical institution or a coroner if death occurs elsewhere. Death certificates are distributed to the Center for biostatistics and computer science of the Institute for Public Health in Kragujevac where the data is entered into a specially designed access database.

The data needed for calculating the mortality rates are obtained from the *Health statistical yearbook of Republic of Serbia* published by The Institute of Public Health of Serbia "Dr Milan Jovanovic Batut" and the annual publications *Municipalities and regions of the Republic of Serbia* published by Statistical Office of the Republic of Serbia.

Statistical analysis

The data are first described by descriptive methods and then they are analysed with adequate methods of analytic statistics. Among descriptive methods, the study uses proportions, as an indicator of the structure for categorical data and measures of variability (mean values, standard deviation) for continual data. χ^2 test with contingency tables is used in examining the significance of the differences in categorical data frequency. Data standardization was made in relation to the standard population of Serbia. The results are presented in tables and charts. The data presentation contains p values with $p \leq 0.05$ considered as statistically significant. The data are processed with SPSS (Statistical Package for the Social Sciences) 21.0 computer program.

RESULTS

The number of deaths in Sumadija District during the selected time period equals 34,681. The trend analysis does not indicate any significant variations during the given time span. The gender analysis reveals that there is a significantly higher number of deceased persons among male population (52.2% against 47.8%, $p<0.05$).

The analysis of the standardised general and gender-specific mortality shows a mild decrease in mortality over time (Chart 1). The analysis of the mortality with respect to age reveals that almost 80% of death cases were people who were 65 or more years old.

The life span of the Sumadian population has been increasing over time. The average age of the deceased was 72.8±14.2 in 2010 and 74.8±14.1 in 2017. The average age of the deceased females (76.2±13.4) was higher than the average age of males (73.8±14.1) ($p<0.05$) throughout whole time period of observation.

The cause-specific analysis shows that natural causes of death dominate absolutely (96.5%) over violent deaths (2.7%) and undetermined causes (0.8%). Non-communicable diseases are the most frequent causes of death ($p<0.05$). However, there is a mild upward trend in non-

communicable diseases as a death cause within male population (Table 1).

The distribution of death causes according to ICD 10 shows that the most frequent causes of death are heart and blood vessel diseases, respiratory and neoplasm diseases. The further analysis indicates that heart and blood vessel diseases are recently becoming more frequent as causes of death in female population while respiratory and neoplasm diseases are becoming more frequent with male population (Chart 2).

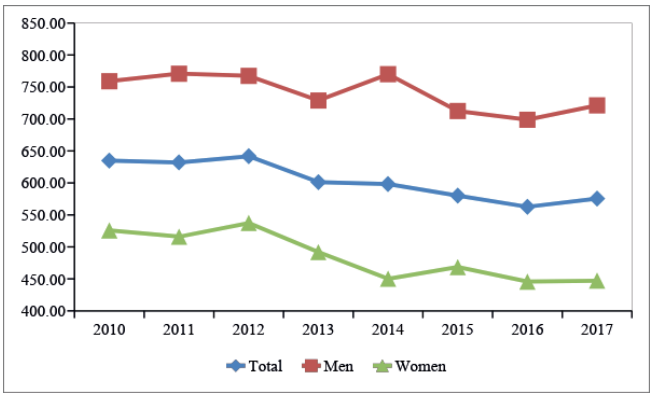
The leading causes of death during the given time period with respect to diagnoses were: Cardiomyopathia congestiva (I42.0, 23.6%), Insufficientia respiratoria, non specificata (J96.9, 5.3%), Neoplasma malignum bronchi et pulmonum, non specificatum (C34.9, 3.8%), Infarctus myocardii acutus, non specificatus (I21.9, 3.7%), Infarctus cerebri, non specificatus (I63.9, 2.9%), Hypertensio arterialis essentialis (primaria) (I10, 2.6%), Morbus pulmonum obstructivus chronicus, non specificatus (J44.9, 2.4%), Apoplexia cerebri ut haemorrhagia sive infarctus, non specificata (I64, 1.9%), Insufficientia cordis, non specificata (I50.9, 1.7%) and Morbus renalis chronicus, non specificatus (N18.9, 1.7%).

Table 1. Distribution of causes of natural death by type and sex, Sumadijski district, 2010-2016

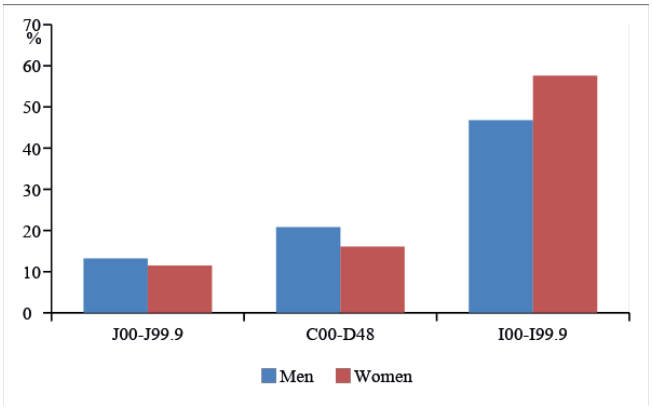
Years	Non-communicable diseases		Infectious diseases	
	Men n (%)*	Women n (%)*	Men n (%)**	Women n (%)**
2010	2050 (49.7)	2076 (50.3)	4 (57.1)	3 (42.9)
2011	2095 (50.4)	2060 (49.6)	4 (50)	4 (50)
2012	2172 (50.6)	2123 (49.4)	1 (11.1)	8 (88.9)
2013	2073 (51.1)	1982 (48.9)	4 (57.1)	3 (42.9)
2014	2263 (53.3)	1977 (46.6)	4 (80)	1 (20)
2015	2186 (52.5)	1977 (47.5)	5 (83.3)	1 (16.7)
2016	2134 (51.9)	1981 (48.1)	2 (50)	2 (50)
2017	2200 (51.6)	2065 (48.4)	2 (100)	0

* - percentage representation of non-communicable diseases by gender for a given year

** - percentage representation of infectious diseases by gender for a given year



Graph 1. Standardized general and full specific mortality of the Sumadija district per 100,000 inhabitants per population of Serbia



Graph 2. Comparative mortality in in men and women

DISCUSSION

The examinations of mortality patterns have huge socio-medical significance and are important in improving public health conditions of a population. Based on the mortality patterns, countries can create their own health policies which would aim at improving health state of the population and prolonging the life span. However, the main goal is not to prolong life expectancy, but to prolong life time without diseases and incapability (7).

Mortality analyses most commonly use a transversal method of analysis (8). With this method, mortality is analysed in shorter time intervals (two, three years). The basic disadvantage of this method is a short time span. This analysis has overcome this disadvantage by prolonging the time period of observation.

The expected life length is calculated based on the mortality tables and it represents an average number of years a person of certain age may live, assuming an unchanged age-specific mortality from a year in which it is calculated. The study, which analyzed the life span of the populations of 15 developed countries over the last 20 years, reveals an increase in life expectancy (9). According to the data for the period 2010 – 2012, the average life expectancy in Serbia equalled 72.3 years for male population and 77.3 for female population. According to *Eurostata* data for 2012, among European countries the life expectancy of males was the highest in Iceland (81.6), and the lowest in Ukraine (66.1). Female population lived longest in Spain (85.4) and shortest in Moldova (74.9).

Vital statistics provides the data about deceased persons, not only with respect to age, but also to gender, nationality, educational level, occupation, marital status, etc. It can thus provide a detailed differential analysis of mortality. In Serbia, the general mortality levels were fluctuating from 1418.1 to 1428.6 from 2011 to 2016. During the same time period, there was a decreasing trend in standardised mortality in both Serbia and Sumadija District. In Sumadija, the values of standardised mortality

fell from 586.4 in 2011 to 539.3 in 2016 (in comparison to global population). Among the former Yugoslav republics, the general mortality rate is highest in Serbia, followed by Croatia, Montenegro, FYR of Macedonia, Slovenia and Bosnia and Herzegovina. Among the neighbouring countries, the most favourable general mortality rate is recorded in Albania (7.1‰), and the least favourable in Bulgaria (15.1‰). According to the 2014 data, Bulgaria has the highest mortality rate in Europe, followed by Ukraine (14.6) and Latvia (14.3%).

The mortality analysis with respect to age groups reveals that the highest number of deceased persons is aged 65 or more. In Sumadija District, mortality within the population of this age group went up from 77% in 2010 to 80% in 2017. In Europe, this share is 60%. The highest mortality with persons older than 65 is recorded in Moldova and Kazakhstan (10).

One of the main reasons for relatively high number of deaths in Serbia is unfavourable age structure of the population, i.e. intensive population ageing. Based on the 2011 consensus, the average age of the Serbian population was 42.2 years with an ageing index of 1.25. In Sumadija District, the ratio of the elderly (above 65) and the population aged 0 – 19 was 0.8. The boundary level is 0.4 which means that Serbian population can be classified as an old population (11). The mortality during the initial years of life is specially examined. Namely, the mortality of babies has been long considered as an indicator of socio-economic life circumstances, and today it is still one of the indicators of general development of some region. Mortality shows certain regularities in gender-specific mortality – males have higher mortality rates throughout whole life. The exceptions may sporadically occur with respect to certain categories, i.e. age groups, like with females in fertile life period.

Cause-specific analyses in the world show the absolute domination of natural causes of death. However, in certain countries the numbers of violent deaths may be high. In 2016, according to World Health Organisation, a half a million people died from violent causes (murders, wars, etc.) (12).

Cause-specific mortality and the development of International Classification of Diseases originates from the seventeenth century publication *Natural and Political Observations made upon the Bills of Mortality* by John Grant who tried to classify and describe death causes with children aged 0 – 6 based on the available death reports. Today, in developed countries, there is a trend of grouping death causes into several leading noncommunicable diseases. In 2016, among 56.9 million of the deceased in the world, 54% died from "The top 10 causes of death". The most common causes depend on socioeconomic development, so they vary among the countries. In developed countries, noncommunicable diseases dominate, including ischemic heart disease, stroke, dementia, neoplasms. In Europe, 80% of deaths are caused by non-communicable diseases, which are responsible for the death of every third person under the age of 65 (13). In underdeveloped countries, there is a slight change in the trends of death causes – there is still an absolute dominance of infectious diseases but there is also a mild increase in non-communicable diseases. The most common causes of death are: respiratory system infection, diarrhea, stroke, ischemic heart disease and HIV infection (14).

The most common causes of death in Serbia are circulatory system disorders, neoplasm, respiratory system diseases and injuries and poisoning (78.9% of deaths). A comparison between 2010 and 2016 shows that there is an upward trend in the number of deaths due to neoplasm and diseases of respiratory system from 20.9% to 21.8% and from 4% to 4.8% (respectively). At the same time, there is a downward trend in the number of deaths from circulatory system diseases (from 54.7% to 51.7%) and injuries and poisoning (from 3.3% to 2.8%).

CONSLUSION

The life expectancy of the inhabitants of Sumadija District is increasing over time.

In Sumadija District there was a slight decrease in the mortality rates during the observed time period.

The highest number of the deceased people is 65 or more years old.

Men have higher mortality rates throughout their lives.

Natural death is dominant and the most common causes of death are non-communicable diseases.

The most common causes of death are heart and blood vessels diseases, in women, and respiratory and neoplasm diseases, in men.

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THE EVALUATION OF SALIVA OXIDATIVE AND ANTIOXIDATIVE MARKERS' LEVELS IN ADOLESCENTS WITH GINGIVAL INFLAMMATION

Vesna Obradovic
Department of Dentistry, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia

PROCENA NIVOA OKSIDATIVNIH I ANTIOKSIDATIVNIH MARKERA PLJUVAČKE KOD ADOLESCENATA SA ZAPALJENJEM GINGIVE

Vesna Obradović
Katedra za stomatologiju, Fakultet medicinskih nauka Univerzitet u Kragujevcu, Kragujevac, Srbija

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ABSTRACT

Periodontal disease is highly prevalent and affects 10%–15% of the world population. Salivary markers of oxidative stress and antioxidant status represent promising tool for research of oral diseases. Given the importance of reactive oxygen species (ROS) in the pathogenesis of periodontal disease, the aim of this study was to determine the association between oxidative stress parameters and periodontal disease gingivitis in adolescents. The study included a consecutive sample of 80 male and female participants referred to the Institute of Dentistry, Kragujevac, Serbia, as a result of periodontal problems or for routine control, aged 18 to 21, with definitive dentition as an inclusion criterion. Patients were divided into three groups depending on their gingival inflammation status. The first group was control group (n=20) with no gingival inflammation, the second group was with mild gingival inflammation (n=19), the third group was with moderate gingival inflammation (n=21) and the fourth group was with severe gingival inflammation (n=20). Oxidative stress parameters were measured in unstimulated whole saliva samples (superoxide anion radical, hydrogen peroxide, nitric oxide, index of lipid peroxidation, reduced gluthatione, catalase and superoxide dismutase). We have found increased levels of hydrogen peroxide and reduced glutathione in the saliva of patients with moderate levels of gingival inflammation, while the other markers were not significantly affected. In conclusion, oxidative stress plays a central role in the pathogenesis and the determination of oxidative and antioxidative levels could be a potent tool in controlling the development of gingivitis.

Keywords: *gingivitis, adolescents, oxidative and antioxidative markers, unstimulated saliva.*

SAŽETAK

Periodontalna oboljenja su veoma česta i javljaju se kod 10%-15% svetske populacije. Pljuvačni markeri oksidativnog stresa i antioksidativnog statusa su od velike pomoći u istraživanju oralnih oboljenja. Zbog važnosti reaktivnih kiseoničnih vrsta (ROS) u patogenezi periodontalnih oboljenja, cilj ovog istraživanja je bio da se odredi povezanost parametara oksidativnog stresa sa periodontalnim oboljenjem gingivitisom, kod adolescenata. Uzorak od 80 muškaraca i žena pregledanih u Zavodu za stomatologiju Kragujevac, ili zbog periodontalnih problema, ili zbog rutinske kontrole , uzrasta izmedju 18 i 21godina i kompletiranom stalnom denticijom kao uključujućim kriterijumom, je korišćen u istraživanju. Pacijenti su bili podeljeni u tri grupe u zavisnosti od stepena izraženosti zapaljenja gingive.Prva grupa je bila kontrolna grupa (n=20) bez zapaljenja, druga je imala blago zapaljenje (n=19), treća grupa je imala zapaljenje srednjeg stepena(n=21), i četvrta je bila sa najtežim stepenom inflamacije (n=20). Parametri oksidativnog stresa su bili izmereni u uzorcima nestimulisane pljuvačke (superoksid anjon radikal, vodonik peroksid, azot monoksid, indeks lipidne peroksidacije , redukovani glutation , katalaza i superoksid dizmutaza). Otkrili smo povišene nivoe vodonik peroksida i redukovanog glutationa u pljuvački pacijenata sa srednjim stepenom zapaljenja, dok drugi markeri nisu bili mnogo izmenjeni. U zaključku, oksidativni stres ima veliku ulogu u patogenezi i odredjivanje oksidativnih i antioksidativnih nivoa bi moglo biti moćno orudje u kontrolisanju razvoja gingivitisa.

Ključne reči: *gingivitis, adolescenti, oksidativni i antioksidativni markeri, nestimulisana pljuvačka.*

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INTRODUCTION

Periodontal disease is highly prevalent and affects 10%–15% of the world population (1). The inflammatory and immune response induced by subgingival plaque is the most important factor in the development of this disease. Subgingival plaque composition and biota have been the subject of several studies, since the presence of different bacteria subtypes has been found to be associated with periodontal status deterioration, greater pocket depth, and higher bleeding indices (2, 3).

Gingivitis and periodontitis are two distinct chronic inflammatory processes belonging to the spectrum of periodontal diseases of the oral cavity affecting the tooth supporting tissues in response to bacterial accumulation. In contrast to periodontitis, gingivitis is initiated only after a few days of inadequate oral hygiene procedures by local plaque deposits adjacent to the highly vascularized gingival tissues. Gingivitis is a superficial inflammatory affection and is not destructive towards the surrounding connective and bone tissues and completely declines with the initiation of adequate oral hygiene procedures (1-3).

Evidence has shown an association between ROS and gingivitis (4-8). This is important because both ROS in the pathogenesis of periodontal disease and the different composition of the biotain periodontal pockets are related to periodontitis (4,5). Periodontal disease can be described as one of the predominant polymicrobial infections of humans (6). Strong evidence of periodontal etiology has been demonstrated for Porphyromonas gingivalis (PG), Aggregatibacter actinomycetemcomitans (Aa), Treponema denticola (TD) and Tannerella forsythia (TF) (7).

Still, gingivitis development mechanisms are not well understood. The disorder is probably multifactorial, and it is characterized by the generation of ROS (4-6) by activated phagocytes at the gingival sulcus (5-7) which have the ability to initiate the destruction of connective tissue. ROS are generated during mitochondrial oxidative metabolism as well as in cellular response to xenobiotics, cytokines and bacterial invasion. Oxidative stress refers to the imbalance due to excess ROS or oxidants over the capability of the cell to mount an effective antioxidant response (8).

Salivary markers of oxidative stress and antioxidant status represent promising tools for research of oral diseases (9). Oxidative stress represents disbalance between the production of various ROS and the activity of endogenous antioxidative defense system (ADS) (9). These reactive molecules are reported to be capable of inducing periodontal tissue destruction. The ability of the host to scavenge ROS is regarded as a key protective mechanism against inadvertent ROS mediated host tissue damage (9, 10). Oxidative stress has been involved in pathogenesis of more than two hundred chronic and acute diseases (9-11). However, the connection between the increased production of ROS and gingival inflammation in adolescents is still very poorly investigated. Given the importance of ROS in the pathogenesis of periodontal disease, the aim of this study was to determine the association between oxidative stress parameters and periodontal disease gingivitis in adolescents.

PATIENTS AND METHODS

Compliance with Ethical Standards

All patients signed an informed consent before the initiation of the study. The study was approved by the Ethics Committee of the local institution (Institute for Dentistry, Kragujevac) in compliance with ethical standards.

Study Group

A consecutive sample of 80 male and female individuals referred to the Institute of Dentistry, Kragujevac, Serbia, as a result of periodontal problems or for routine control, between 18 and 21 years old and with definitive dentition, as inclusion criteria, were included in the study. Patients were divided into three groups depending on their gingival inflammation status. First group was control group (n=20) with no gingival inflammation, second group was with mild gingival inflammation (n=19), third group was with moderate gingival inflammation (n=21) and fourth group was with severe gingival inflammation (n=20). The degree of gingival inflammation was evaluated and have had clinically proven gingivitis, by commonly applied index of WHO (12). This study included adolescents with gingival inflammation except the examinees in the control group, and excluded adolescents without any other acute or chronic disease. The study also excluded patients who, for any reason, did not have completed permanent dentition. None of the patients wore orthodontic appliance, because orthodontic therapy could change the composition of saliva significantly. All clinical measurements were performed by the same investigator.

Collection of unstimulated whole saliva

Oxidative stress parameters were measured in unstimulated whole saliva samples, which were collected in the morning, after at least 12 hours of fasting before clinical examinations and bacterial collection. Subjects were instructed to allow saliva to pool in the bottom of the mouth and drain it into a collection tube when necessary and not to swallow any saliva for the duration of the collection. Before the analysis, saliva was centrifuged at 4.000 ×g for 10 min at 4°C to eliminate cell debris and the supernatant was aliquoted and stored at −80°C until the analysis (13).

Oxidative markers from saliva (determination of superoxide anion radical (O2-), hydrogen peroxide (H2O2), index of lipid peroxidation (TBARS) and nitrites (NO2-))

The level of superoxide anion radical (O2-) was measured NBT (nitroblue tetrazolium) reaction in TRIS-buffer combined with saliva samples and read at 530 nm (14).

The protocol for measurement of hydrogen peroxide (H2O2) was based on oxidation of phenol red in the presence horseradish peroxidase (POD). 200 µl sample with 800 µl PRS (phenol red solution) and 10 µl POD were combined (1:20). The level of H2O2 was measured at 610 nm (15).

Nitrite (NO2-) was determined as an index of nitric oxide production with Griess reagent. 0.1 ml 3N PCA (perchloric acid), 0.4 ml 20 mM EDTA (ethilenediaminetetraacetic acid) and 0.2 ml saliva were put on ice for 15 min and then centrifuged 15 min at 6000 rpm. After pouring supernatant, 220 µl K2CO3 was added. Nitrites were measured at 550 nm. Distilled water was used as a blank probe (16).

The degree of lipid peroxidation in saliva was estimated by measuring thiobarbituric acid reactive substances (TBARS) using 0.4 ml 1% TBA (thiobarbituric acid) in 0.05 NaOH mixed with 0.8 ml of saliva, incubated at 100°C for 15 min and measured at 530 nm. Distillated water was used as a blank probe. TBA extract was obtained by combining 0.8 ml saliva and 0.4 ml TCA (trichloroacetic acid) and then samples were put on ice for 10 min, and centrifuged for 15 min at 6000 rpm (17).

Antioxidative markers from saliva (determination of reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT))

The level of reduced glutathione (GSH) was determined based on GSH oxidation with 5.5- dithio-bis-6.2-nitrobenzoic acid, using method by Beutler (18).

SOD activity was determined by the epinephrine method of Beutler. A hundred µl supernatant and 1ml carbonate buffer were mixed, and then 100 µl of epinephrine was added. Detection was performed at 470nm (19).

CAT activity was determined according to Aebi. Supernatants of saliva were diluted with distilled water (1:7 v/v) and treated with chloroform-ethanol (0.6:1 v/v) to remove haemoglobin and then 50 µl CAT buffer, 100 µl sample and 1 ml 10 mM H2O2 were added to the samples. Detection was performed at 360 nm (20).

Statistical analysis

Results are expressed as means ± standard deviation (SD). All results shown are expressed as mean and 95% confidence interval. Statistical comparisons between groups were assessed by Mann-Whitney or Kruskal-Wallis tests. The independent variables were age, gender (as confounding variables) and the different oxidative markers. All p values were two-tailed and probability values of less than 0.05 were considered to be statistically significant. Statistical analyses were performed using SPSS software (IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.).

RESULTS

Basic characterics of study population

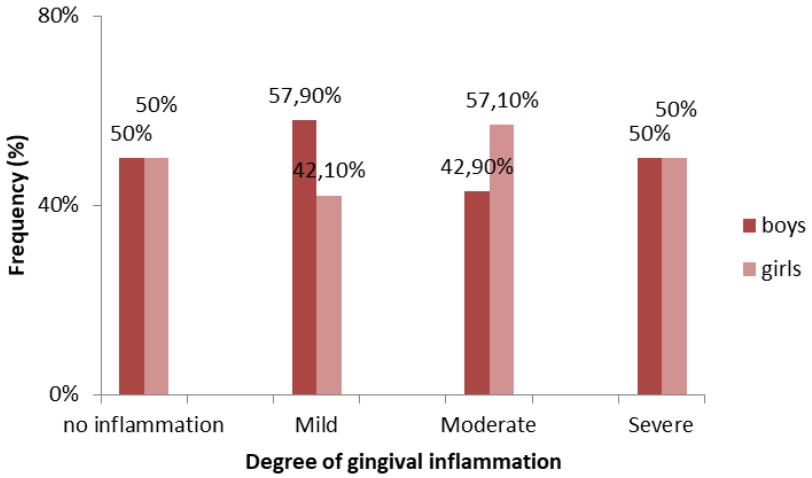
Among 80 subjects in this study, 40 participants (50%) were female, and 40 participants (50%) were male. The study included adolescents from 18 to 21 years old, and the average age was 19.15±0.66 in the first group, 19.10±0.66 in the second group, 19.26±0.70 in the third group and 19.18±0.60 in the fourth group (Table 1). The distribution of participants according to the degree of gingival inflammation is shown in Table 1.

Table 1. Basic characteristics of the study population and the distribution of the degree of gingival inflamma-tion among the study population

		Without	**Mild**	**Moderate**		
Number of participants		20	19	21	**Degree of gingival inflammation**	*p*
Gender	Male	10 (50%)	11 (57.9%)	**Severe**	10 (50%)	[b]*p*=0,825
	Female	10 (50%)	8 (42.1%)	12 (57.1%)	10 (50%)	
Age		19.15±0.66	19.10±0.66	19.26±0.70	19.18±0.60	[a]*p*=0,857
(X±SD (Med, min-max)		(19.25; 18-27)	(19.0; 18-21)	(19.50; 18-21)	(19.1; 18.1-21)	

**p; at-test; bχ 2-test*

Figure 1. Frequency of basic characteristics of the study population and the distribution of the degree of gingival inflammation among the study population in percent (%)



O_2^- values

Values of superoxide anion radical were not significantly higher in the experimental groups compared to the control ($p>0.05$). Also, in comparing levels of this marker among groups with present inflammation, statistical difference was not proved ($p>0.05$) (Table 2).

H_2O_2 values

Values of H_2O_2 were significantly higher in the group with moderate inflammation comparing to the control, mild and severe inflammation group ($p<0.05$). When comparing experimental groups among themselves, the values of this marker were again higher in the group with moderate gingival inflammation compared to the groups with mild and severe inflammation ($p<0.05$) (Table 2).

NO_2^- values

Values of nitrites were not significantly higher in the experimental groups compared to the control ($p>0.05$). When comparing experimental groups among themselves, the values of this marker were higher in the group with severe inflammation compared to the group with mild and moderate inflammation and compared to the control group, but not significantly ($p>0.05$) (Table 2).

TBARS values

Values of TBARS were not statistically affected in the groups with gingival inflammation compared to the control ($p>0.05$). In the group of patients with severe inflammation levels of TBARS were increased but not significantly ($p>0.05$) (Table 2).

Table 2. Levels of prooxidative and antioxidative markers from saliva samples during different of gingival inflammation among the study population

Parameters	Degree of gingival inflammation				*P value*
	Without	Mild	Moderate	Severe	
TBARS (µmol/ml)	3.53±2.36 (3.73; 0.11-7.25)	3.42±2.21 (3.89; 0.17-7.22)	3,63±2,86 (4,09; 0,10-7,22)	4,38±2,20 (4,90; 0,61-7,01)	[a]p=0,594
NO (nmol/mol)	14,45±8,11 (11.46; 2.47-33.69)	14,23±7,05 (13.11; 5.55-28.68)	13,59±6,16 (11.52; 5.89-25.43)	14,96±5,92 (14.93; 6.56-33.70)	[a]p=0.935
O_2^- (nmol/mol)	2.39±2.78 (1.48; 0.33-10.22)	2.81±3.78 (1.98; 0.33-17.78)	1.68±0.99 (1.65; 0.33-4.28)	1.93±1.36 (1.65; 0.0-5.60)	[b]p=0.639

Parameters	Degree of gingival inflammation				*P value*
	Without	**Mild**	**Moderate**	**Severe**	
H_2O_2 (nmol/mol)	0.47±0.27 (0.53; 0.023-0.967)	0.31±0.34 (0.14; 0.046-1.16)	0.30±0.25 (0.23; 0.023-0.967)	0.28±0.30 (0.18; 0.023-1.382)	**[b]p=0.047***
SOD (U/mg protein)	35.39±40.52 (20.20; 0.0-146.52)	35.13±83.19 (16.28; 0.0-374.40)	56.20±106.29 (16.28; 0.0-463.98)	25.64±45.92 (16.28; 0.0-211.64)	[b]p=0.714
CAT (U/mg protein)	140.45±86.54 (125.6; 28.75-408.5)	173.52±122.91 (134.4; 25.75-545.0)	173.60±80.59 (157.75;96.0-381.0)	192.05±130.47 (158.12; 25.75-564.5)	[a]p=0.493
GSH (U/mg protein)	478.74±391.28 (356.14; 83.73-1842)	367.98±225.27 (293.06; 83.73-711.7)	707.73±201.73 (586.12; 83.73-2051)	537.97±445.41 (397.72; 83.73-1716)	**[b]p=0.012***

**p<0.05; [a]ANOVA; [b]Kruskal Wallis test*

GSH values

Values of GSH were significantly changed in the group with moderate inflammation compared to the control, and compared to the groups with mild and severe inflammation ($p<0.05$). Also, among all experimental groups, levels of this marker were significantly changed ($p<0.05$) (Table 2).

SOD values

Values of SOD were not significantly changed in the group with inflammation compared to the control ($p>0.05$). Also, among all experimental groups, levels of this marker were not significantly changed ($p>0.05$) (Table 2).

CAT values

Similarly to SOD, values of CAT were not significantly changed in the group with inflammation compared to the control ($p>0.05$). Also, among all experimental groups, levels of this marker were not significantly changed ($p>0.05$) (Table 2).

DISCUSSION

The present study was aimed at assessing potential connection between oxidative stress and gingival inflammation at different clinical stages. This topic was very poorly investigated in reliable databases, especially having in mind that our study population consisted of adolescents.

The medical significance of oxidative stress has become increasingly recognized to the point that it is now considered to be a component of virtually every disease process. More recently, evidence has also emerged for a crucial role of ROS in periodontal tissue destruction (1-5). ROS are described as oxygen free radicals and other non-radical oxygen derivatives involved in oxygen radical production (7). They are involved in normal cellular metabolism and continuously generated by the cells in most tissues. Another category of substances called antioxidants exist in the cells and can effectively delay or inhibit ROS-induced oxidation (2). Under physiological conditions, ROS are effectively neutralized by antioxidants, which prevent ROS-mediated tissue damage. When an inflammation happens, ROS production is drastically increased mainly due to cells of innate immune system, e.g., neutrophils and macrophages during the process of phagocytosis via the metabolic pathway of the "respiratory burst" (9). Subsequently, high levels or activities of ROS cannot be balanced by the antioxidant defense system, which leads to the oxidative stress and tissue damage (8). ROS can directly cause tissue damage, involving lipid peroxidation, DNA damage, protein damage, and oxidation of important enzymes; meanwhile, they can function as signaling molecules or mediators of inflammation (1-9).

In the first part of the study, we examined the potential connection between superoxide anion radical and hydrogen peroxide production during different levels of gingival inflammation in adolescents aged 18 to 21. O_2^- and H_2O_2, as a very reactive oxygen species, are responsible for various oxidative stress-related damages of organs and tissues. It is well-known that neutrophils are the most abundant blood white cells and belong to the first defense line against bacterial infection. During an infection, after the initiation of the host response by pathogenic biofilm, neutrophils become the most common inflammatory cells gathering in periodontal

tissue and gingival sulcus and they are believed to be the predominant source of ROS in periodontitis (8, 9). Following the stimulation by pathogens, neutrophils produce O_2^- via the metabolic pathway called "respiratory burst" catalyzed by NADPH oxidase during phagocytosis (4, 9). O_2^- can be released into phagosomal and extracellular environment and then converted to different radical and non-radical derivatives, such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), hydroxyl radical (OH•) and singlet oxygen ($^1O_2$).

In our study, values of O_2^- were not significantly higher in the experimental groups compared to the control ($p>0.05$). Also, in comparing levels of this marker among groups with present inflammation, statistical difference was not proved ($p>0.05$) (Table 2). On the other hand, values of H_2O_2 were significantly higher in the group with moderate inflammation compared to the control, mild and severe inflammation group (Table 2). Previous clinical study also confirmed connection between increased ROS production and gingival inflammation. Given the close relation between inflammation and oxidative stress, the role of ROS and antioxidant systems in the pathogenesis of periodontal tissue injury and gingival inflammation has regained attention in the last years (21-23).

Furthermore, we examined the influence of gingival inflammation on index of lipid peroxidation and nitric oxide in saliva samples of participants. None of the mentioned markers were influenced in patients with gingival inflammation compared to patients without gingivitis (Table 2). In our study, local oxidative response to mild and severe inflammation was weak or undetected, but moderate inflammation induced significant increase of hydrogen peroxide in saliva.

Lamster and Novak described that oxidants can also lipid peroxidation, induction of pro-inflammatory cytokines, and hydrolytic enzymes, such as, MMPs, as well as inactivation of protease inhibitors. Additionally, H_2O_2 overproduced extracellularly can even pass through biologic membranes freely and act as intracellular second messengers, activating a variety of signal transduction pathways (24).

Various studies have pointed toward the axiom that the reactive oxygen species and antioxidants are in dynamic equilibrium and any disturbance in one would lead to changes in the other (25).

Studies have confirmed that the inflammatory response in gingivitis is associated with an increased local and systemic oxidative stress and compromised antioxidant capacity (25). The results of our study partially confirmed previous research, and during the elevated levels of hydrogen peroxide, we found elevated levels of GSH in the same group of participants and during the moderate degree of gingivitis.

Numerous studies showed higher level of antioxidant markers in saliva of gingivitis patients compared with that of healthy controls as well as their significant association with clinical periodontal parameters (26).

One of the few studies about oxidative status in the state of gingival inflammation in adolescents is the study by *Marton et al*. They reported about the level of malondialdehyde, a stable end-product of lipid peroxidation which was induced by reactive oxygen intermediates and the activity of two potent antioxidant enzymes, superoxide dismutase and glutathione peroxidase from tissue homogenates of 22 surgical periapical granuloma specimens. In their study, malondialdehyde levels were significantly higher and glutathione peroxidase activity was significantly lower in periapical granuloma samples than in healthy gingival tissue homogenates, which were used as controls. The activity of superoxide dismutase was similar in periapical granuloma and in the control samples. The results of the mentioned study indicated an altered balance between the production and the elimination of toxic oxygen metabolites in chronic apical periodontitis, and it was concluded that reactive oxygen intermediates, which are being produced by activated phagocytic cells abundantly present in periapical granulomas, can contribute to periapical tissue injury and bone loss in this disease (27).

Brock et al reported that the levels of antioxidant markers were significantly higher in adult gingivitis patients than in healthy periodontal controls. They concluded the involvement of ROS in periodontal pathology and reported that it would be modulated by *in vivo* antioxidant defense systems (28).

Literature data did not suggest differences of oxidative status in youth and adult period, but suggested potential mechanisms by which gingivitis induces ROS production. Namely, ROS are able to induce the activation of the key matrix myeloperoxidases (MMPs) in periodontal tissues, such as MMP-8 and MMP-9, through direct enzyme oxidation, although indirect mechanisms involving intracellular signaling cannot be precluded. MMP-8 and MMP-9 are both promising periodontal and apical disease biomarkers which cooperatively hydrolyze type I collagen, a key step in periodontal supporting tissue loss. PMN-derived myeloperoxidase (MPO) catalyzes HOCl release and besides its antimicrobial effects, it has been reported to oxidatively activate latent proMMP-8 and -9 in vitro and inactivate tissue inhibitor of metalloproteinase (TIMP)-1. Ex vivo studies suggest that oxidative activation of MMP-8 and MMP-9 represents the dominant mechanism in destructive periodontal lesions as well as in gingivitis (29-32).

Previous clinical studies, as well as our results, suggest that the antioxidant capacity of gingivitis patients is both qualitatively and quantitatively distinct from that of saliva, plasma and serum. Whether changes in the redox status is, in gingivitis is undoubtedly present predisposition to or the results of ROS-mediated damage. Probably, the type of sample for determination of markers of redox status may be a reason for partial differences of our results with the results of some previous studies (33, 34).

Also, it is important to notice that presence of different types of bacteria could differently influence on markers of oxidative stress by different mechanism (35).

Limitation of our study is that our study is focuses on the increased local oxidative stress in gingivitis, and specifically on the relationship between the local and systemic biomarkers of oxidative stress. Also, the relationship between gingivitis and systemic inflammation, and the effects of periodontal therapy on oxidative stress parameters are not still evaluated and discussed in this stage of our study.

CONCLUSION

In conclusion, oxidative stress plays a central role in the pathogenesis and the determination of oxidative and antioxidative levels could be a potent tool in controlling the development of gingivitis. Further studies are needed to unravel the complex effects of ROS in gingival tissue breakdown and their associated systemic diseases, as well as the potential contributions of adjuvant antioxidant therapies.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interests relevant to the manuscript.

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FACTORS ASSOCIATED WITH THE OCCURRENCE OF DEATH OUTCOME IN CHILDREN WITH NEONATAL RESPIRATORY DISTRESS SYNDROME

Aleksandar G. Kočović[1], Gordana D. Kostić[2], Dragana M. Savić[2], Milica M. Stanojević[3], Miloš N. Milosavljević[1], Slobodan M. Janković[4] , Milica N Milosavljević[5] and Srdjan M. Stefanović[1]

[1]*University of Kragujevac, Faculty of Medical Sciences, Department of Pharmacy, Serbia*
[2]*University of Kragujevac, Faculty of Medical Sciences, Department of Pediatrics, Serbia*
[3]*College of Applied Health Sciences Cuprija*
[4]*University of Kragujevac, Faculty of Medical Sciences, Department of Pharmacology and Toxicology, Serbia*
[5]*University of Pristina, Medical Faculty, Pristina*

FAKTORI UDRUŽENI SA POJAVOM SMRTNOG ISHODA KOD DECE SA NEONATALNIM RESPIRATORNIM DISTRES SINDROMOM

Aleksandar G. Kočović[1], Gordana D. Kostić[2], Dragana M. Savić[2], Milica M. Stanojević[3], Miloš N. Milosavljević[1], Slobodan M. Janković[4] , Milica N Milosavljević[5] i Srdjan M. Stefanović[1]

[1]*Univerzitet u Kragujevcu, Fakultet medicinskih nauka, Katedraza farmaciju, Kragujevac, Srbija*
[2] *Univerzitet u Kragujevcu, Fakultet medicinskih nauka, Katedra za pedijatriju, Kragujevac, Srbija*
[3]*Visoka škola primenjenih zdravstvenih nauka, Ćuprija, Srbija*
[4]*Univerzitet u Kragujevcu, Fakultet medicinskih nauka, Katedra za farmakologiju i toksikologiju, Kragujevac, Srbija*
[5]*Univerzitet u Prištini, Medicinski fakultet, Priština*

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ABSTRACT

Neonatal respiratory distress syndrome (NRDS) is a consequence of immaturity at birth and it is still associated with relatively high mortality rate. The aim of this study was to identify the factors associated with the occurrence of fatal outcome in newborns with neonatal respiratory distress syndrome.The research was designed as a case-control study nested in a retrospective cohort, and it enrolled newborns treated during 2015 at Pediatric Clinic of Clinical Center in Kragujevac. Diagnosis of NRDS and decision about the treatment were left at the discretion of attending pediatricians. The cases were patients with fatal outcome, while controls were randomly selected from the pool of survivors and matched with each case by gender in a ratio of 4:1. The study included 371 newborns, of whom 201 (54.2%) were male and 170 (45.8%) female. Lethal outcome occurred in 36 newborns (9,7%). Significant association was found between death and APGAR score ($OR_{adjusted}$: 0.516, 95% CI: 0.322-0.827), weight on delivery ($OR_{adjusted}$: 0.996, 95% CI: 0.993-0.999), duration of hospitalization ($OR_{adjusted}$: 0.901, 95% CI: 0.835-0.972) and mechanical ventilation ($OR_{adjusted}$: 165.256, 95% CI: 7.616-3585.714). Higher gestational age, higher birth weight, higher APGAR score and longer duration of hospitalization were singled out as protective factors, while use of mechanical ventilation increased the risk of death. Major limitations of the study were retrospective nature and relatively small number of identified cases. Postponing delivery and delivery in institution with neonatal intensive care unit are crucial for survival of newborns with NRDS.

Keywords: *neonatal respiratory distress syndrome, risk factors, mechanical ventilation.*

SAŽETAK

Neonatalni respiratorni distres sindrom se javlja kao posledica nedovoljne zrelosti deteta na rođenju i još uvek se često završava smrtnim ishodom. Cilj rada je bio da se identifikuju faktori koji su udruženi sa pojavom smrtnog ishoda kod novorođenčadi sa neonatalnim respiratornim distres sindromom.

Istraživanje je dizajnirano kaostudija tipa slučaj-kontrola u retrospektivnom kohortu, a sprovedeno je na novorođenčadi koja su tokom 2015. godine lečena na Pedijatrijskoj klinici Kliničkog centra u Kragujevcu. Postavljanje dijagnoze neonatalnog respiratornog distres sindroma, kao i odluke o terapijskim procedurama bili su u nadležnosti dežurnog pedijatra. Slučajevi su bili pacijenti sa letalnim ishodom, dok su kontrole nasumično odabrane iz grupe preživelih pacijenata, a povezivane sa svakim od slučajeva po polu u odnosu 4:1. U studiju je uključeno 371 novorođenče od čega je 201 (54,2%) novorođenče bilo muškog pola, a 170 (45,8%) ženskog pola. Kod 36 (9,7%) novorođenčadi nastupio je smrtni ishod. Značajna povezanost u prilagođenom regresionom modelu uočena je između smrtnog ishoda i APGAR skora (OR: 0.516, 95% CI: 0.322-0.827), telesne težine na porođaju (OR: 0.996, 95% CI: 0.993-0.999), trajanja hospitalizacije (OR: 0.901, 95% CI: 0.835-0.972) i primene mehaničke ventilacije (OR: 165.256, 95% CI: 7.616-3585.714). Veća gestacijska starost, veća telesna masa na rođenju, veća vrednost APGAR skora i duža hospitalizacija su se izdvojili kao protektivni faktori, dok je primena mehaničke ventilacije fakor rizika za smrtni ishod. Glavna ograničenja studije su retrospektivni karakter istraživanja i relativno mali broj identifikovanih slučajeva. Odlaganje porođaja i porođaj u ustanovi koja poseduje jedinicu neonatalne intenzivne nege su ključni faktori za preživljvanje dece sa neonatalnim respiratornim distres sindromom.

Ključne reči: *Neonatalni respiratorni distres sindrom, faktori rizika, mehanička ventilacija.*

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Corresponding author:
Milos N Milosavljevic
Faculty of Medical Sciences
University of Kragujevac
69 Svetozara Markovica Street
34000 Kragujevac, Serbia
+381669047886
milosavljevicmilos91@gmail.com

INTRODUCTION

Neonatal respiratory distress syndrome (NRDS), also known as the disease of the hyaline membranes, is characterized by respiratory failure that occurs after the birth or during the next 48 hours. This syndrome is potentially life-threatening and difficult to treat, with sudden onset and progressive course during the first hours after birth (1). It could be also associated with serious acute and chronic complications, which may further contribute to deterioration of the infants' health and their quality of life (2-4).

Main reason for occurrence of NRDS is lack of surfactant, which occurs as a result of the immaturity of the enzymatic systems necessary for its synthesis (5-7). However, some authors suggest that development of NRDS has a genetic foundation (8). Based on previous studies, the most important risk factor for the occurrence of NRDS is a premature birth. The other risk factors include maternal diabetes mellitus, perinatal asphyxia, history of NRDS occurrences in the family and delivery by caesarean section (9-10).

In recent years, progress was made in understanding pathophysiology as well as in the treatment of this condition, which led to certain reduction in morbidity and mortality rates (11). However, an emerging problem is an increasing number of premature newborns, especially those with gestational age between 34 and 37 weeks (12). Although risk factors for neonatal respiratory distress syndrome were well documented, the data linking certain factors with the occurrence of fatal outcome in these patients are still scarce.

The aim of this study is to identify factors associated with the occurrence of death in newborns with NRDS, and to analyze the degree of their relative impact on the observed outcome.

PATIENTS AND METHODS

A total of 371 newborns with NRDS treated in Center for Neonatology at Pediatric Clinic of Clinical Center Kragujevac between January 1st and December 31st, 2015, were enrolled in a retrospective cohort. Relevant demographic and clinical data were extracted from the patients' histories. The diagnosis of NRDS and decision about the treatment were left at discretion of attending pediatricians. The newborns with incomplete files as well as those referred from other hospitals (where they have been previously diagnosed and treated) to Neonatal Intensive Unit of Pediatric Clinic in Kragujevac were excluded from the study. This study was approved by Ethics Committee of Clinical Centre Kragujevac.

Based on outcome of interest, i.e. death due to NRDS, a case-control study was nested in aforementioned retrospective cohort. Cases (n=36) were patients with fatal outcome, while controls (n=144) were participants who survived and whose treatment was successfully completed. These two groups were individually matched by gender, and for each case there were four matched controls randomly selected from survived patients enrolled in the cohort study, as previously mentioned. Cases and controls were then compared in terms of factors assumed to have an important association with death, such as gestational age (in weeks), type of birth (vaginal delivery or caesarean section), APGAR score, weight on delivery, duration of hospitalization, mechanical ventilation, duration of mechanical ventilation, oxygen therapy, surfactant therapy and the development of acute complications of NRDS such as pneumonia, pneumothorax and pulmonary hemorrhage.

Sample size calculation

In order to determine required sample size for this research, a following calculation for categorical/dichotomous variables were performed (13):

$$N = 4 \cdot (z_{1-\frac{\alpha}{2}})^2 \cdot \frac{p(1-p)}{GP^2}$$

Where N stands for– number of patients in the sample; p – is the proportion of characteristic in the sample; GP – Confidence interval width; $(z_{1-\frac{\alpha}{2}})$ – 1.96 (with probability of 95%). The effect size was taken from the study by Smolarova et al. (14), who had found the incidence of lethal outcome in 27% of newborns with NRDS with 95% confidence interval of 20-35%. According to previously mentioned parameters, minimum necessary sample size was 134 newborns.

Statistical analysis

The baseline characteristics of the patients were summarized by descriptive statistics. Means ± standard deviations were used for presenting continuous data, and frequencies (percentages) for presenting categorical variables. After the normality of the data distribution for continuous variables had been checked by Kolmogorov-Smirnov test, an appropriate parametric or nonparametric test (i.e. Student's T test for independent samples or Mann-Whitney U test) was used to evaluate the observed differences. Significance of differences in the rates of categorical variables were tested by the Chi-square test with Yates continuity correction in 2*2 contingency tables, or in a case of low prevalence of particular categories by Fisher's test. The influence of independent variables on the dichotomous outcome (i.e. patient was dead or alive) was tested using univariate and a stepwise backwards conditional multivariate logistic regression analysis. The results were shown as crude and adjusted odds ratios (ORs) with corresponding 95% confidence intervals (CI). The level of significance was 0,05 in all analyses, while stepwise regression model removed all variables with an additional probability (p value) of 0.1 and above. The association between observed risk/protective factors and death was considered significant if 95% CI of adjusted OR did not include the value of 1. All calculations were performed by statistical program for social sciences (SPSS version 18).

RESULTS

The study included 371 newborns in total, of whom 201 were male (54.1%) and 170 female (45.8%). In total, 36 newborns died (9,7%), 13 male (36.1%) and 23 female (63.9%). Average gestational age was 35.3±4.6 weeks and average birth weight was 2500.8±1039.8 grams. Complications (pneumonia, pneumothorax and pulmonary hemorrhage) were identified in 82 newborns (22.1%) and a total number of complications was 89. There were 60 cases of pneumonia, 18 cases of pneumothorax and 11 cases of pulmonary hemorrhage. Seven newborns (1.9%) had two complications.

Baseline characteristics of cases and controls are shown in Table 1. Highly significant differences between cases and controls were observed in the following features: gestational age ($p<0.001$), APGAR score ($p<0.001$), weight on delivery ($p<0.001$), duration of hospitalization ($p<0.001$), mechanical ventilation ($p<0.001$), duration of mechanical ventilation ($p<0.001$), surfactant therapy ($p<0.001$), presence of pneumonia ($p=0.010$) or pulmonary hemorrhage ($p=0.001$).

Table 1. Baseline characteristics of cases and controls.

Variable	Cases (n=36)	Controls (n=144)	Test value and probability of null hypothesis
Gestational age (weeks)	28.4±5.2	37.1±3.4	U=604.000 p=0.000*
Type of birth - vaginal delivery - cesarean section	 24 (66.7%) 12 (33.3%)	 80 (55.6%) 64 (44.4%)	χ^2=1.457 p=0.227
APGAR score	3.9±2.6	8.0±1.7	U=622.500 p=0.000*
Weight on delivery (grams)	1242.8±929.2	2918.5±826.1	U=531.500 p=0.000*
Duration of hospitalization (days)	6.8±12.6	15.3±9.1	U=750.500 p=0.000*
Mechanical ventilation	35 (97.2%)	45 (31.3%)	χ^2=50.766 p=0.000*
Duration of mechanical ventilation (days)	6.8±12.6	2.4±5.8	U=1131.500 p=0.000*
Oxygen therapy	34 (94.4%)	303 (84.0%)	χ^2=2.613 p=0.106
Surfactant therapy	27 (75.0%)	63 (16.0%)	χ^2=50.019 p=0.000*
Pneumonia	0 (0.0%)	23 (16.0%)	χ^2=6.592 p=0.010*
Pneumothorax	5 (13.9%)	13 (6.9%)	χ^2=1.818 p=0.178
Pulmonary hemorrhage	4 (11.1%)	1 (0.7%)	χ^2=11.571 p=0.001*
Results are presented as mean ± standard deviation, or n (%); *Significant difference			

The results of the both univariate and multivariate binary logistic regression with a model of acceptable quality (Cox & Snell R square 0.527 Nagelkerke R square 0.833, Hosmer-Lemeshow Chi square 2.148, df = 8, p = 0.976) are shown in Tables 2 and 3. After adjustment for potential confounders and other independent variables it was shown that death was more likely in patients with lower APGAR score and weight on delivery, shorter period of hospitalization and use of mechanical ventilation support (Table 2). Although crude ORs for gestational age, surfactant therapy and the development of acute complications such as pneumothorax and pulmonary hemorrhage pointed out on an important association with death, after adjustment, their influence did not reach a level of statistical significance.

Table 2. Crude and adjusted odds ratios (OR) of the risk factors for death in neonates due to NRDS

Risk factors	**Univariate model Crude OR (95% CI)**	**Multivariate model Adjusted# OR (95% CI)**
Gestational age (weeks)	0.685 (0.609-0.771)	1.870 (0.942-3.715)
Type of birth (referent category vaginal delivery)	0.591 (0.275-1.272)	0.101 (0.009-1.181)
APGAR score	0.465 (0.369-0.584)	0.516 (0.322-0.827)
Weight on delivery (grams)	0.998 (0.998-0.999)	0.996 (0.993-0.999)
Duration of hospitalization (days)	0.830 (0.774-0.890)	0.901 (0.835-0.972)
Mechanical ventilation	105.000 (13.884-794.091)	165.256 (7.616-3585.714)
Duration of mechanical ventilation (days)	1.042 (1.004-1.081)	0.000 (0.000-/)
Oxygen therapy	1.795 (0.431-9.065)	0.000 (0.000-/)
Surfactant therapy	14.280 (5.989-34.049)	0.173 (0.009-3.299)
Pneumonia	0.000 (0.000-/)	0.000 (0.000-/)
Pneumothorax	3.710 (1.064-12.937)	0.863 (0.040-18.544)
Pulmonary hemorrhage	5.875 (1.253-27.552)	4.865 (0.232-102.024)
p – Statistical significance; CI – Confidence interval; * Statistically significant # Adjusted for gestational age, type of birth, APGAR score, weight on delivery, duration of hospitalization, mechanical ventilation and surfactant therapy.		

Table 3. Multivariate model quality characteristics

Parameter	**Value**	**df**	**p**
Cox & Snell R square	0.527	8	0.976
Nagelkerke R square	0.833		
Hosmer-Lemeshow Chi square	2.148		
df – Degrees of freedom; p – Statistical significance			

DISSCUSION

Our study identified higher gestational age, higher birth weight, higher APGAR score and longer duration of hospitalization as protective factors for death outcome in newborns with NRDS, while the use of mechanical ventilation increased the risk of death.

Respiratory distress syndrome is one of the most common causes of infant death worldwide. Incidence data vary from country to country, but it is common for incidence rates to be higher when gestational age is lower (1). In our study, of the 371 patients, 36 patients died, which makes about 10%. This result is consistent with the data provided by the World Health Organization and the European Neonatology Association (15-16).

The results of this study indicate a statistically significant difference in gestational age in children who suffered from respiratory distress syndrome in comparison with those who did not, which is consistent with previous studies and data obtained in the last extensive study in which the incidence ranged from 92% when the gestational age was around 24-25 weeks and gradually decreased to 57% at gestational age of 30-31 weeks (1, 17). It should also be noted that gestational age loses significance as a protective factor when viewed in combination with other observed factors. Possible cause of this phenomenon is that gestational age was not determined precisely in all pregnant women, as observed in other studies (12).

Higher APGAR score and longer hospitalization were identified as protective factors, like in other similar studies (18-20). In general, the APGAR score over 7 is associated with better survival. Duration of hospitalization ranges between 10 and 32 days, depending on a study sample (21-22). A longer stay in intensive care units and more frequent survival are associated with the fact that severe patients' fatal outcomes occur within 48 hours, while the patients with a milder clinical picture require longer treatment, and consequently there is much greater chance of survival. Average number of hospitalization days in our cases was 6.8±12.6.

Regarding decreased death rate in patients who were not treated by surfactants, care should be taken when drawing conclusions. It is important to note that the surfactant is administered in patients with the severe clinical picture who initially have greater likelihood of fatal outcome. Besides, studies examining possibilities of less invasive use of surfactants (via nasal cannula or aerosol), which would reduce the probability of lung injury, are underway, but none of these techniques entered routine clinical practice (23-25).

In most cases, mechanical ventilation is necessary to increase chances of survival of patients with respiratory distress syndrome. Although we had information about the number of days that a patient spent on mechanical ventilation, the details about characteristics of the procedure itself were not available (whether the pressure was positive or negative, what modality was used, what was the number of respirations, complications, etc.) (26-28), which makes explanation of the impact of mechanical ventilation on lethal outcome very difficult. Respiratory complications of respiratory distress syndrome occur either as a result of the primary respiratory disorder or more often as a result of therapy, and in most cases as a result of intubation and mechanical ventilation (1). The patients who developed pneumonia were treated by antibiotics (29-31), and those who experienced pneumothorax by surgical drainage. Pulmonary hemorrhage occurred only in a few cases, which is consistent with other studies. Treatment of pulmonary hemorrhage implied theuse of antifibrinolytics such as a tranexamic and aminocaproic acid (32-33). Other acute complications in this study were not monitored.

Evidence of a chronic complications of respiratory distress syndrome (34-37) was not found in the patients' files, although ophthalmologists (due to retinopathy) and child's neurologists (due to neurological disorders) were regularly consulted, which was in line with current treatment guidelines for respiratory distress syndrome (1). A possible reason why these complications were not observed was that the patient's follow-up period was too short, or that the data from the control visit were not available.

One of major limitations of this study was its retrospective nature, so certain details were missing for some patients and the data about follow-up after discharge were not available. Also, data concerning maternal health and course of pregnancy were not always adequately noted. Small number of cases is another important limitation, which decreased number of factors that could have been included in the regression model. In future research, more attention should be paid to acute complications not covered by this study and treatment procedures themselves. Besides, overall maternal health and occurrence of hypertension and diabetes in pregnancy should be taken into account. A prospective study should also be considered, which would reduce the amount of missing data in patients and enable identification of currently unknown risk factors.

CONCLUSION

In conclusion, higher gestational age, higher birth weight, higher APGAR score and longer duration of hospitalization are protective factors, while use of mechanical ventilation increases the risk of death. Postponing delivery as much as possible and conducting delivery in an institution with neonatal intensive care unit are crucial for ensuring survival of newborns with neonatal respiratory distress syndrome.

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THE EFFECTS OF DIRECT RED BULL ADMINISTRATION TO ISOLATED HEARTS OF TRAINED AND UNTRAINED RATS WHO REGULARLY CONSUMED OR DID NOT CONSUME ENERGY DRINK: FOCUS ON CARDIODYNAMICS AND OXIDATIVE STRESS

Irena Pusica[1], Ivan Srejovic[2], Jovana Bradic[2], Jelena Smigic[2], Stefani Bolevich[3], Sergey Bolevich[4], Vladimir Jakovljević[2,4] and Dusica Djordjevic[2]
[1]*Primary Health Centre New Belgrade, Belgrade, Serbia*
[2]*Department of Physiology, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia*
[3]*Department of Pathophysiology, 1st Moscow State Medical University I.M. Sechenov, Russian Federation*
[4]*Department of Human Pathology, 1st Moscow State Medical University I.M. Sechenov, Russian Federation*

EFEKTI DIREKTNE ADMINISTRACIJE RED BULL-A U IZOLOVANOM SRCU TRENIRANIH I NETRENIRANIH PACOVA KOJI SU REDOVNO KONZUMIRALI ILI NISU KONZUMIRALI ENERGETSKO PIĆE: FOKUS NA KARDIODINAMIKU I OKSIDATIVNI STRES

Irena Pušica[1], Ivan Srejović[2], Jovana Bradić[2], Jelena Šmigić[2], Stefani Bolevich[3], Sergey Bolevich[4], Vladimir Jakovljević[2,4] i Dušica Đorđević[2]
[1]*Dom zdravlja Novi Beograd, Beograd, Srbija*
[2]*Katedra za fiziologiju, Fakultet medicinskih nauka, Univerzitet u Kragujevcu, Kragujevac, Srbija*
[3]*Katedra za patofiziologiju, Prvi moskovski državni medicinski univerzitet I.M. Sečenov, Ruska federacija*
[4]*Katedra za humanu patologiju, Prvi moskovski državni medicinski univerzitet I.M. Sečenov, Ruska federacija*

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ABSTRACT

Energy drinks (EDs) contain caffeine and other active ingredients which affect cardiovascular system. The aims of this study were to examine direct effects of Red Bull (RB) on cardiodynamics and oxidative stress in isolated hearts of rats. The rats were divided into four groups: untrained rats who never consumed ED (dED-UT); untrained rats who consumed ED 5 days a week during 4 weeks (ch+dED-UT); rats trained 5 times a week for 4 weeks, but did not consume ED (dED-T); rats trained and consumed ED 5 times a week for 4 weeks (ch+dED-T). After sacrificing, hearts were isolated and perfused according to Langendorff technique. Through the isolated heart of all rats in each group, RB was administered. The parameters of cardiac function were recorded, and the levels of prooxidants were measured in the coronary effluent during coronary autoregulation. Rats in ch+dED-UT group had significantly lower rates of myocardial contraction and relaxation compared to rats in dED-UT group. The same effect was recorded in the dED-T group compared to dED-UT group. The levels of hydrogen peroxide were significantly higher in trained rats. Rats in ch+dED-T group also had significantly higher levels of superoxide anion radical and index of lipid peroxidation, as well as lower levels of nitrites when compared to ch+dED-UT group, while opposite effect was recorded in rats in dED-T group compared to dED-UT group. The RB could have a potentially negative inotropic effect in chronic consumers. Prooxidative effect of RB was most pronounced in trained chronic consumers.

Keywords: *cardiovascular system, energy drinks, oxidative stress, rats, swimming*

SAŽETAK

Energetska pića (EP) sadrže kofein i druge aktivne sastojke koji utiču na kardiovaskularni sistem. Ciljevi ovog istraživanja bili su da se utvrde direktni efekti Red Bull-a (RB) na kardiodinamiku i oksidativni stres u izolovanim srcima pacova. Pacovi su bili podeljeni u četiri grupe: netrenirani pacovi koji nikada nisu konzumirali EP (dED-UT); netrenirani pacovi koji su konzumirali EP, 5 dana nedeljno tokom 4 nedelje (ch+dED-UT); trenirani pacovi 5 puta nedeljno, tokom 4 nedelje, koji nisu konzumirali EP (dED-T); trenirani pacovi koji su konzumirali EP, 5 puta nedeljno tokom 4 nedelje (ch+dED-T). Nakon žrtvovanja, srca pacova su izolovana i perfundovana prema tehnici po Langendorff-u. Kroz izolovana srca svih pacova u svakoj grupi, administriran je RB. Određivani su parametri funkcije srca, kao i nivo prooksidativnih vrsta u koronarnom efluentu tokom koronarne autoregulacije. Pacovi u grupi ch+dED-UT imali su značajno niže stope kontrakcije i relaksacije miokarda u poređenju sa pacovima u grupi dED-UT. Isti efekat zabeležen je u grupi dED-T u odnosu na grupu dED-UT. Nivoi vodonik peroksida bili su značajno viši u grupi treniranih pacova. Pacovi u grupi ch+dED-T, imali su takođe značajno više nivoe superoksid anjon radikala i indeksa lipidne peroksidacije, kao i niže nivoe nitrita u poređenju sa grupom ch+dED-UT, dok je suprotan efekat zabeležen kod pacova u dED-T grupi u poređenju sa dED-UT grupom. RB bi mogao imati potencijalno negativan inotropan efekat kod hroničnih konzumera. Prooksidativni efekat RB bio je najizraženiji kod treniranih hroničnih konzumera.

Ključne reči: *kardiovaskularni sistem, energetska pića, oksidativni stres, pacovi, plivanje*

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Corresponding author:
Irena Pušica;
Primary Health Centre New Belgrade,
Bulevar maršala Tolbuhina 30, 11 070 New Belgrade, Serbia.,
Tel.: +381 11 2222 100; +381 63 620 976; Fax: +381 11 2222 190.
E-mail: irenapetrovic83@yahoo.com

ABBREVATIONS:

ED - energy drink, **RB** - Red Bull, **CPP** - coronary perfusion pressure,
dp/dt max - maximum rate of left ventricular pressure development,
dp/dt min - minimum rate of left ventricular pressure development,
SLVP - systolic left ventricular pressure, **DLVP** - diastolic left ventricular pressure,
HR - heart rate, **CF -** coronary flow, O_2^- - superoxide anion radical,
H_2O_2 - hydrogen peroxide, **NO** - nitrogen monoxide,
TBARS - thiobarbituric acid reactive substances

INTRODUCTION

The term "energy drink" (ED) is used for caffeinated beverages, which are consumed in order to improve physical and mental performance (1). Extensive advertising, the colourful packaging and noted short-term improvement in performance after consumption have resulted in the high popularity of EDs. They are most popular among athletes, drivers and young people (2).

The effects of EDs are associated with the frequency and amount of consumption, as well as with the concentration and interaction of ingredients (3). The health hazard data on EDs are found to be limited; therefore the assessment of their safety is based on the impact of individual ingredients. Red Bull (RB) is one of the most commonly consumed EDs (4). The ingredients of the original RB are as follows: caffeine, taurine, glucuronolactone, carbohydrates, and B-group vitamins (5). Caffeine, a methylxanthine, increases sympathetic nerve activity (6). Taurine, a derivative of the amino acid cysteine (6), increases muscle strength, improves endurance, reduces physical exercise-induced DNA damage and accelerates recovery after training (7). The consumption of glucose or other carbohydrates before and during physical activity delays the onset of fatigue, conserves muscle glycogen and improves performance (8). Glucuronolactone is a naturally occurring substance (5), which is formed from glucose in the liver (5, 9) and it is added to EDs to fight fatigue and provide a sense of well-being (10). The B-group vitamins belong to a group of water-soluble vitamins that have the role of coenzyme and are important for the proper function of cells, especially mitochondrial function and energy production (11).

EDs exhibit a maximum effect 30-60 minutes after ingestion (12). It is assumed that most of the biological effects of EDs are mediated by a positive inotropic effect, which implies increase in heart rate, cardiac output, myocardial contractility, stroke volume and arterial blood pressure (13). Particularly important is the effect of EDs on the changes in ventricular repolarization (14). The results of meta-analysis have shown that acute consumption of EDs significantly raises systolic and diastolic arterial pressure, while there is no significant effect on the heart rate (15). In athletes, acute consumption of RB had a positive inotropic effect (enhancement of left ventricular and left atrial contractility) in the recovery period after physical exercise (16). But also, the case series of ED-associated acute adverse cardiovascular events were described (17). ED consumption may lead to the increased cardiomyocytes apoptosis, which could be the cause of cardiovascular disorders in the ED consumers (2). Chronic ED consumption may be the cause of hypertensive heart disease, coronary artery disease, cerebrovascular disease and peripheral arterial disease (18). EDs affect the increase in blood glucose level, total cholesterol, triglycerides and low-density lipoprotein cholesterol, which all contribute to an increase in cardiovascular risk (19, 13). Endothelial dysfunction and increased risk of myocardial ischaemia, which may occur due to the loss of nitrogen monoxide (NO) activity in the blood vessel wall, have also been linked to ED consumption (20). However, it has also been demonstrated that acute RB consumption may exhibit a positive effect on the endothelial function (21, 22). Furthermore, results of previous studies have shown that acute ED administration induced lipid peroxidation and oxidative stress in the liver and brain of rats (23), and that the chronic use of energy drinks leads to toxicity, an inflammatory response and oxidative stress (24-30). However, it was shown that RB administration in rats for 14 days caused the acceleration of soft tissue healing and that was explained by the antioxidant effects of the RB ingredients (31).

Previous studies have shown various results regarding the effects of EDs on heart and cardiovascular system, depending on the dose, population, existence of risk factors and other protocol variables. To our knowledge, there is currently no published research examining the direct effect of ED on the heart. Thus, the main aim of this study was to examine direct effects of RB on cardiodynamics, coronary flow and oxidative stress in isolated rat hearts. Furthermore, the aim of the study was to examine if regular training or daily ED consumption change the effects that direct ED administration has on previously mentioned parameters.

MATERIALS AND METHODS

The study was conducted in the Laboratory for Cardiovascular Physiology at the Faculty of Medical Sciences, University of Kragujevac. It was approved by the Ethics Committee of the Faculty. The conception, design and performance of the study were followed by Good Laboratory Practice criteria and the European Council Directive (86/609EEC).

Subjects

The Wistar albino rats were used in this study. The sample size calculation, based on a study published by Barcelos et al. (32), revealed that 24 rats were required to perform the study. At the beginning of the study, rats were eight weeks old and their weight was 200-250g. They were housed in conventional cages in groups of 8 animals per cage. They were fed with commercial rat food (20% protein food, Veterinary Institute Subotica) and water ad libitum. Room temperature was set to 25 °C and 12 hours of light were provided.

ED consumption and training protocol

The study lasted 4 weeks. The rats were divided into four groups (six rats in each group) depending on chronic ED consumption during the study period (rats who did and did not consume ED every day) and depending on whether they were subjected to the training protocol or not. After sacrificing the animals, ED was administered to the isolated heart of all rats in each group (all hearts were perfused with ED, as explained bellow).

Thus, groups were as follows:

dED-UT group: untrained (sedentary) rats who never consumed ED,

ch+dED-UT group: untrained rats who consumed ED 5 days a week during 4 weeks,

dED-T group: trained rats 5 times a week for 4 weeks, which did not consume ED,

ch+dED-T group: trained rats which consumed ED 5 times a week for 4 weeks.

The rats of two groups were subjected to a swimming practice (1h per day, 5 days a week) in a 80x60x100cm pool for experimental animals. An electric heater was used to keep the water temperature at 34 C°. During swimming, the pump installed in the pool made constant waves, in order to prevent the rats from floating. Rats were monitored the whole time during swimming. The rats in other two groups were untrained (they were not subjected to a swimming practice).

The ED was administered to rats in two groups by an intragastric gavage (p.o.). RB was used in the amount of 3.75 ml/kg, as determined on the basis of the previously published studies (32, 33). The indicated dose corresponds to a dose of caffeine close to the maximum recommended (about 6 mg /kg). A standard can of 250 ml RB contains: 80 mg of caffeine, 1000 mg of taurine, 21.5 g of sucrose, 5.25 g of glucose, 600 mg of glucuronolactone, 20 mg of vitamin B3 (niacinamide), 5 mg of vitamin B5 (calcium pantothenate), 5 mg of vitamin B6 (pyridoxine hydrochloride), 50 mg of inositol, 5 µg of vitamin B12 (cyanocobalamin), 100 mg of sodium citrate, as well as natural and artificial flavors and colors (caramel, riboflavin) (34,11,35).

After one month, rats were sacrificed by short ketamine/xylazine narcosis. After that, their hearts were excised and attached to the Langendorff apparatus via aortic cannula. Krebs–Henseleit buffer was used during the performance of retrograde perfusion according to the Langendorff technique. First, an equilibration period, during which coronary perfusion pressure (CPP) was kept at 70 cmH_2O, was performed. After that, CPP was changed in the following order: 1) 60 cmH_2O, 2) 80 cmH_2O, 3) 100 cmH_2O, 4) 120 cmH_2O, and 5) 40 cmH_2O. Through the isolated heart of all rats in each group, Krebs–Henseleit buffer, in which 150 µmol of ED was dissolved, was perfused by the Langendorff retrograde perfusion method.

Cardiodynamic parameters

Parameters of myocardial function were measured using the pressure sensor (transducer BS4 73-0184, Experimentria Ltd, Hungary) which was attached to the latex ballon, filled with bubble-free saline, which was inserted into the left chamber (36). Cardiodynamic parameters were continuously measured. The following parameters of myocardial function were recorded: 1) maximum and minimum rate of pressure development in LV (dp/dt max and dp/dt min), 2) systolic and diastolic left ventricle pressure (SLVP and DLVP) and 3) heart rate (HR). Furthermore, coronary flow (CF) was measured flowmetrically.

Oxidative stress

Coronary flow, which was collected during each CPP, was used to measure the levels of oxidative stress in coronary venous effluent. Spectrophotometer (Analytic Jena Specord S 600, UK) was used to determine the levels of 1) superoxide anion radical (O_2^-), 2) hydrogen peroxide (H_2O_2), 3) nitrogen monoxide (NO) and 4) index of lipid peroxidation (thiobarbituric acid reactive substances, TBARS). The exact protocols for measurement of those prooxidative species may be found in our previously published paper (37) or in the original sources (38-41).

Statistics

SPSS 23.0 was used to perform the statistical analysis. Comparison of groups was performed using the parametric (t-test for independent samples) or nonparametric test (Mann-Whitney U test), depending on the results of the Shapiro-Wilk test for data distribution. The results on the figures are shown as the mean ± standard error of the mean (X ± SE).

RESULTS

Cardiodynamic parameters of isolated rat hearts in four groups (dED-T, ch+dED-T, dED-UT, ch+dED-UT) are shown in Figures 1-6. Prooxidative parameters in the effluent during coronary autoregulation of isolated rat hearts in four groups (dED-T, ch+dED-T, dED-UT, ch+dED-UT) are shown in Figures 7-10.

1) The direct effect of the ED on the heart of untrained rats who chronically consumed the ED and those who did not consume ED

Cardiodynamics

In relation to the dED-UT group, the following were recorded in the ch+dED-UT group: 1) at all CPPs, level of dp/dt max was lower, but statistically significant only at CPP 60-100 cmH_2O ($p < 0.05$); 2) at all CPPs, level of dp/dt min was lower (more positive), but statistically significant only at CPP 60-120 cmH_2O ($p < 0.05$); 3) at all CPPs, level of CF was higher, but statistically significant only at CPP 80-120 cmH_2O ($p < 0.05$); at all CPPs, statistically significantly lower level of SLVP ($p < 0.05$) and statistically significantly higher level of DLVP ($p < 0.05$); 4) at all CPPs, higher level of HR, but only statistically significantly higher at CPP 60-80 cmH_2O ($p < 0.05$).

Oxidative stress

In relation to the dED-UT group, the following were recorded in the ch+dED-UT group:

1) at all CPPs, level of O_2^- was lower, but statistically significantly only at CPP 40, 80-120 cmH_2O ($p < 0.05$); 2) at all CPPs, level of TBARS was statistically significantly lower ($p < 0.05$); 3) at all CPPs, levels of nitrites (NO) were higher, but statistically significant only at CPP 80-120 cmH_2O ($p < 0.05$); 4) at all CPPs, level of H_2O_2 was higher, but without statistical significance ($p > 0.05$).

2) The direct effect of the ED on the heart of trained rats who chronically consumed the ED and those who did not consume ED

Cardiodynamics

Although at all CPP levels dp/dt max, dp/dt min, CF and SLVP were lower in ch+dED-T than in dED-T group, and levels of DLVP higher, no statistical significance was observed in any cardiodynamic parameter between the groups ($p > 0.05$). Also, there was no statistically significant difference in the level of HR between these two groups ($p > 0.05$).

Oxidative stress

Levels of H_2O_2 at CPP 60, 100-120 cmH_2O were significantly higher in ch+dED-T when compared to dED-T group ($p < 0.05$). There was no statistically significant difference between those two groups in the levels of other prooxidative species.

3) The direct effect of the ED on the heart of trained and untrained rats who did not consume ED

Cardiodynamics

In relation to the dED-UT group, the following were recorded in the dED-T group:1) at all CPPs, statistically significantly lower level of dp/dt max, dp/dt min and SLVP ($p < 0.05$); 2) at all CPPs, level of HR was lower, but statistically significant only at CPP 40, 60 and 100 cmH_2O ($p < 0.05$); 3) at all CPPs, level of CF was higher, but statistically significant only at CPP 60-120 cmH_2O ($p < 0.05$). There was no statistically significant difference in the level of DLVP between these two groups ($p > 0.05$).

Oxidative stress

In relation to the dED-UT group, the following were recorded in the dED-T group: 1) at all CPPs, higher level of O_2^-, but without statistical significance ($p > 0.05$); 2) at all CPPs, level of H_2O_2 was higher, but statistically significant only at CPP 60-120 cmH_2O ($p < 0.05$); 3) at all CPPs, level of TBARS was lower, but statistically significant only at CPP 40-80 cmH_2O ($p < 0.05$); 4) at all CPPs, levels of nitrites (NO) were higher, but statistically significant only at CPP 80-120 cmH_2O ($p < 0.05$).

4) The direct effect of the ED on the heart of trained and untrained rats who chronically consumed the ED

Cardiodynamics

In relation to the group ch+dED-UT, the following were recorded in the group ch + dED-T: 1) at all CPPs, lower level of dp/dt max, CF and SLVP but without statistical significance ($p > 0.05$); 2) at all CPPs, level of dp/dt min was lower, but statistically significant only at CPP 40-100 cmH_2O ($p < 0.05$); 3) at all CPPs, level of DLVP was lower, but statistically significant only at CPP 40, 80-120 cmH_2O ($p < 0.05$); at all CPPs, statistically significantly lower level of HR ($p < 0.05$).

Oxidative stress

In relation to the group ch+dED-UT, the following were recorded in the group ch + dED-T: at all CPPs, level of O_2^- was higher, but statistically significant only at CPP 40, 100-120 cmH_2O ($p < 0.05$); at all CPPs, statistically significantly higher level of H_2O_2 and TBARS ($p < 0.05$); at all CPPs, levels of nitrites (NO) were lower, but statistically significant only at CPP 60-100 cmH_2O ($p < 0.05$).

FIGURES

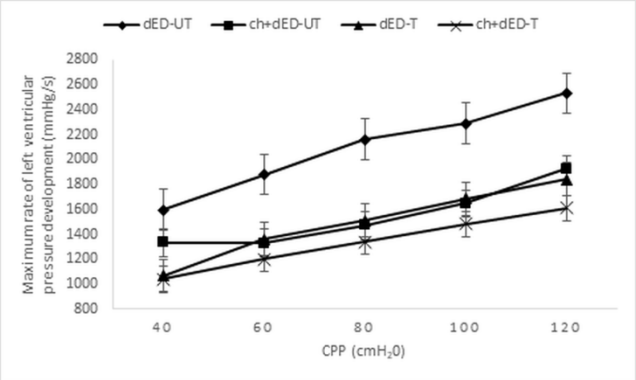

Figure 1.

Values of maximum rate of left ventricular pressure development during coronary autoregulation of the isolated trained rat hearts in the following groups: dED-UT, ch+dED-UT, dED-T, ch+dED-T. CPP, coronary perfusion pressure. Data are means ± SE.

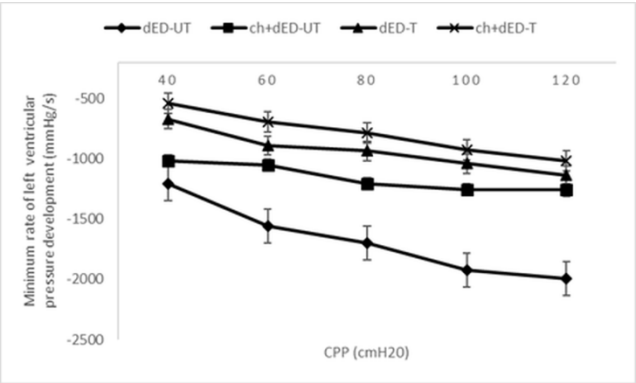

Figure 2.

Values of minimum rate of left ventricular pressure development during coronary autoregulation of the isolated trained rat hearts in the following groups: dED-UT, ch+dED-UT, dED-T, ch+dED-T. CPP, coronary perfusion pressure. Data are means ± SE.

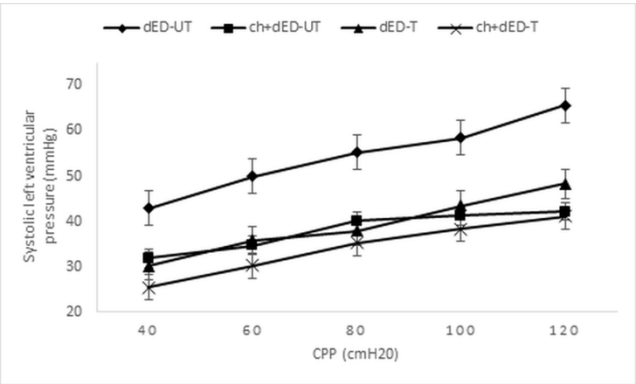

Figure 3.

Values of systolic left ventricular pressure during coronary autoregulation of the isolated trained rat hearts in the following groups: dED-UT, ch+dED-UT, dED-T, ch+dED-T. CPP, coronary perfusion pressure. Data are means ± SE.

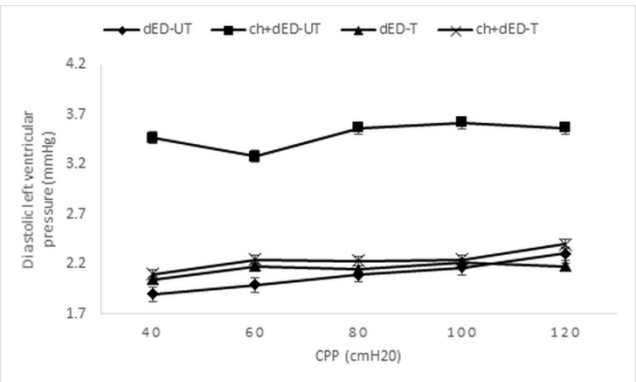

Figure 4.

Values of diastolic left ventricular pressure during coronary autoregulation of the isolated trained rat hearts in the following groups: dED-UT, ch+dED-UT, dED-T, ch+dED-T. CPP, coronary perfusion pressure. Data are means ± SE.

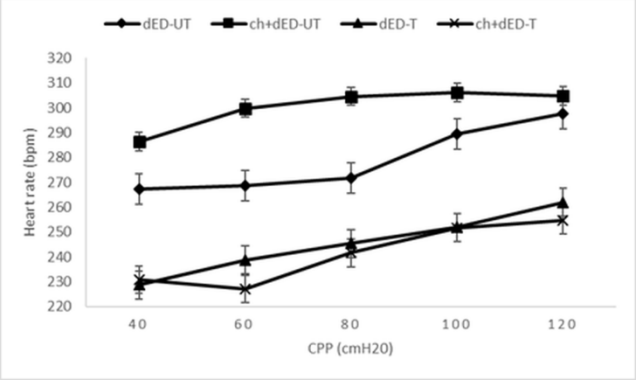

Figure 5.

Values of heart rate during coronary autoregulation of the isolated trained rat hearts in the following groups: dED-UT, ch+dED-UT, dED-T, ch+dED-T. CPP, coronary perfusion pressure. Data are means ± SE.

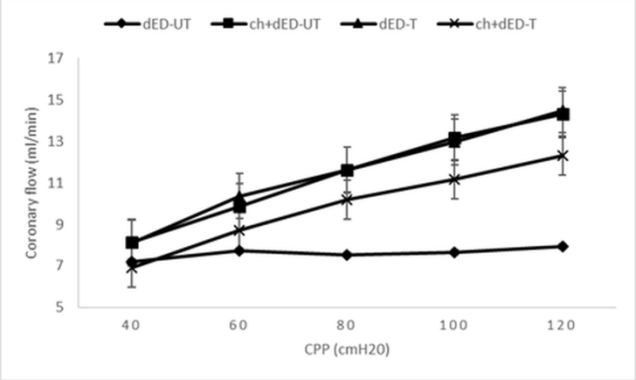

Figure 6.

Values of coronary flow during coronary autoregulation of the isolated trained rat hearts in the following groups: dED-UT, ch+dED-UT, dED-T, ch+dED-T. CPP, coronary perfusion pressure. Data are means ± SE.

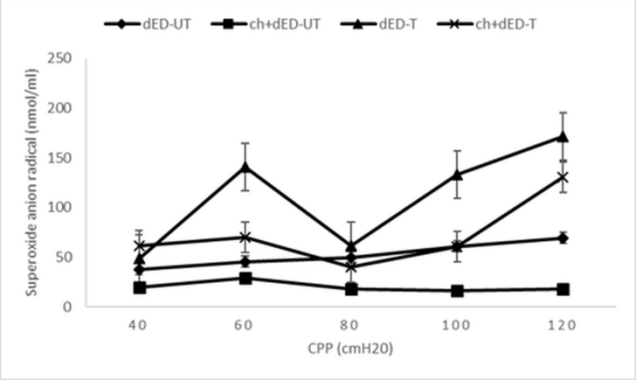

Figure 7.

Values of superoxide anion radical in effluent, during coronary autoregulation of the isolated trained rat hearts in the following groups: dED-UT, ch+dED-UT, dED-T, ch+dED-T. CPP, coronary perfusion pressure. Data are means ± SE.

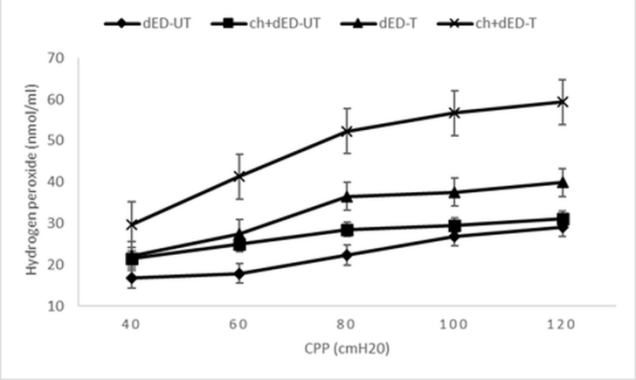

Figure 8.

Values of hydrogen peroxide in effluent, during coronary autoregulation of the isolated trained rat hearts in the following groups: dED-UT, ch+dED-UT, dED-T, ch+dED-T. CPP, coronary perfusion pressure. Data are means ± SE.

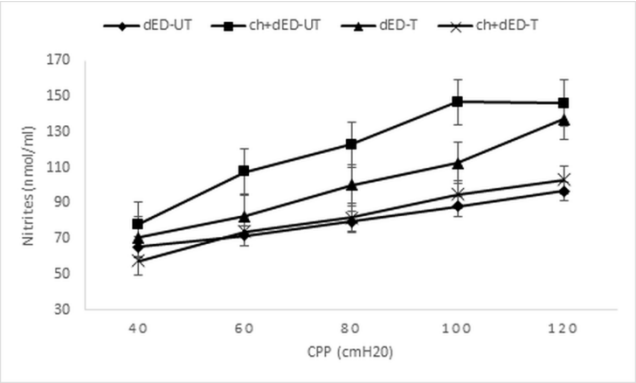

Figure 9.

Values of nitrites in effluent, during coronary autoregulation of the isolated trained rat hearts in the following groups: dED-UT, ch+dED-UT, dED-T, ch+dED-T. CPP, coronary perfusion pressure. Data are means ± SE.

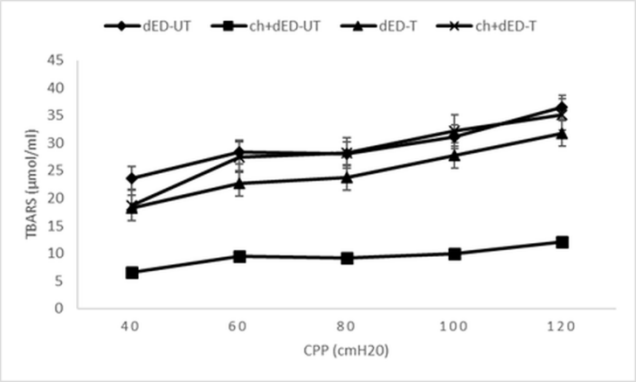

Figure 10.

Values of index of lipid peroxidation in effluent, during coronary autoregulation of the isolated trained rat hearts in the following groups: dED-UT, ch+dED-UT, dED-T, ch+dED-T. CPP, coronary perfusion pressure. Data are means ± SE.

DISCUSSION

In this research, we studied the direct effects of RB on cardiodynamics, coronary flow and oxidative stress in isolated hearts of trained and untrained rats, who chronically consumed RB in comparison with those who did not consume RB.

The results of our study showed that, after direct RB administration to the heart, untrained rats who chronically consumed RB had statistically significantly lower levels of dp/dt max, dp/dt min and SLVP, while levels of DLVP were significantly higher, compared to untrained rats who never consumed RB. The same was observed when trained rats regular RB consumers were compared to trained rats non-consumers of RB, except that no statistically significant difference between groups was found. This could be interpreted as either a potentially harmful effect of RB on the myocardial contraction and relaxation in chronic consumers, or their decreased reactivity to RB ingredients due to adaptation induced by daily consummation. Trained rats that never consumed RB had statistically significantly lower levels of dp/dt max, dp/dt min and SLVP when compared to untrained rats that never consumed RB, while trained rats that chronically consumed RB had only statistically significantly lower levels of dp/dt min when compared to untrained chronic RB consumers. This might suggest that trained rats respond less to direct RB administration, as well as that chronic RB consumption decreases the effects that acute/direct consummation has on the heart, both in trained and untrained rats. In chronic untrained RB consumers, DLVP significantly increased after direct RB administration to the heart when compared to untrained non-consumers, while this change was not so pronounced in trained rats. Regarding the HR, untrained rats that chronically consumed RB responded to direct RB administration with higher HR than those who did not use to consume RB, while both trained groups, chronic RB consumers and non-consumers of RB, had lower levels of HR when compared to their untrained matched controls. Trained rats also had higher CF than untrained rats. This also suggests that regular training depresses the effects of direct RB on the heart cardiodynamics, while CF is preserved.

As we have previously mentioned, there are no other studies that included administration of ED directly to the isolated rat heart, except for the study that has been recently published by our team (42). In that paper, we have shown that, in trained rats, acute consummation of the RB had a positive inotropic effect (manifested as significantly higher level of dp/dt max and dp/dt min compared to the levels measured in control rats), while chronic administration affected the isolated increase in SLVP, which could be considered the potentially negative impact chronic ED consumption (42). There were no significant differences in cardiodynamic parameters after acute RB consummation (30min before sacrificing) between trained rats that regularly drank RB and those who did not (42), which is in consent with the results presented in this paper (direct instead of acute RB administration). Those results

suggest that acute/direct RB administration affects cardiodynamics to the greater extent in untrained, than in trained rats, i.e. that regular training affects the effects of both acute and chronic ED consumption.

The results regarding the levels of prooxidant species in the coronary effluent showed that, after direct RB administration to the isolated heart, levels of H2O2 were the highest in trained rats: both trained chronic RB consumers and non-consumers had higher H_2O_2 levels than their untrained matched controls, and trained chronic consumers had higher levels of H_2O_2 in comparison to trained non-consumers. Prooxidative effect of direct RB administration was most prominent in trained chronic RB consumers, since they had significantly higher levels of O_2 H_2O_2, TBARS and lower levels of NO when compared to untrained chronic RB consumers. Except in the case of H_2O_2, opposite was observed when trained non-consumers of RB were compared to untrained non-consumers of RB: levels of TBARS were lower and levels of NO higher in trained rats. This suggests that positive effects of regular training on redox state (43) may be diminished by chronic ED consumption. Pusica et al. (42) have shown that both rats who chronically consumed RB, and rats who consumed RB acutely, had significantly increased levels of lipid peroxidation in coronary effluent when compared to control rats, as well as that acute RB consummation increased the levels of TBARS to the greater extent in rats who chronically consumed RB than in those who have not consumed it before, which suggests that in chronic consumers acute ED consumption continues to deteriorate redox status. Interestingly, in this research, untrained rats who consumed RB on a daily basis had lower levels of O_2, TBARS and higher levels of NO in coronary effluent after direct RB administration to the isolated rat heart than untrained rats who did not used to consume RB. Thus, in untrained rats, chronic RB consumption did not negatively affect cardiac oxidative state, which is the opposite to the results related to the cardiodynamics. Finally, the relationship between cardiodynamics and oxidative stress in coronary effluent in our study may be discussed in terms of the relationship between NO levels and CF: in groups that had increased levels of CF levels of NO in coronary effluent were also significantly higher than in their matched controls. This supports the role of NO in endothelial function and vasodilatation (44).

CONCLUSIONS

The conclusion of this study is that the RB could have a potentially negative inotropic effect in chronic consumers. However, it may also be considered as their decreased reactivity to RB ingredients due to adaptation induced by daily consummation. It seems that trained rats respond less to direct RB administration, as well as that chronic RB consumption decreases the effects that direct administration has on the heart, both in trained and untrained rats. Our results suggest that cardiac prooxidative effect of direct RB administration was the most pronounced in trained chronic RB consumers, while chronic RB consumption did not deteriorate oxidative status in isolated hearts of untrained rats.

ACKNOWLEDGEMENTS

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

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HOSPITALIZATION CHARACTERISTICS OF PATIENTS WITH MULTIPLE SCLEROSIS IN THE CLINICAL CENTER OF KRAGUJEVAC

Dragana Andric[1], Snezana Radovanovic[2], Svetlana Radevic[2], Ivana Simic Vukomanovic[2], Aleksandra Arnaut[3], Sanja Kocic[2]
[1] *Clinical Center Kragujevac, Department for organization, planning, evaluation and medical informatics, Kragujevac, Serbia*
[2] *University of Kragujevac, Serbia, Faculty of Medical Sciences, Department of Social medicine*
[3] *University of Kragujevac, Faculty of Medical Sciences, Department of Dentistry, Kragujevac, Serbia*

KARAKTERISTIKE HOSPITALIZACIJE PACIJENATA OBOLELIH OD MULTIPLE SKLEROZE U KLINICKOM CENTRU KRAGUJEVAC

Dragana Andrić[1], Snežana Radovanović[2], Svetlana Radević[2], Ivana Simić Vukomanović[2], Aleksandra Arnaut[3], Sanja Kocić[2]
[1] *Klinički centar Kragujevac, Služba za organizaciju, planiranje, evaluaciju i medicinsku informatiku, Kragujevac, Srbija*
[2] *Univerzitet u Kragujevcu, Fakultet medicinskih nauka, Katedra za Socijalnu medicinu, Kragujevac, Srbija*
[3] *Univerzitet u Kragujevcu, Fakultet medicinskih nauka, Odsek za stomatologiju, Kragujevac, Srbija*

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ABSTRACT

Introduction/Goal: The goal of this paper is to analyze the hospitalization patterns of MS patients in the central and western Serbiain terms of hospitalization days, average length of inpatient stay, hospitalization rate, rehospitalizationpractices and treatment outcomes. Method: The research is designed as a retrospective descriptive epidemiological study. The study uses hospitalization reports from the Clinical Center of Kragujevacfor the time period ranging from January, 2007–December, 2014. Results: The study has revealed that during the given time period, 1.109 MS patients were hospitalized (745 female and 364 male). Women were significantly more numerous (67.2%) (p<0.05).The average rate of hospitalization equaled 70.5 days per 100,000 citizens and was higher with women (94.8 per 100,000) than with men (45.4 per 100,000). The study has revealed a declining trend in hospitalization rates, inpatient days and average length of hospitalization. The number of standard inpatient days decreased while the number of hospitalizations in the day hospital increased (r =-0,905, p=0.002). In terms of age, the study has revealed that the number of hospitalizations decreased and hospitalization length increased with age (p<0.05).There was a statistically significant inverse correlation between age and rehospitalization (r=-0.138, p=0.000).In respect to treatment outcomes, in 93% there was an improvement of the condition. Conclusion: The obtained results indicate that there is a need for establishing a register which could enable long-term monitoring of patients with MS which could eventually provide certain insights into the different aspects of the illness.

Keywords: *multiple sclerosis, hospitalization report, length of inpatient stay, hospitalization rate, rehospitalization*

SAŽETAK

Uvod/Cilj: Cilj ovog rada je analiza hospitalizacije pacijenata obolelih od multiple skleroze u centralnoj i zapadnoj Srbiji u pogledu broja bolničkih dana, prosečne dužine hospitalizacije, stope hospitalizacije, rehospitalizacije i ishoda lečenja. Metode: Istraživanje je dizajnirano kao retrospektivna deskriptivna epidemiološka studija. Istraživanje koristi izveštaje o hospitalizaciji Kliničkog centra Kragujevac za vremensko razdoblje od januara 2007. do decembra 2014. godine. Rezultati: Istraživanje je pokazalo da je u toku datog vremenskog perioda hospitalizovano 1109 pacijenata sa MS-om (745 ženskog i 364 muškog pola). Žene su bile znatno brojnije (67,2%) (p<0.05). Prosečna stopa hospitalizacije iznosila je 70,5 dana na 100.000 stanovnika i bila je viša za žene (94.8 na 100,000) nego za muškarce (45.4 na 100,000). Istraživanje je pokazalo opadajući trend u stopama hospitalizacije, broju bolničkih dana i prosečnoj dužini hospitalizacije. Broj standardnih bolničkih dana se smanjio dok je broj hospitalizacija u dnevnoj bolnici porastao (r=-0,905, p=0.002). U pogledu starosti pacijenata, studija je pokazala da se broj hospitalizacija smanjuje, a dužina povećava kako se povećava starost pacijenata (p<0.05). Utvrđena je i statistički značajna negativna korelacija između starosti i rehospitalizacije (r=-0.138, p=0.000). U pogledu ishoda lečenja zabeleženo je poboljšanje stanja pacijenata kod njih 93%. Zaključak: Dobijeni rezultati pokazuju da postoji potreba za uspostavljanjem registra koji bi omogućio dugoročno praćenje pacijenata sa MS-om, a onda i uvid u različite aspekte bolesti.

Ključne reči: *multipla skleroza, izveštaj o hospitalizaciji, dužina hospitalizacije, stopa hospitalizacije, rehospitalizacija*

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Corresponding author:
Svetlana Radević
University of Kragujevac, Faculty of Medical Sciences,
Department of Social Medicine, Kragujevac, Serbia
Phone: +381 34 306 800
Fax: +381 34 306 800
E-mail: cecaradevic@yahoo.com

INTRODUCTION

Multiple sclerosis (MS), an autoimmune, chronic, and progressive disease, is the most common cause of non-traumatic neurological disability in young and middle-aged adults and thus it places a substantial burden on patients, families and the healthcare system (1,2). According to WHO, there are more than 2.5 million MS patients worldwide with wide prevalence variations (3). The average prevalence of MS in the world for both genders equals 33 on 100,000 citizens with the highest prevalence rate in North America (140 on 100,000 citizens) and Europe (108 on 100,000), and the lowest prevalence rate in Africa (2.1 on 100,000) (4). It is estimated that MS affects 5,000 people in Serbia and the estimated prevalence rate is 65 MS patients on 100,000 citizens (5).

Hospital admission rates in MS patients are higher than in general populations (6). In fact, they are more than twice as likely to visit a medical professional or to be hospitalized despite a significant downward trends over the past 25 years (2). The annual frequency of hospitalizations in the MS population reportedly ranges from 2.7% to 25.8%. Some changes in MS care have occurred over the last 20 years. Diagnostic criteria have changed, diagnostic delays are now shorter, and disease-modifying therapies have been introduced (7). Despite these changes, patients with MS are relatively high users of the healthcare system (8). However, little is known about one of the most financially burdensome elements of their healthcare use – hospitalizations. The deeper understanding of their hospitalization patterns is an imperative in facilitating appropriate resource allocation; it can also help in the evaluation of disease-management strategies and can provide an indication of drug effectiveness (2).

The goal of this paper is to analyze hospitalization patterns of patients with MS in the Clinical Center Kragujevac with special emphasis on the number of hospitalization days, the length of inpatient stay, hospitalization rate, rehospitalizationpractices and treatment outcomes.

METHODOLOGY

Study population, sample and instruments

The research is designed as a retrospective, descriptive epidemiological study. The study uses hospitalization reports from the Clinical Center of Kragujevac which provides medical treatment for patients coming from the central and western Serbia. The selected time period ranges from the 1st of January, 2007 – the 31st of December, 2014 as a basic sample. The hospitalization reports have been used to create an electronic data basis in the Center for biostatistics and informatics at the Institute for Public Health Kragujevac. The total amount of hospitalization reports from the selected time periods equals 420,960. 8,017 hospitalization reports with multiple sclerosis (G35 group VI according to ICD-10) as a primary cause for hospitalization have been isolated.

Taking into consideration the flow of the illness, one part of the patients received medical treatment in the day hospital of the Clinic for Neurology to make sure that the therapy which modifies the natural flow of the illness was administered on regular basis (590 patients, i.e. 6,378 hospitalization reports). Those patients have been excluded from the study and only those patients, who were hospitalized due to certain impairments in their condition, i.e. relapse, have been included. The final sample equals 1,639 hospitalization reports or 1,109 hospitalized patients.

The study uses the following variables: gender, age, birth month, the number of hospitalization days, the number of rehospitalizations and treatment outcomes.

Statistical analysis

All statistical calculations have been performed using a commercial, standard software package SPSS, version 18.0. (The Statistical Package for Social Sciences, SPSS Inc, version 18.0, Chicago, IL). All the data are presented and analyzed by adequate mathematical-statistical methods appropriate for the data type. Chi-square ($\chi 2$) has been used to contrast differences in the prevalence of categorial variables. The correlation between numerical variables has been tested with Pearson or Spearman correlation. Chi-Square, One-Variable test has been used to examine the correspondence between two distributions, the so-called correspondence test. Binomial test has been used to determine whether proportions of one category out of two are different from the specific value (50%). All the results where the probability is less than 5% are considered to be statistically significant.

RESULTS

In the Clinical Center Kragujevac, during the given eight-year time period, 1.109 patients were hospitalized with neurologist-confirmed diagnosis of definite MS (745 female and 364 male). They achieved a total number of 1,639 hospitalizations. There is no statistically significant difference in the number of hospitalized patients per year of observation (χ^2=13.445, df=7, p=0.061) while the difference in the number of hospitalizations is statistically significant (χ^2 =55.277, df =7, p=0.005).

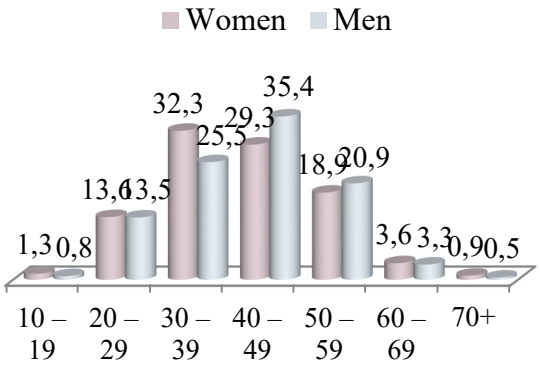
In terms of gender, among hospitalized patients women were significantly more numerous (67.2%) than men (32.8%) with the ratio of 2.04 : 1 ($p<0.05$).

The average age of hospitalized patients equals 41.5 years (SD=10.948). The youngest patient was 15 while the oldest was 83 years old. The average age of hospitalized women was 41 (SD=11.094) while the average age for men was 42 (SD=10.618).

In terms of age groups, the majority of hospitalized patients were in the age group from 40 – 49 (31.3%), then from 30 – 39 (30.1%). The lowest number of patients was in the age group 70+ (0.8%). The highest number of females belonged to an age group from 30 to 39 (32.3%) while the

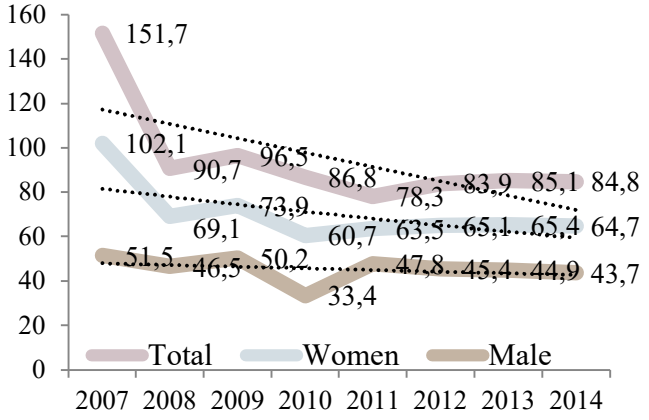
highest number of males was in the age group 40 to 49 years old (35.4%) (Chart 1). The difference in the distribution of patients based on their gender in comparison to the age groups is not statistically significant (χ^2=8.308, df=6, p=0.21).

Chart 1. The distribution of hospitalized patients according to gender and age



The average rate of hospitalization in the given period equaled 70.5 days per 100,000 citizens where the average rate of hospitalization of women (94.8 per 100,000) was significantly higher in respect to hospitalization rate of men (45.4 per 100,000). The hospitalization rate analysis has revealed that there was a declining trend which was more emphasized in case of females (Chart 2).

Chart 2. Hospitalization rate per 100,000 citizens, total and according to gender, 2007 – 2014.



From the total number of 1,109 patients, 757 patients had only one hospitalization (503 women and 254 men), while 352 patients had more than one hospitalization (242 women and 110 men). Women, in average, were hospitalized 1.5 times while men were hospitalized 1.4 times. There is no statistically significant difference in genders in terms of the number of hospitalizations (p>0.05).

However, there is a statistically significant inverse correlation between age and rehospitalization (r=-0.138, p=0.000). As the age of the patients grew, the number of their hospitalizations decreased, i.e. the younger patients were more frequently rehospitalized. The most commonly hospitalized patients belonged to the age groups from 30 – 39 and from 40 – 49.

The total number of inpatient days equaled 19,264. Women had 13,548 and men had 5,716 inpatient days. The number of hospitalization days was the highest during the first year of the observation (4,942 days) and the lowest during the last year (1,767 days). The difference is statistically significant (χ^2= 92.985 df =7, p=0.000). The decline in the number of inpatient days has been also observed in respect to gender. The hospitalization length was reduced from 16.5 days in the first year to 11.7 in the last year of the observation (Table 1).

Table 1. The number of inpatient days (IP days) with average length of hospitalization (AHL)

	Total		Women		Men	
Year of observation	IP days	AHL	IP days	AHR	IP days	AHL
2007	4.942	16,5	3.817	16,8	1.125	15,4
2008	2.265	11,3	1.533	11,4	732	11,1
2009	2.225	10,4	1.460	10,3	765	10,7
2010	2.089	11,9	1.544	12,1	545	11,6
2011	1.941	10,7	1.250	10,9	691	10,3
2012	2.061	10,9	1.324	10,7	737	11,3
2013	1.974	10,4	1.393	11,1	581	9,1
2014	1.767	9,4	1.227	9,8	540	8,7
Total	19.264	11,7	13.548	12,1	5.716	11,1

The average length of hospitalization for females was 12.1 days and for males 11.1 days. There is no statistically significant correlation between gender and hospitalization length (p>0,05).

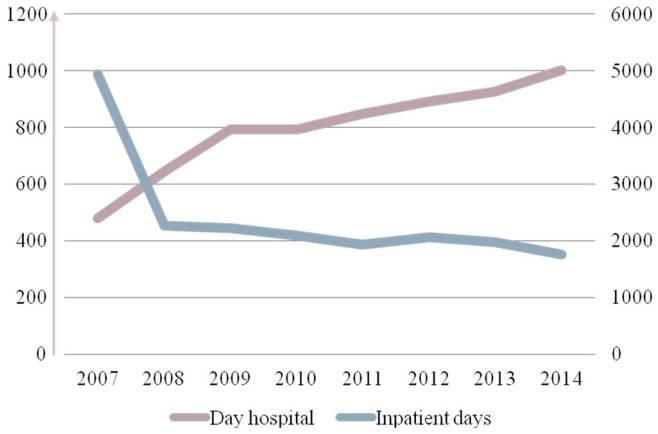
There is a statistically significant correlation between age and hospitalization length (p<0.05). Hospitalization length increases with age. The longest hospitalizations have been recorded with patients who are 80 years old or more, while the shortest hospitalization length has been recorded in the age group from 10 – 19 (Table 2).

The ratio of standard inpatient days and hospitalization days in the day hospital was inversely proportional. During the years, the number of standard inpatient days was decreasing while the number of hospitalizations in the day hospital was increasing (r=-0,905, p=0.002) (Chart 3).

Table 2. The average hospitalization length (AHL) according to age groups

	Total		Women		Men	
Age groups	IP days	AHL	IP days	AHL	IP days	AHL
10-19	176	8,8	124	8,4	52	7,4
20-29	3073	11,9	2307	12,7	766	9,9
30-39	6505	12,6	4915	12,8	1590	11,9
40-49	5556	11,2	3541	11,4	2015	10,7
50-59	3319	11,4	2244	11,5	1075	11,3
60-69	516	11,5	327	10,5	189	13,5
70-79	74	9,25	59	9,5	15	15
80+	45	22,5	31	31	14	14
Total	19.264	11,7	13.548	12,1	5.716	11,1

Chart 3. The ratio of standard inpatient days and hospitalization days in the day hospital



In respect to treatment outcome, in majority of cases the study has detected an improvement of the condition of the patients (93.7%).

DISCUSSION

The first goal of the study was to examine demographic characteristics of hospitalized patients (gender and age) like important indicators of MS occurrence. In the Clinical Center Kragujevac, 67.2% of women and 32.8% of men were hospitalized with the ratio of 2.04:1. The almost similar ratio of hospitalized patients in respect to gender has been detected on the level of the whole country where 4,447 people were hospitalized in 2012 with 67.8% of females and 32.2% of males (the ratio is 2:1). The results of this study in terms of gender distribution are in accordance with the results obtained for neighboring countries and the world: Croatia (2.7:1), Slovenia (2.3:1), Romania (2.1:1) and Bosnia and Herzegovina (2.3:1) (9,10). In Canada, where the prevalence of MS is the highest in the world, during the time period ranging from 1996 to 2006, the ratio of hospitalizations between women and men was 2.6: 1 (11).

The average age of females treated at the Clinical Center Kragujevac was 41, and 42 for male patients. The result of the studies conducted for the other countries of the world are similar to the result obtained through this study. Namely, the average age of hospitalized patients ranges from 33 – 42 for women and from 39 – 46 for men (10-12). The results of the study indicate that hospitalized women are younger in respect to men which is completely expected taking into consideration that females get affected by MS at earlier age in comparison to males (13). The analysis of the age groups according to gender reveals that the highest percentage of hospitalized women is from 30 – 39 years old (32.3%) while the highest percentage of men is aged between 40 – 49 (35.4%). The distribution according to age groups correlates with the data obtained on the national level. In Serbia, in 2012, the highest percentage of hospitalized women was 30 – 39 years old (33%) while for men it was the age group from 40 – 49 (27.2%). The distribution of hospitalized patients according to their age and gender varies among countries. In Croatia and Romania the highest number of hospitalized men belongs to the same age group as in Serbia but the highest number of women belongs to older age group (50 – 59). In Slovenia, the majority of women were in the same age group as female MS patients in Serbia, but hospitalized male MS patients were in the younger group (30 – 39 years) (9).

The second goal of this paper was to analyze hospitalization with special emphasis on hospitalization rate, rehospitalization, the number of inpatient days, hospitalization length and treatment outcomes.

The average hospitalization rate in the given time period was higher in female patients (94.8 per 100,000 citizens) than in male patients (45.4 per 100,000 citizens). In a large study conducted in England which dealt with geography of MS hospitalizations on the sample of 56,681 patients (39,006 women and 17,562 men) hospitalized during the period ranging from 1999 – 2005, the obtained rates were higher in female (22 on 100,000 citizens) than in male patients (10.4 on 100,000) (14). This is similar to our results. Contrary to our results, a study conducted in Canada on the sample of 5,138 hospitalized patients from 1998 – 2006 has revealed that women have lower hospitalization rate than men (2). It is possible that faster progression of the illness detected in men is the reason for such results, i.e. there are bigger chances of physical comorbidity with male MS patients. According to the data from 2012 in the Republic of Serbia, the hospitalization rate was 61.8 per 100,000 citizens (women 81.6 and men 40.l8) while the hospitalization rate in the surrounding countries is lower: Croatia – 51.9; Slovenia – 12.9; Romania – 15.3. The similar rates have been detected in the Scandinavian countries like Denmark (35.5) and Finland (18.9) (9,15).

One of the aims of the study was to determine the correlations between gender/age with rehospitalization patterns. The study has revealed that there is no correlation between gender and rehospitalization while there is a correlation between age and rehospitalization. Namely, younger patients are more often rerhospitalized. The possible explanation may

lie in the fact that there were certain patients who had been diagnosed with MS recently and those patients with MS in its early stages are more prone to relapses which then gradually decline over time (13). There are no studies on the possible correlations between gender/age and rehospitalization frequency so there is no data to compare with the results of this study. This is the additional significance of this research.

In the given time period, every patient was hospitalized 1.5 times in average which is close to an average on the global level (16). The results of a study conducted in Germany reveal that every MS patients is hospitalized 0.57 times (17) and in England the same value is 1.96 times in average (2).

The average length of hospital treatment in the given time period was 11.7 days. In respect to gender, our results reveal that women spent 12.1 days while men spent 11.1 days in average. The study has revealed that there are no correlations between gender and hospitalization length. However, the results show that there is a positive correlation between age and hospitalization length. Older patients tend to be hospitalized longer which can be explained by higher comorbidity and higher functional addiction of older patients. The longest hospitalization have been detected in the age group 80+ (women 31 and men 14 days) and the shortest in 10 – 19 (women 8.4 and men 7.4 days). The similar results were obtained in a study conducted in Canada where older people, who have MS longer, stayed in hospitals longer amounts of time; gender and clinical form of the illness proved not to be influential in terms of hospitalization length in this study (2). The results show that the values of average length of hospitalization in the Clinical Center Kragujevac (the population of the Central and West Serbia gravitates towards it) are more similar to the results in surrounding countries and some countries of the Western Europe (France – 4.9; Bulgaria – 5; Spain – 6.7; Romania – 6.9; Slovenia – 7.1; Hungary – 10.7; Croatia – 13.6 and Finland – 18.6) than to the results on the national level– 23 days (9,15).

The differences in hospitalization rates and hospitalization length between countries may be the result of the differences in economic level and the introduction of the new system of financing medical institutions through the diagnostically-related groups (DRGs). The advantage of DRGs is that it motivates the reduction of costs per patient through: the reduction of hospital treatment length, diagnostic and treatment procedures and the focus on day hospitals.

One of the aims of this study was to examine the relations between standard inpatient days and a day hospital treatments. The increase in day hospital visits is directly linked to the decrease in inpatient days. It is a result of the fact that patients who are already diagnosed with MS during the further flow of the illness are more commonly treated in day hospitals where during the relapse they are administered pulse corticosteroid and immune-modular therapy. In any case, treatments in day hospitals have positive impact on life quality of patients and reduce the costs of standard hospital treatments.

This study examined hospitalization patterns of patients with MS in Clinical Center Kragujevac. This may be a limitation in generalizing the findings. However, the main limitation of this study is that there is no registry of patients with multiple sclerosis that could be used as a gold standard for making comparisons to the data from hospitalization reports. Despite these limitations, this is the first study conducted in MS hospitalization patterns in Serbia. Electronic hospitalization reports are very important data source about hospitalized patients. The possibility of making errors while entering data is reduced to a minimum because there are control mechanisms in the application forms.

In Serbia, there are no regional or national Registers for MS which makes long-term monitoring difficult. Such registers have been founded in Denmark, Norway, Sweden, Italy (The Multiple Sclerosis Database Network), USA (NARCOMS Register and the National Register for MS) and Germany (18,19). The registers for MS follow the medical condition of the diseased during time and take care of the implementation of guides for their care and treatment, the evaluation of their progress and strive to detect and overcome all the lacks and deficiencies in providing medical treatment to people suffering from MS.

CONCLUSION

Based on the results obtained through this study, the following conclusions can be drawn:

1. Demographic characteristics of MS patients, like gender and age, are strong indicators of MS occurrence. Hospitalized patients are more commonly women and middle-aged people.
2. The average hospitalization rate for women is twice higher than for men.
3. Gender has no influence on rehospitalization. Younger patients are more commonly hospitalized.
4. The average inpatientdays is similar for both genders.
5. Hospitalizations of older people last longer.
6. The increase in treating patients in day hospitals is directly related to the reduction in hospitalization length.
7. In majority of cases,a treatment results in an improvement of a medical condition.

The obtained results indicate that there is a need for establishing a register which could enable long-term monitoring of patients and deepen the understanding of MS by providing certain insights into the different aspects of the illness. This could further help in making decisions relevant for MS to different institutions included in the process.

CONFLICT OF INTEREST

All authors declare no support from any organisation for the submitted work; no financial relationships with any organisations that might have an interest in the submitted work; no other relationships or activities that could appear to have influenced the submitted work. The authors declare that they have no conflict of interest.

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OLANZAPINE - FOCUS ON THE CARDIOMETABOLIC SIDE EFFECTS

Miroslav Mitrovic [1], Tamara Nikolic [2], Marko Turnic [3] and Dusan Djuric
[1] Pharmanova doo Beograd, Serbia
[2] University of Kragujevac, Faculty of Medical Sciences, Department of Pharmacy, Serbia
[3] Adoc doo, Beograd, Serbia

OLANZAPIN - FOKUS NA KARDIOMETABOLIČKE EFEKTE

Miroslav Mitrović [1], Tamara Nikolić [2], Marko Turnić [3] i Dušan Đurić
[1] Pharmanova doo, Beograd, Srbija
[2] Univerzitet u Kragujevcu, Fakultet Medicinskih nauka, Katedra za farmaciju, Srbija
[3] Adoc doo, Beograd, Srbija

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ABSTRACT

In this article, we review the recent findings concerning weight gain, diabetes mellitus (DM), hyperlipidemia, cardiovascular side effects in patients receiving olanzapine. It will consider the OLZ is associated with an increase in metabolic syndrome or cardiovascular events, and knowledge of these risks is crucial for further monitoring of patients with OLZ-treatment. Although it is one of the most commonly prescribed and effective AATPs, olanzapine causes the most weight gain and metabolic impairments in humans. As noted with glucose abnormalities and antipsychotics, olanzapine has the greatest propensity for causing proatherogenic hyperlipidemia. The mechanism of dyslipidemia with OLZ is poorly understood, but OLZ has been shown to increase lipogenesis, reduce lipolysis, and enhance the antilipolytic effects of insulin in adipocytes. Olanzapine can induce cardiomyopathy in selected patients.

Taken together, all mentioned data indicate that interventions aimed at the amelioration of obesity and cardiovascular illness need to be as multipronged and complex as the contributing psychosocial, behavioural, and biological factors that make obesity and cardiovascular illness more likely in patients with severe mental illness, including schizophrenia.

Keywords: *olanzapine, weight gain, dyslipidemia, cardiovascular disease*

SAŽETAK

U ovom članku, razmatramo nedavna saznanja u vezi dobijanja u težini, šećerne bolesti (DM), hiperlipidemije i kardiovaskularnih neželjenih efekata kod pacijenata koji su na terapiji olanzapinom. Uz pretpostavku da je olanzapin u vezi sa povećanim rizikom za nastanak metaboličkog sindroma i kardiovaskularnih događaja, od presudnog je značaja poznavanje potencijalnih rizika kako bi se sproveo monitoring ovih pacijenata. Iako olanzapin (OLZ) predstavlja jedan od najčešće propisivanijih i najefektnijih atipičnih antipsihotika, ipak nosi i najvišu stopu rizika za nastanak metaboličkih smetnji kod ljudi. Olanzapin uzrokuje poremećaj metabolizma glukoze, povećava lipogenezu, smanjuje lipolizu, povećava antilipolitičke efekte insulina u adipocitima što uzrokuje dislipidemiju i doprinosi visokom proaterogenom potencijalu olanazapina. Opisani su i slučajevi kardiomiopatije usled primene olanzapina.

Sumarno posmatrano, literaturni podaci ukazuju na neophodnost složenih preventivnih i terapijskih protokola kod pacijenata sa mentalnim poremećajima, uključujući i shizofreniju, a koji su na terapiji olanzapinom, usmerenih na smanjenje psiholoških i bioloških faktora rizika za kardiovaskularne bolesti.

Ključne reči: *olanzapin, povećanje telesne težine, dislipidemija, kardiovaskularna oboljenja*

INTRODUCTION

Antipsychotics were first introduced into clinical practice in the 1950s and approved in 1996 by the FDA.

Antipsychotics are now frequently used beyond their core indications of schizophrenia and bipolar disorder. Off-label use of antipsychotics is frequent in major depressive disorder and other mood disorders, anxiety disorders and dementia (1-4). In recent years, the atypical antipsychotics or second-generation antipsychotics have become the drugs of choice for acute psychoses. They are "atypical" as they are differentiated from "conventional" or first-gen-

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Corresponding author:
Dusan Djuric, PhD, Assoc. Professor,
Department of Pharmacy, University of Kragujevac,
Faculty of Medical Sciences,
Svetozara Markovica 69. 34000 Kragujevac, Serbia,
phone: +38134306800,
E-mail: duca1duca@gmail.com.

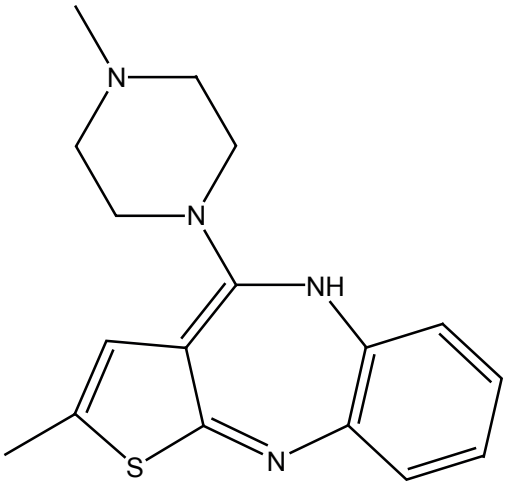

Figure 1. Olanzapine ($C_{17}H_{20}N_4S$) structure

eration antipsychotics based on their clinical profile. They have fewer side effects regarding extrapyramidal symptoms when compared to typical antipsychotics. Schizophrenia is a devastating illness that affects up to 1% of the population; it is characterized by a combination of positive symptoms, negative symptoms, and cognitive impairment. The atypical antipsychotic (APs) drugs have become the most widely used agents to treat a variety of psychoses because of their superiority with regard to safety and tolerability profile compared to conventional/'typical' APs (1-4).

Olanzapine (Figure 1) (OLZ; $C_{17}H_{20}N_4S$; 2-methyl-4-(4-methyl-1-piperazinyl)-10H-thieno[2,3-b][1,5]benzodiazepine) is an antipsychotic drug of the thienobenzodiazepine class that is effective in treating schizophrenia and acute manic episodes, and in preventing the recurrence of bipolar disorders (5). It is as has been shown to have some therapeutic advantages over other classic antipsychotics in terms of symptom reduction and its adverse event profile. It has a low propensity to cause extrapyramidal effects or sustained increases in prolactin levels (6, 7). Nevertheless, treatment with OLZ is associated with a higher risk of weight gain and, more extensively, metabolic syndrome than other typical and atypical antipsychotics (8).

Olanzapine is known as MARTA (multi-acting receptor targeted antipsychotics). Proposed mechanisms of action of atypical antipsychotics as well as olanazapine, is dopaminergic and serotonergic modulation and induction

Table 1. Potential clinical efficacy, benefits and possible effects related to the mechanisms of action of olanzapine. EPS, extrapyramidal symptoms

Mechanism of action	Clinical efficacy	Possible effects
D_2 antagonism	↓ positive symptoms	EPS ↓negative symptoms ↑ cognitive symptoms hyperprolactinaemia
D_2 partial agonism	↓ positive symptoms ↓ negative symptoms ↓ cognitive symptoms	little or no EPS behavioral activation
5-HT_2A antagonism	↓ negative symptoms	↓ EPS ↑ weight gain hyperphagia and obesity ↑metabolic syndrome
5-HT_1A partial agonism	↓ negative symptoms ↓ cognitive symptoms ↓ anxiety symptoms ↓ depressive symptoms	No adverse effects
Muscarinic antagonism	↓ EPS	↓ anticholinergic symptoms e.g. dry mouth, constipation, tachycardia
Muscarinic agonism	↓ psychotic symptoms ↓ cognitive symptoms	No adverse effects
Adrenergic α_1 and α_2 antagonism	No effects on negative and positive buhevior symptoms	↓adrenergic symptoms e.g. orthostatic hypotension and consequently induced tachycardia hyperphagia and obesity ↑metabolic syndrome
Histamine H_1 antagonism	↓ positive symptoms	↑ sedation ↑ weight gain hyperphagia and obesity ↑metabolic syndrome
Glutamate modulation	↓ positive symptoms ↓ negative symptoms ↓ cognitive symptoms ↓ illness progression	No adverse effects

of neuroplasticity. OLZ shares higher affinity to 5-HT_2A receptors than D_2 receptors (high 5-HT_2A/D_2 ratio). In comparison to the other atypicals, olanzapine presents high affinity for serotoninergic 5-HT_2A, 5-HT_2C, 5-HT_3, and 5-HT_6 receptors, medium affinity for dopaminergic D_1, D_2, D_3, D_4, D_5, and muscarinic M_1–M_5 receptors, low affinity for adrenergic α_1 and α_2 receptors, and the highest affinity for histamine H_1 receptors (olanzapine is the most potent histamine H_1 antagonist known) (*Table 1*) (9, 10).

Although the usual dose range for olanzapine is 5–15 mg/d, there are no standard reference values with respect to the expected concentrations of olanzapine after therapeutic administration. In clinical studies, steady state blood (plasma) concentrations of olanzapine are rarely over 150 ng/mL, but the potential for toxicity has been suggested at concentrations as low as 100 ng/mL (11).

Approximately 85 % of an oral OLZ dose is absorbed but, as about 40 % is inactivated by first-pass hepatic metabolism, its oral bioavailability is about 60 %. OLZ has a mean half-life in healthy individuals of 33 hours (range 21-54 hours). Peak plasma concentrations are reached within six hours. The drug is approximately 93 % bound to plasma proteins, mainly albumin (90 %) and alpha 1-acid glycoprotein (77 %). Its distribution volume is 16.4 ± 5.1 L (X±SD). Mean apparent plasma clearance is 26 L/h (range 12-47 L/h). After the administration of [14C]-OLZ in a single load pharmacokinetic study, approximately 87 % of the radioactivity was excreted, with 30 % appearing in the faeces and 57 % in the urine (10-12).

Psychiatrists have gradually prescribed antipsychotic drugs in come to reduce psychiatric symptoms, and extrapyramidal symptoms and tardive dyskinesia occur less frequently with atypical agents. Beside that, these medications may present a different set of adverse effects (10-12).

In this article, we review the recent findings concerning weight gain, diabetes mellitus (DM), hyperlipidaemia, cardiovascular side effects in patients receiving olanzapine. Evident increase in cardio-metabolic side effects during olanzapine administration inevitably leads to a question of its effect on the cardiovascular system of diseased psychotic patients. Bearing in mind that the treatment of these diseases is most often lifelong, it is clear that the degree of exposure of treated patients with olanzapine is long lasting, and for these reasons, not only the positive therapeutic effects of the drug must be seriously analyzed, but also the degree of impact of its adverse effects on the general health of the diseased.

Atypical antipsychotics are responsible for increasing cardiovascular risk by more than 30% in schizoid patients (10). Similar results were also seen in the increased risk for the development of metabolic syndrome and the risk of diabetes. Given that these drugs have a significant place in consumption, their impact on the health budgets in the countries where they are used is high (10). The treatment of cardiovascular and metabolic complications caused by the use of atypical antipsychotics is inevitable and this cost represents additional pressure on health funds (11, 12).

It will consider the OLZ is associated with an increase in metabolic syndrome or cardiovascular events, and knowledge of these risks is crucial for further monitoring of patients with OLZ-treatment. Recognizing these complications in addition to the necessary monitoring, opens space for the development of new drugs or procedures that need to eliminate or at least significantly reduce the consequences of cardiometabolic complications caused by the application of atypical antipsychotics, in this case olanzapine.

WEIGHT GAIN

Weight gain and obesity are critical issues in patients with schizophrenia. The abnormal nutritional status and 'developmental' obesity in schizophrenia have been described more than half-century ago. To date, there are over 2600 papers indexed by Medline on the topic of weight gain and obesity in schizophrenia. Patients with schizophrenia consume unhealthy food (13-15). A recent meta-analysis of 31 studies about dietary patterns identified a high consumption of saturated fat and low intake of fruit and dietary fiber. Also, controlled investigation indicated that patients with schizophrenia had higher daily intake of calories and protein per kilogram of body weight, which was independent of BMI. Social isolation, low interest in social achievement, and unmarried and unemployed status are common in patients with schizophrenia and lead to decreased levels of participation in sports and other mainstream physical activities (14).

The importance of neurotransmitter and hormonal effects in the weight accrual of patients with schizophrenia has been studied for olanzapine. Leptin levels were similar in schizophrenia patients and healthy control subjects with comparable BMIs (15). An inverse association was observed for baseline weight and leptin levels with the extent of weight gained during 3–6 months of antipsychotic monotherapy (16). This study suggesting a drug--mediated disruption of the hypothalamic appetite control, as well as prevous animal study, also indicated that olanzapine increased the orexigenic NPY mRNA and decreased the anorexigenic POMC in the arcuate nucleus (17) and upregulated ghrelin and ghrelin signaling, leading to hyperphagia.

Histaminergic transmission is involved in energy homeostasis and also seems to be relevant to antipsychotic-related weight gain, as the extent of histamine H1 receptor (H1R) antagonism of antipsychotics was the best predictor of the degree of weight gain in clinical studies. 5-HT2c antagonism has been implicated in antipsychotic drug-related weight gain too, and most second-generation antipsychotics, especially for olanzapine, which is a potent 5-HT_2c antagonist. Synergistic effects between the blockade of D_2 receptors and 5-HT_2a or 5-HT_2c receptors might play a key role in triggering a cascade of events that lead to increased energy intake and weight gain (18-22).

In a retrospective analysis of 1191 patients diagnosed with schizophrenia or schizoaffective disorder treated with olanzapine (23), approximately 15% of subjects had a rapid change of ≥7% body weight during the first 6 weeks of treatment, with a mean weight gain of 1.8–3.2 kg (about 4% of the baseline body weight) during the first 2 weeks. Increasing evidence indicates that antipsychotics have greater orexigenic weight gain potential in children and adolescents than in adults (24) and that young patients receiving antipsychotics are at increased risk of being or becoming overweight or obese. A recent comparison of pooled long-term studies (median followup = 201 days) of patients treated with olanzapine indicated a mean weight gain of 4.8 kg in adults, but 11.2 kg for adolescents (22-24). A debate is continuing with regard to the inverse relationship between baseline BMI and antipsychotic- induced weight gain. Pooled longitudinal data in patients treated with olanzapine (mean modal dose = 13.3 mg/day) indicated that the slowing in the rate of weight gain observed after 2–4 months of treatment was greatest for patients who were obese at baseline (25, 26).

METABOLIC SYNDROME

Also, atypical antipsychotics such as olanzapine often induce excessive weight gain and type 2 diabetes. In the past decade there have been numerous case reports, retrospective studies, and epidemiological investigations suggesting that certain OLZ may be associated with a great risk of DM. Although it is one of the most commonly prescribed and effective AATPs, olanzapine causes the most weight gain and metabolic impairments in humans. By World Health Organization criteria, 10.1% of patients developed diabetes mellitus (DM) after only 6 weeks of antipsychotic therapy (P =0.016) (27-28).

However, the mechanisms underlying these drug-induced metabolic perturbations remain poorly understood. Clinical studies have suggested the involvement of multiple genes, including those that encode the histamine, α-adrenergic, and serotonin (5-HT) receptors. Among them, *Htr2c* encodes the 5-HT 2C receptor, which acts in the brain to regulate food intake, body weight, and glucose metabolism (29, 30). Blockade of HTR2C signaling in mice leads to hyperphagia and obesity (31) that resemble AATP-induced metabolic symptoms in humans. Rates of metabolic syndrome are significantly higher in schizophrenia than in the general population. OLZ, as an atypical antipsychotic, has been associated with detrimental effects on metabolic risk factors. The pathomechanisms that underlie metabolic syndrome as a complication of antipsychotic treatment are not fully understood. Probably, the effects of OLZ on histamine H1, serotonin 5-HT2c and muscarinic M3 receptors are thought to play a central role. In addition, antipsychotics may have direct effects that cause leptin insensitivity as well as on appetite regulation (30-33).

Olanzapine, after clozapine, shows the strongest association with the risk for diabetes. Other studies have demonstrated significant changes in blood glucose levels with antipsychotic therapy despite not measuring other markers of glucose-insulin homeostasis. Lindenmayer and colleagues randomized 157 patients with schizophrenia to 14 weeks of therapy with clozapine, haloperidol, olanzapine, or risperidone. Fasting blood glucose was measured at baseline, at 8 weeks, and at end point (34, 35).

Olanzapine was associated with a significant increase in fasting glucose at end point (mean change from baseline 14 mg/dL, P < .02). Glycosylated hemoglobin (HbA1c) has been used as a surrogate marker for insulin resistance and glycemic control in the assessment of some antipsychotic medications. Olanzapine is associated with elevations in HbA1c levels. In some patients, a direct effect of olanzapine on pancreatic β-cell function may be present (36) but more commonly the accumulation of body weight with central adiposity, and the resultant increase in insulin resistance, would explain the development of diabetes mellitus over time.

Using the Food and Drug Administration (FDA) adverse events database, the risk of diabetes mellitus was increased for olanzapine, risperidone, clozapine and quetiapine, whereas a decreased risk was found for haloperidol, aripiprazole and ziprasidone (34, 36).

Both typical and atypical antipsychotics can cause significant increases in cholesterol, triglycerides and low-density lipoprotein cholesterol. The risk of hyperlipidaemia differs for individual antipsychotics. The risk of hyperlipidaemia appears higher for patients under treatment with clozapine and olanzapine (37) particularly for younger patients. *Simpson* and colleagues found that olanzapine, but not ziprasidone, significantly increased total cholesterol (median change from baseline to end point at 6 months, 13 mg/dL, P = .03) and low-density lipoprotein (LDL) cholesterol (median change from baseline to end point at 6 months, 17 mg/dL, P = .04) (37, 38).

In a further study, lipids were measured at multiple time points over 28 weeks, and olanzapine was associated with significant increases in total cholesterol. Olanzapine has been shown to be associated with unfavourable lipid derangements compared with aripiprazole. As noted with glucose abnormalities and antipsychotics, olanzapine has the greatest propensity for causing proatherogenic hyperlipidaemia. The mechanism of dyslipidaemia with OLZ is poorly understood, but OLZ has been shown to increase lipogenesis, reduce lipolysis, and enhance the antilipolytic effects of insulin in adipocytes (37-40).

CARDIAC DYSFUNCTION

Schizophrenia is associated with increased mortality and reduced life expectancy, with cardiovascular disease being the most frequent cause of death. Antipsychotics have detrimental effects on different risk factors for cardiovascular disease (41).

Patients with schizophrenia are at high risk of metabolic syndrome, a cluster of risk factors for cardiovascular disease. Prevous cohort study confirmed that 40% percent of 3470 French patients with schizophrenia (mean age at inclusion 39.3 years) died during an 11-year follow-up period. In the Olmstead County study, patients with schizophrenia had a significantly increased mortality, in particular from cardiovascular disease (42-44). Patients with schizophrenia frequently have multiple risk factors for cardiovascular disease. Firsty, excess prevalnce od obesity and increased BMI in patients with mental disorder in one of the major factor for develpment of cardiovascular disease (44). Monitoring glucose is crucial, and patients with risk factors for diabetes mellitus (e.g., obesity, family history of diabetes) who are starting treatment with olanzapine should undergo fasting blood glucose testing at the beginning of treatment and periodically during treatment. Beside abdominal obesity, dyslipidemia, hypertension and diabetes mellitus and have additive effects on an individual's risk of developing diabetes mellitus and cardiovascular disease. Rates of smoking are higher in schizophrenia patients than in the general population. Schizophrenic patients who smoke are at higher risk of death as well as death from cardiovascular disease than schizophrenic patients who do not smoke (45, 46).

Furthermore, typical and atypical antipsychotics are associated with a significant dose-related increase in the risk of sudden cardiac death. Some of the cases of sudden cardiac death have been associated with cardiac arrhythmia, in particular torsade de pointes, possibly secondary to a prolongation of the QT interval (45-47). But, OLZ was initially linked to potential QTc prolongation. Extensive studies have shown modest Qtc interval prolongations for these patients that are most likely not clinically relevant and with no evidence for increased mortality by disturbed Ot-changes by OLZ (48). But, study conducted by Morissette indicate that olanzapine possesses direct cardiac electrophysiological effects. They demonstrated that olanzapine can prolong cardiac repolarization in a reverse frequency- dependent manner by blocking time-dependent outward potassium current involved in cardiac repolarization. In fact, they shown that olanzapine 5.7 μM caused a significant prolongation of cardiac repolarization (13%) (49).

Adverse hemodynamic effects are possible with olanzapine, particularly orthostatic hypotension, bradycardia and tachycardia (50), which are most likely owing to adrenergic α1 blockade. Because of the antagonism of α1-receptors, OLZ is associated with orthostatic hypotension and consequently induced tachycardia, but in lowpotency. This risk of a OLZ-hypotension is not pronounced for olanzapine. Especially in eldery, OLZ is associated with increased risk of cardiovascular disease and also, this drug has also been associated with venous thromboembolism and pulmonary embolism. Again, OLZ seems to be associated with a low risk (51, 52).

Interestingly, some of previous clinical study, reported about the effects of olanzapine on inducing of specific cardiac disorders, such as myocarditis and cardiomyopathy. These rare but potentially fatal complications of antipsychotic treatment, myocarditis and cardiomyopathy are associated with antipsychotics are most frequently seen under treatment with clozapine, but can also occur with olanzapine treatment (53). Malays reported about 28-year-old male patient with bipolar disorder who taking olanzapine and lorazepam for almost 10 years and presented with weight gain, diabetes, and anasarca. Evaluation of the patient revealed he was in heart failure. The reason for his heart failure was ambiguous and an investigation into it revealed negative results. Literature search conducted showed a few reported cases of putative olanzapine induced cardiomyopathy and this is one of them. Well, cardiomyopathy is a less known side effect of OLZ (54). The main proposed mechanism for cardiomyopathy is myocarditis and myopericarditis by direct toxicity or allergic reaction. In animal studies, three months of olanzapine treatment was shown to induce ventricular hypertrophy of the heart. Cardiac lesions induced by neuroleptic drugs in the rabbit (55).

In practice, olanzapine induced cardiac disorder should be considered in a patient who develops dyspnoea or other signs of the heart failure (56). Olanzapine should be withdrawn in those cases and treatment of heart failure should be done on a routine basis. Olanzapine can induce cardiomyopathy in selected patients. Early recognition and cessation of the drug is required to prevent irreversible myocardial damage. Cardiac functional assessment is periodically required for the patients taking antipsychotics. Cautious use is required in patients with known heart disease.

CARDIOMETABOLIC MONITORING OF PATIENTS WITH OLZ-TREATMENT

Patients with severe psychiatric disorders and with antipsyhotic therapy are at increased risk of cardiovascular disease, although some of this risk may be conferred by the psychiatric disease or lifestyle. Weight gain, obesity, metabolic and cardiovascular disorders in patients with schizophrenia and other mental disorders are associated with a host of adverse physical and psychiatric outcomes, as well as with OLZ treatment (*Table 1*). Therefore, body weight and related metabolic indices need to be monitored routinely and targeted as part of a comprehensive and integrated care programme in patients with OLZ-treatment (33, 35, 57, 58).

Ideally, a treatment algorithm should start with healthy lifestyle education/instruction and with lower cardiometabolic risk antipsychotic than OLZ. It is reccomendation that only consider higher risk agents, such as olanzapine, when it has become clear that the physically safer medication is not sufficiently effective or tolerated. Psychiatric care providers should aim for balancing acute

and long-term efficacy as well as tolerability, and engage other medical specialists as needed to improve the overall well-being of patients with schizophrenia. American Diabetes Association and American Psychiatric Association suggested that optimal management of patients with schizophrenia should include baseline assessment on their weight, waist circumference, blood pressure, blood glucose level and lipidogram and family history on obesity, diabetes, dyslipidemia, hypertension and cardiovascular illness (33, 35). During the first three months, weight gain should be monitored on monthly basis, while biochemical analysis should be performed after the first three months, and then once a year. In patients with significant weight gain, increase of blood glucose level or dyslipidemia, the first intervention should be switch to another antipsychotic. If necessary, a patient should be referred to an endocrinologist and advised on changing their life style (57).

Suggested algorithm for cardiometabolic monitoring of patients treated with OLZ is precisely described by *Manu* and coworkers. Suggested algorithm for managing antipsychotic-related weight gain is power tool for prevention of cardiovascular disease and for decreasing of mortality in patients with psychotic disorders. Nevertheless, it is also important to consider that antipsychotics are currently the only medication class with evidence for effective treatment of psychosis (58-60).

CONCLUSION

Taken together, all mentioned data indicate that interventions aimed at the amelioration of obesity and cardiovascular illness need to be as multipronged and complex as the contributing psychosocial, behavioural, and biological factors that make obesity and cardiovascular illness more likely in patients with severe mental illness, including schizophrenia. The use of olanzapine in the treatment of psychosis, especially schizophrenia, has revolutionized the treatment of these diseases, but has led to the opening of a question and price that we have to pay in terms of the development of cardio-metabolic complications and their impact on the quality of life of the diseased. By clearly recognizing the complications and mechanism of their emergence, we are given the opportunity to better implement new therapeutic procedures, by introducing drugs of similar therapeutic potential, but with a significantly lower impact on the development of cardiometabolic complications by applying adequate hygienic dietary regimes and changing lifestyle habits.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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PRIMARY PERCOUTANEOUS CORONARY INTERVENTION ON UNPROTECTED LEFT MAIN CORONARY ARTERY WITH STAGED COMPLEX BIFURCATIONAL TREATMENT: A CASE REPORT

Ivan Simic[1,2], Vladimir Zdravkovic[1,2]
[1] Department of Internal medicine, Faculty of medical sciences, University of Kragujevac, Kragujevac, Serbia
[2] Department of Cardiology, Clinical Center Kragujevac, Kragujevac, Serbia

PRIMARNA PERKUTANA KORONARNA INTERVENCIJA NA NEPROTEKTOVANOM GLAVNOM STABLU LEVE KORONARNE ARTERIJE SA ODLOŽENIM KOMPLEKSNIM BIFURKACIONIM TRETMANOM: PRIKAZ SLUČAJA

Ivan Simić[1,2], Vladimir Zdravković[1,2]
[1] Katedra za internu medicine, Fakultet medicinskih nauka, Univerzitet u Kragujevcu, Kragujevac, Srbija
[2] Klinika za kardiologiju, Klinički centar Kragujevac, Kragujevac, Srbija

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ABSTRACT

We present the case of patient with ST elevation myocardial infarction in cardiogenic shock with primary percutaneous coronary intervention of Left anterior descending coronary artery and Left main coronary artery with staged complex procedure on Left anterior descending/Diagonal branch bifurcation in Culotte manner. This case shows that "the simpler, the better" approach of only infarct related artery revascularization may be applied in acute patients with cardiogenic shock and optimal clinical and hemodynamic response on revascularization and intra-aortic balloon pump. But, complete revascularization should be done in staged procedure and later, a control coronary angiography with intravascular ultrasound assistance is mandatory.

Key words: *cardiogenic shock, PPCI, left main, IABP, IVUS*

SAŽETAK

Predstavljamo slučaj pacijenta sa akutnim infarktom miokarda sa elevacijom ST segmenta u kardiogenom šoku i urađenom primarnom perkutanom koronarnom intervencijom na prednjoj levoj silaznoj koronarnoj arteriji i glavnom stablu leve koronarne arterije kao i odloženom kompleksnom procedurom na bifurkaciji prednje leve silazne arterije i dijagonalne grane i to Culotte tehnikom. Ovaj slučaj pokazuje da 'što jednostavnije, to bolje' pristup u smislu revaskularizacije samo infarktne arterije može biti primenjen kod akutnih pacijenata u kardiogenom šoku i optimalnim kliničkim i hemodinamskim odgovorom na revaskularizaciju i primenu intraaortne balon pumpe. Ali, kompletna revaskularizacija bi trebalo da bude učinjena u odloženoj proceduri sa kasnijom kontrolnom koronarnom angiografijom obavezno praćenom intravaskularnim ultrazvukom.

Ključne reči: *kardiogeni šok, PPCI, glavno stablo, IABP, IVUS*

ABBREVIATIONS

DES-drug eluting stent;
DG-diagonal branch coronary artery;
EBU-extra backup catheter;
FFR-fractional flow reserve;
IABP-intraaortic balloon pump;
IVUS-intravascular ultrasound;
LAD-left anterior descending coronary artery;
LCA-left coronary artery;
LCX-left circumflex coronary artery;
LM-left main coronary artery;
NC-noncompliant balloon
PCI-percutaneous coronary intervention;
RCA-right coronary artery;
RI-intermedial branch;
STEMI-ST elevation myocardial infarction

INTRODUCTION

Case report

A 62 year old man was admited to our Coronary Care Unit with chest pain lasting for 2 hours. ECG showed anterior ST elevation myocardial infarction (STEMI). The following risk factors for coronary artery desease were present: hyperlypidaemia, smocking, heredity. Physical examination revealed silent heart sounds, pulmonary crepitant rales and hypotension (TA=100/60 mmHg), prograding to cardiogenic shock. He was quickly trasfered to our Catheterisation Laboratory. Coronary angiography (radial approach) showed (Fig. 1) significant stenosis (70%) of left main (LM), occluded left anterior descending coronary artery (LAD) in proximal part, ostialy reduced intermedial

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Corresponding author:
Ivan Simic,
address: Vase Carapica 6/22, 34 000 Kragujevac,
Tel: 0641619940,
E-mail: ivansimickg@gmail.com.

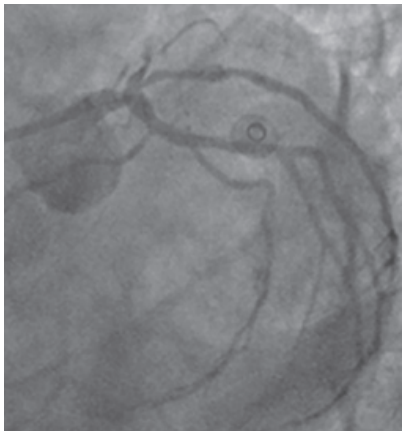

Fig. 1 Initial coronary angiography LAO/ CAUD projection

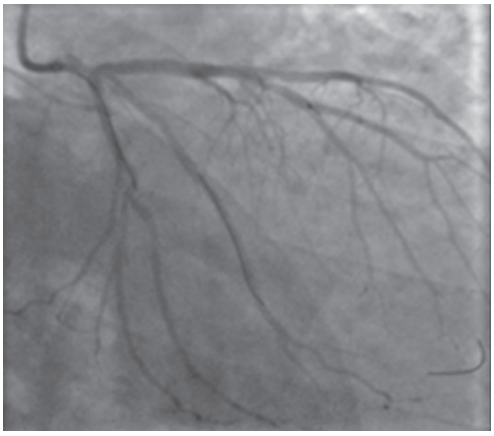

Fig. 2 Suspected hasenes in ostial LAD/ LM

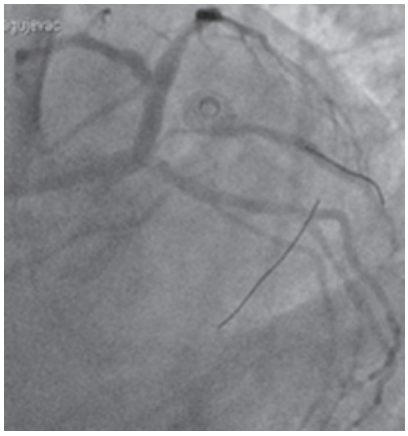

Fig. 3 Post stenting coronary angiography LAO/CAUD projection

branch (RI) (70%), relatively desease free left circumflex (LCX) and borderline stenosis on proximal right coronary artery (RCA). We decided to instantly perform Primary percutaneous coronary intervention (PPCI) on LAD: we placed Intraaortic balloon pump (IABP) via left femoral artery due to cardiogenic shock. We canulate Left coronary artery (LCA) with Extra backup guiding catheter (EBU GC) 3.5 6 Fr. Runthrough guide wire (GW) succesfuly passed occlusion on LAD. Second Runthrough GW was placed in a big diagonal branch (DG). We implanted the stent Liberte Monorail 3.5x20 mm in LAD/DG at 14 atm. Due to suspected hasenes (Fig. 2) in ostial LAD/ LM we implanted another bare-metal stent (in the absence of adequate drug eluting stent), Liberte Monorail 4.5x16mm at 16 atm., with overllap, to the ostium of the Left main and postdilated it with the noncompliant (NC) balloon 4.5x15 mm at 22 atm. The final angiography showed seriously pinchted LAD and RI (Fig. 3, Fig.4). We decided to perform percutaneous coronary intervention (PCI) of these two bifurcations in staged procedure before discharge.

After 3 days in Coronary Care Unit, IABP was removed.

We performed planed PCI on 10th day of hospitalisation, after clinical stabilisation.

Via left femoral access we cannulated LCA with EBU 4.0 7 Fr GC. After numerous atempts Runthrough GW passed through the stents struts and was placed in distal LAD. Second Runthrough GW protects DG. After postdilatation of the first implantet stent in proximal optimisation technique (POT) manner with NC baloon Sprinter 3.75x15mm at 18 atm, we succeded to put the small 1.5 mm balloon in LAD. Then we performed the stent fenestration with Sprinter 2.75x15mm NC baloon, and implanted drug eluting stent (DES) Resolute Integrity 3.0x18mm at 14 atm in LAD in Culotte maner. Final kissing balloon inflation was performed with two NC Sprinter baloons: 3.75x15 mm in DG and 3.0x12 mm in LAD at 10 atm (Fig. 5). Control angiography showed optimal result on LAD/ DG bifurcation (Fig. 6). Then, we put the wire in RI from DG and third wire in LCX. Balloon dilatation on ostial RI was performed with Sprinter balloon 2.5x15 mm at 16 atm. Finally, the post dilatation of stent in LM was done with NC balloon Sprinter 4.5x15mm at 20 atm. The final angiographic results was excellent with TIMI 3 flow in treated arteries.

The control angio performed six months later showed borderline in-stent restenosis in LM and "Culotte" with optimal result (Fig. 7).

- We have used Intravascular ultrasound (IVUS) (Fig. 8) for final conclusion – optimal medical therapy:

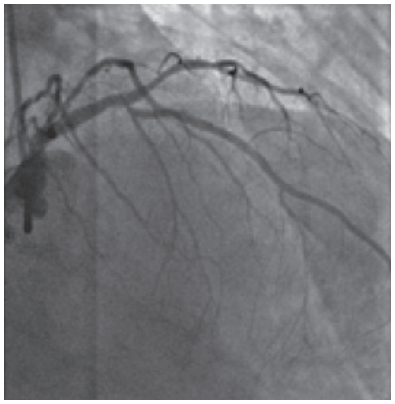

Fig. 4 Post stenting coronary angiography RAO/CRAN projection

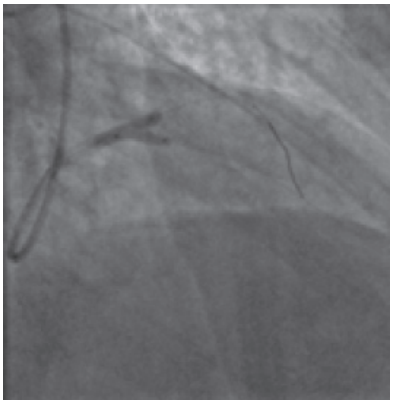

Fig. 5 Final kissing balloon inflation

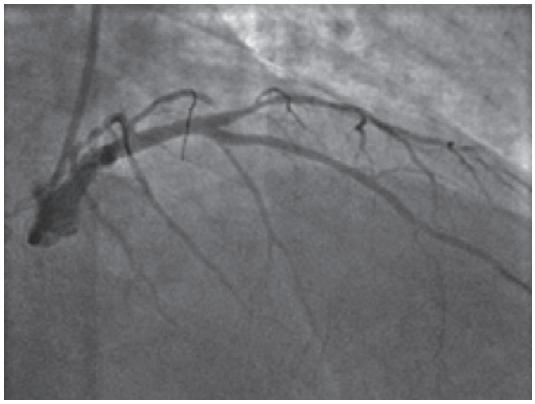

Fig. 6 Final result on LAD/DG bifurcation

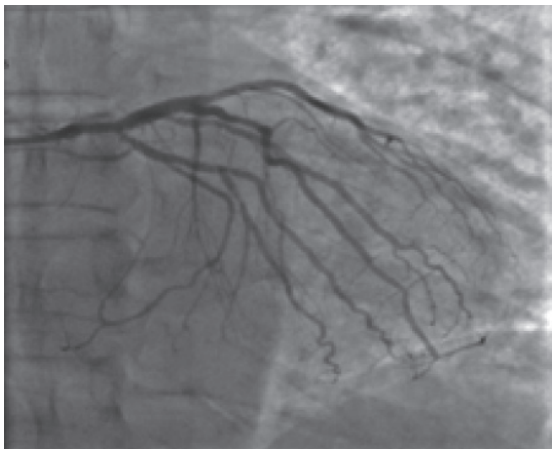

Fig. 7 Control angio - six months later

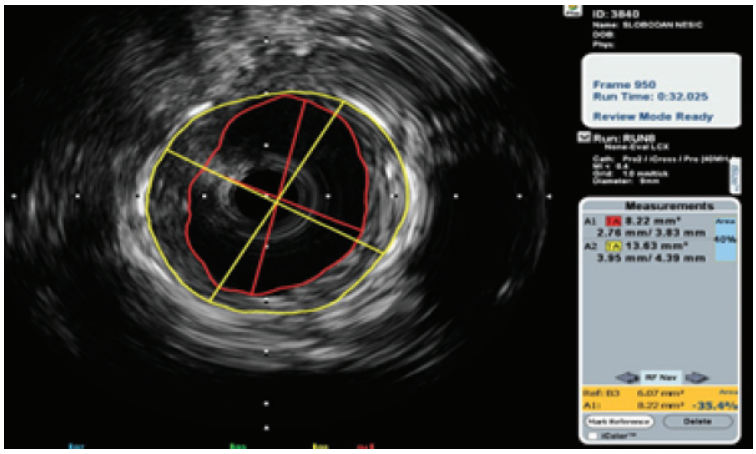

Fig. 8 Intravascular ultrasound measurements

- IVUS LM-LAD: restenosis up to 40%; LM diam. 2.76 mm; min. area 8.22 mm^2
- IVUS LCx: ostial stenosis up to 60%; LM diam. 2.3 mm; min. area 6.07 mm^2

DISCUSSION

First of all, this case study clearly demonstrated the safety and efficacy of radial access for primary percutaneous coronary intervention in patients with ST segment myocardial infarction (STEMI), even when dealing with complex angiographic finding. Radial access is recommended arterial site in PPCI with class of recommendations IIb and level of evidence B [1]. In the RIVAL study and the RIFLE STE ACS Trial, radial access reduced mortality in patients with STEMI [2, 3]. A meta-analysis of randomized controlled trials demonstrated that in STEMI patients undergo PPCI, the radial approach is associated with better outcome and should be preferred access site for experienced operators [4].

According to the current ESC guidelines, 50% of STEMI patients have significant multivessel disease [1]. It is recommended that only the infarct-related artery should be treated during primary PCI [5,6]. Non-culprit stenosis treatment is possible only in STEMI patients with cardiogenic shock with multiple, subocclusive (>90% diameter reduction) stenoses and with no positive responses on PPCI of culprit lesion. Having in mind above mentioned, we initially performed PPCI on culprit lesion only, but with bailout PCI of LM due to coronary artery dissection. In non-urgent patients, coronary artery bypass grafting (CABG) has been recommended treatment option for significant LM stenosis [7]. SYNergy Between PCI With TAXUS and Cardiac Surgery (SYNTAX) Study demonstrated that MACEs were not significantly higher in PCI group of patients compared with CABG group, with higher incidence of TVR [8]. But, in STEMI patients, primary PCI of unprotected left main coronary artery is still controversial. In systematic review on the available literature and a meta-analysis on the treatment of PPCI of ULMCA, Vis et al. [9], demonstrated that the observed 30-day all-cause mortality was higher in patients presenting with cardiogenic shock (55%) compared with patients without cardiogenic shock (15%)(relative risk: 3.74, 95% confidence interval [CI]: 2.95 to 4.76, p <0.001), regardless of stent type.

Since our patient had good hemodynamic response on PCI and IABP, we did not perform complex PCI on LAD/DG bifurcation and staged this procedure. Intra-aortic balloon pump (IABP) is strongly recommended in patients with cardiogenic shock (Class Ib) in the current ESC guidelines for treatment of STEMI [1]. IABP has hemodynamic benefits as a result of afterload reduction and diastolic augmentation with improvement of coronary perfusion.

When patient was clinically stabilized, before discharge, we performed a complex PCI of LAD in Culotte manner, via right femoral artery and 7 French system. After such complex procedures, we performed control coronary angiography after six months with IVUS assistance. Few studies have validated IVUS measurements as anatomic predictors for the hemodynamic significance of left main lesions [10-12]. Repeated revascularization due to restenosis may be deferred in patients with left main minimal luminal cross-sectional area (MLA) ≥ 6.0 mm^2 (in our case 8,22 mm^2). The IVUS MLA value that best predicted fractional flow reserve (FFR) < 0.80 was 4.8-6 mm^2. There was no advantage of using DES in large vessels (≥3,5 mm) for preventing a hard endpoint, whereas the usage of DES resulted in a significant reduction of TVR in patients with STEMI in the ICAS registry [13]. The routine usage of intravascular imaging in primary PCI remains controversial but we strongly support the mandatory use of IVUS for left main percutaneous coronary interventions and even later evaluation.

CONCLUSIONS

- Prompt intervention can reduce the adverse effects of the most devastating myocardial infarctions.
- Quick establishing of the flow in STEMI is crucial.

- "The simpler, the better"- if possible, the complicated procedures should be prolonged until the patient becomes stable.
- In the settings of good hemodynamic response to revascularization and IABP, there is no need for complet urgent revascularisation of STEMI patients in cardiogenic shock. Complex bifurcational treatement should be avoided initialy but carefully planed.
- IVUS can be of a great assistance for the final decision.

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APPLICATION OF NONVASCULAR INTERVENTIONAL RADIOLOGY PROCEDURES IN THE TREATMENT OF IATROGENIC URETERAL INJURIES

Igor Sekulic[1], Jelena Stevanovic[1], Srdjan Prodanovic[2], Jelena Boskovic Sekulic[3], Berislav Vekic[4,5] and Nemanja Rancic[1]
[1]Institute for Radiology, Military Medical Academy, Belgrade, Serbia
[2]Institute for Rheumatology, Belgrade, Serbia
[3]Center for Urgent medicine, Clinical Center Kragujevac, Kragujevac, Serbia
[4] University of Kragujevac, Faculty of Medical Sciences, Kragujevac, Serbia
[5]Department of Surgery, Clinical Centre "Dr Dragiša Mišović- Dedinje", Belgrade, Serbia

PRIMENA NEVASKULARNIH INTERVENTNIH RADIOLOŠKIH PROCEDURA U LEČENJU JATROGENIH POVREDA URETERA

Igor Sekulić[1], Jelana Stevanović[1], Srđan Prodanović[2], Jelena Bošković Sekuluć[3], Berislav Vekić[4,5] i Nemanja Rančić[1]
[1]Institut za radiologiju, Vojnomedicinska akademija, Beograd, Srbija
[2]Institut za reumatologiju, Beograd, Srbija
[3]Urgentni centar, Klinički centar Kragujevac, Kragujevac, Srbija
[4] Univerzitet u Kragujevcu, Fakultet medicinskih nauka, Kragujevac, Srbija
[5]Klinika za hirurgiju, Kliničko bolnički centar "Dr Dragiša Mišović-Dedinje", Beograd, Srbija

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ABSTRACT

Introduction. *He most common ureteral injuries are iatrogenic injuries. Diagnosis of ureteral lesions includes ultrasound, computer tomography, intravenous urography, anterograde and retrograde ureterography. For a definitive diagnosis it is necessary to determine the existence of the extralumination of contrast media from the ureter. Minor ureteral injuries can be treated with nonvascular interventional radiology procedures.* ***Case presentation.*** *We have presented two patients with iatrogenic ureteral injuries. Injury in the first patient occurred at the sigmoid colon resection and partial resection of the bladder, whereas in the second patient the lesion was formed as a result of cesarean section. In both patients, there was a history of previously conducted interventions, clinical picture included fever and pain, a diagnosis was made by intravenous and anterograde urography. Patients were treated with interventional radiology procedures and they have been definitely cured.* ***Conclusion****. Methods of nonvascular interventional radiology can be successfully applied in the treatment of minor iatrogenic ureteral injuries.*

Keywords: *ureter, iatrogenic trauma, anterograde urography, ureteral endoprosthesis*

SAŽETAK

Uvod. *Najčešće povrede uretera su jatrogene prirode. Dijagnoza ovih lezija uključuje primenu ultrazvuka, skenera, intravenske urografije, anterogradne i retrogradne ureterografije. Za definitivnu dijagnozu neophodno je utvrditi postojanje ekstraluminacije kontrastnog sredstva iz uretera. Minimalne lezije uretera mogu se lečiti procedurama nevaskularne interventne radiologije.* ***Prikazi slučajeva.*** *Prikazana su dva slučaja jatrogenih povreda uretera. Kod prvog pacijenta povreda je nastala tokom resekcije sigmoidnog kolona i parcijalne resekcije mokraćne bešike, dok je kod drugog pacijenta povreda nastala tokom carskog reza. Kod oba pacijenta u anamnezi je postojao podatak o prethodnoj intervenciji. Klinička slika je uključivala temperaturu i bol, a dijagnoza je postavljena intravenskom i anterogradnom urografijom. Pacijenti su tretirani procedurama interventne radiologije, kojima su i definitivno izlečeni.* ***Zaključak.*** *Metode nevaskularne interventne radiologije mogu se uspešno primeniti u lečenju minornih jatrogenih povreda uretera.*

Ključne reči: *ureter, jatrogena povreda, anterogradna urografija, ureteralna endoproteza*

ABBREVIATIONS

US - ultrasonography
IVU - intravenous urography
CT - computer tomography
INR - interventional nonvascular radiology

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Corresponding author:
Nemanja Rancic, MD, PhD,
Institute for Radiology Military Medical Academy, Belgrade
Tel: +381638524443
E-mail: nece84@hotmail.com

INTRODUCTION

Injuries to the ureter are one of the most serious complications of any abdominal or pelvic procedure (1). Ureteral injuries can be divided by cause into traumatic (usually the result of traffic accidents) (2) and iatrogenic which are far more common and they are the cause of 75% of ureteral lesions (3, 4).

Pelvic or abdominal surgeries, including laparoscopy and ureteroscopy, are a risk of ureteral injuries (5-11). According to the extensive retrospective studies, the most common interventions that occur as a cause of ureteral trauma are: urological (42%), gynecology and obstetrics (34%), while general surgical interventions are the cause of ureteral lesions in 24% of cases (8). The most common injuries are of the distal ureter (74%) (3). Other authors showed that gynaecological surgery is involved in about 52-82% of all cases with ureteral injuries: it was estimated that the risk of ureteral injuries is approximately from 0.0027 to 0.09% during emergency Caesarean section, 0.5-8% during intra-cesarean hysterectomy, 0.04-3% during abdominal hysterectomy and 0.02-0.47% during vaginal hysterectomy (9, 10).

Clinical symptomatology that occurs includes the pain by type of renal colic, pain in the loins, fever, and weakness (1, 7). This clinical picture including the data on the recently performed surgical, gynecology or any procedure, should arouse suspicion of ureteral lesion.

Ureteral lesion diagnosis is very difficult because clinical manifestations are non-specific. Radiology plays a critical role in diagnosis of these injuries (12). Diagnostic procedures include ultrasonography (US) with a potential findings of ureterohydronephrosis or periureteral pathological fluid collection; intravenous urography (IVU) with the findings of the extralumination of contrast medium; anterograde and retrograde urography in which the extralumination of contrast medium as well as the lesion location are clearly detected; and computer tomography (CT) urography with the findings of contrast medium extravasation, hydronephrosis, ureteric obstruction, urinary ascites, and periureteral collection or localized fluid collections such as urinoma (13-15). If the diagnosis is still equivocal after IVU and/or CT urography, then bilateral retrograde pyelography should be performed. It is the most accurate imaging exam to determine the location, type, and degree of iatrogenic ureteric injury.

Ureteral lesions of I-IV grade (Table 1 (16-18)), that is, those lesions with less than 2 cm can be successfully treated by the method of nonvascular interventional procedure, while for those of V grade, that is, with more than 2 cm, surgical treatment is required.

The aim of case study of these two patients is to show that lesions of the ureter, less than 2 cm, can be quite successfully treated with nonvascular interventional radiology procedures that are comfortable for the patient. One patient previously underwent a general surgery, and the other patient previously underwent gynecological and obstetric surgery. In both patients, as a result of these interventions, ureteral lesions occurred.

Case I

A secretion of urine through the abdominal drains in the postoperative course was detected in the patient of 61 years of age, who underwent sigmoid colon resection and partial resection of the bladder due to cancer of the sigmoid colon. The patient underwent cystoscopic exploration and the insertion of "JJ" endoprosthesis was attempted. The IVU was performed and the extralumination of contrast agent at the level of distal third of the left ureter was detected. The patient was referred to the department of nonvascular interventional radiology of the Institute of Radiology, Military Medical Academy, for further treatment.

Ultrasound shows left kidney without dilatation of pyelocaliceal system (Figure 1). Ultrasound guided anterograde urography shows the puncture site, non-dilated dorsal calyx and pyelocaliceal system without dilatation (Figure 2). As guidance for puncture under ultrasound guidance, dorsal Malpighi's pyramid in the interpolar region was used. After inserting the guide catheter, nephrostomy tube of caliber 8F (French) was placed and nephrotomography showed the left ureter to the prevesical part wherein the extralumination of contrast medium was detected (Figure 3). After 10 days, the control nephrotomography showed the maintenance of the extralumination of contrast medium but to a lesser extent. The guide wire and the manipulation catheter were inserted through the existing nephrostomy tube. After passing by the place of ureteral injury with the guide wire and inserting it into the bladder, the modified ureteral prosthesis was placed as a function of external-internal drainage. Modification of the original 'pig-tail' catheter of caliber 7F was performed by the scalpel and three holes, positioned in the pyelon and the proximal ureter, were made. The distal end of the catheter, with the original holes, was positioned in the bladder. In the next follow-up, anterograde urography showed normal mobility of endoprosthesis without the extralumination of contrast medium (Figure 4). Therefore, a drainage bag was removed, and endoprosthesis was left as a function of the internal derivation of urine.

Case II

This case describes a 30 year old patient who reported the pain in the left lumbar lodge and the increased body temperature after giving a birth by C-section. Three days after the delivery, CT urography was performed and on that occasion the extralumination of contrast agent from the left ureter with the creation of perirenal urine was observed. Female patient was subsequently admitted to the Clinic for Urology Military Medical Academy as an emergency. At the department of nonvascular interventional radiology she underwent antegrade urography and the extralumination of contrast medium was confirmed (Figures 5a and 5b). The guide wire and the manipulation catheter were inserted and after passing by the injury site, the original ureteral endoprosthesis in the function

of internal drainage caliber 8F was placed. (Figures 6a and 6b). In this way, the extralumination of urine was prevented. Percutaneous drainage of urine was performed and it was completed after seven days (Figures 7a and 7b). After a month, the follow-up anterograde urography showed the complete patency of the canal system without the extralumination (Figures 8a and 8b and 9a and 9b).

The extraction of endoprosthesis (Figures 10a and 10b) was performed and the follow-up ultrasound examination did not show any signs of hydronephrosis.

Table 1. Grading ureter lesions

I grade	Hematoma: contusion or hematoma without devascularization
II grade	Laceration: < 50% transaction
III grade	Laceration: > 50% transaction
IV grade	Laceration: complete transection with < 2 cm of devascularization
V grade	Laceration: avulsion with > 2 cm of devascularization

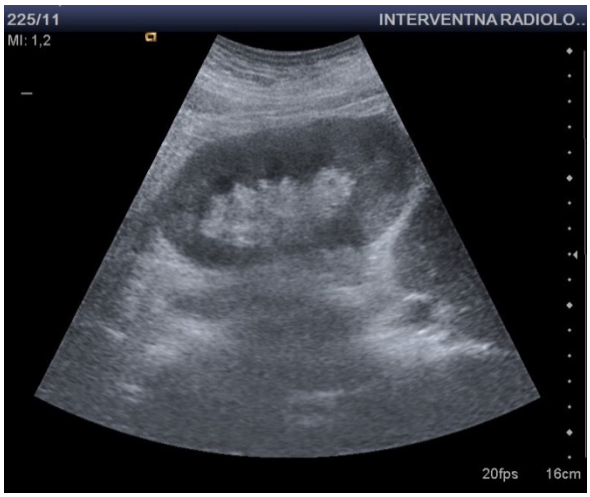


Figure 1. Normal morphology of the left kidney without dilatation of pyelocaliceal system

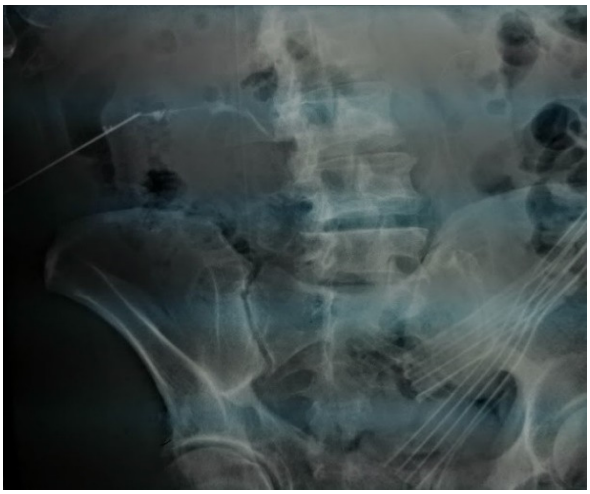


Figure 2. Punctuated non-dilated dorsal calyx

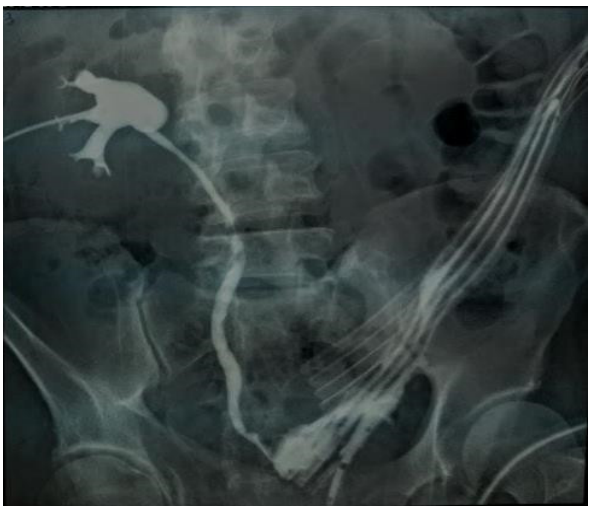


Figure 3. Nephrotomography was showed the location of the extralumination

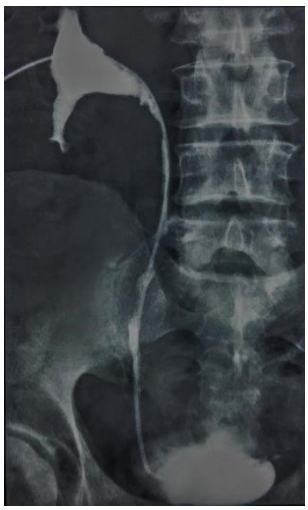


Figure 4. Normal patency of the endoprosthesis without the extralumination

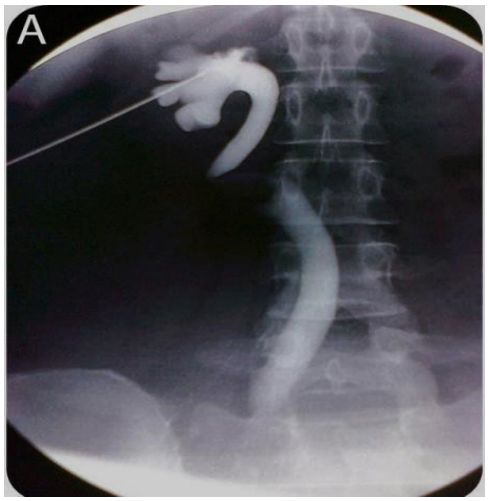


Figure 5a. Dilation of pyelocaliceal system and the ureter of the left kidney

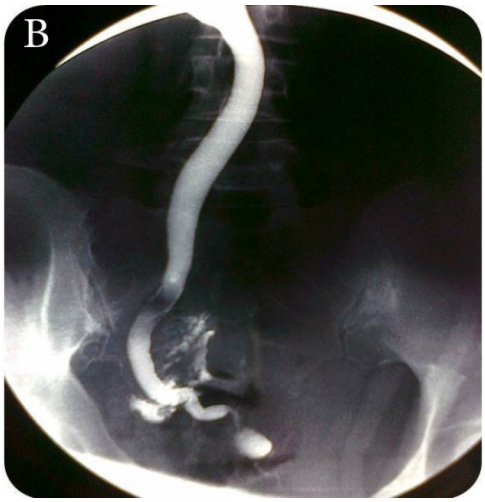
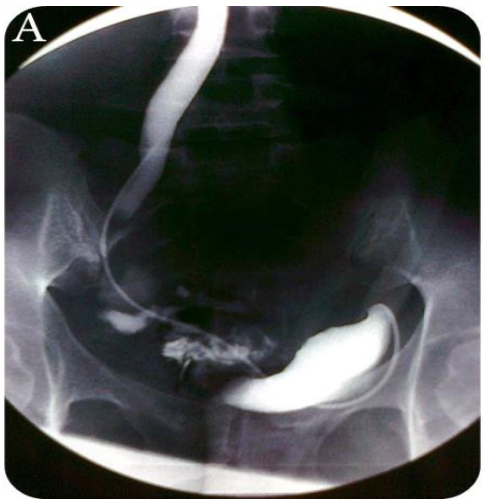
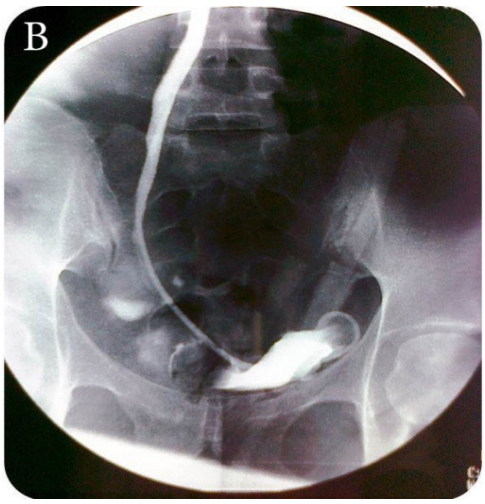
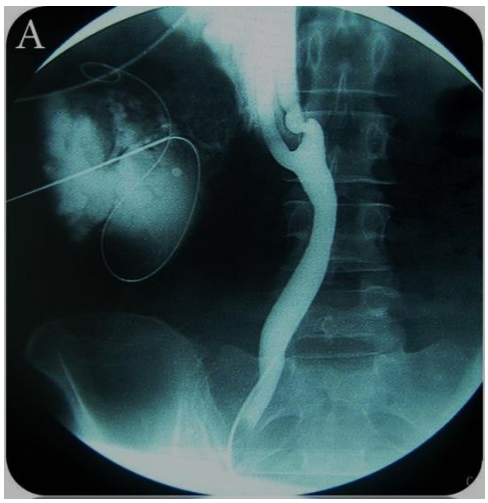


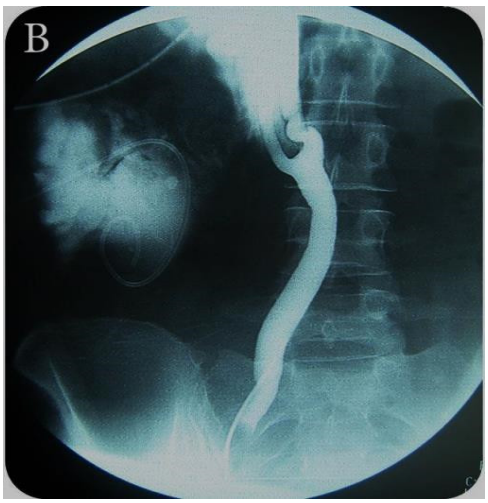
Figure 5b. The extralumination of contrast medium in the prevesical region



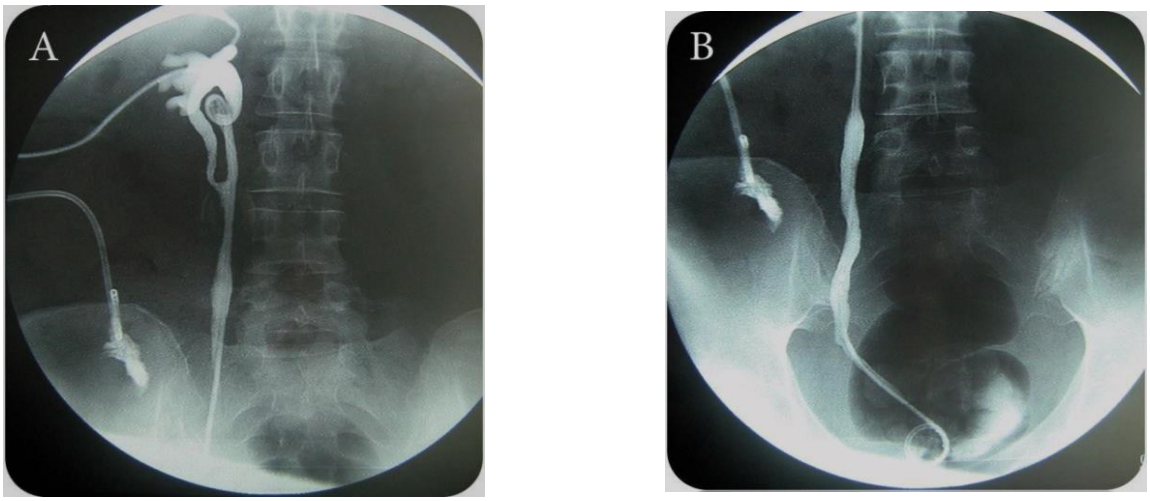


Figures 6a and 6b. Passing by the location of the injury, placing the external-internal ureteral endoprosthesis

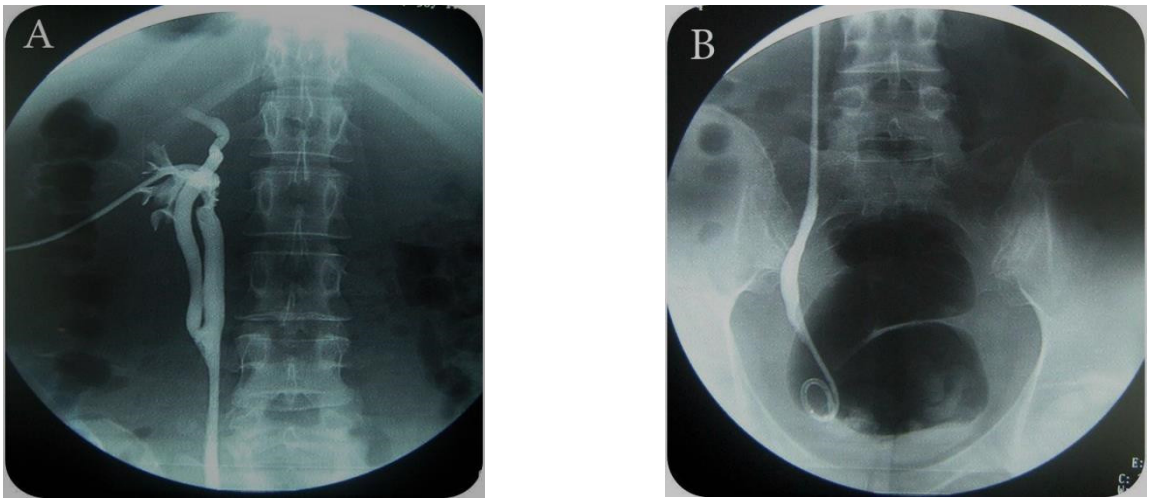




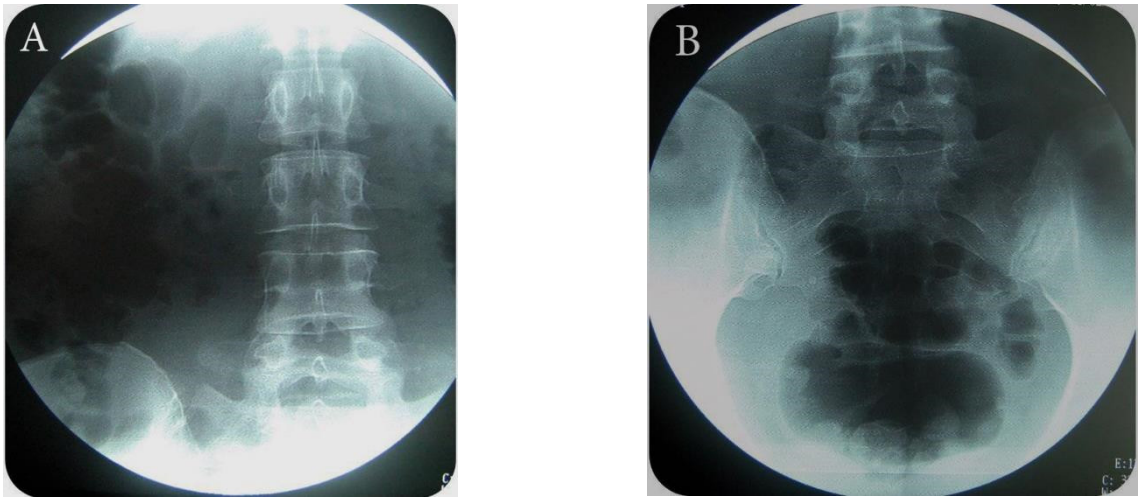
Figures 7a and 7b. Placing catheter of caliber 6F for the purpose of the drainage of urine



Figures 8a and 8b. After a month, the follow-up anterograde urography.
Pyelon duplex et ureter fissus (incidental findings)



Figures 9a and 9b. After a month, follow-up before extraction of endoprosthesis



Figures 10a and 10b. Contrast medium lagging was not detected post-micaceous

DISCUSSION

Ureteral injuries are serious complications of any abdominal or pelvic procedure. These complications can result in high morbidity (increased hospital stay, secondary invasive interventions, reoperation, potential loss of renal function and deterioration patient's quality of life) and mortality for these patients (19-21). In one new study, mortality rate was even 7.3% (1). High mortality rate in this study is attributed to delayed presentation, deranged renal function tests on admission, missed ureteral injuries and presence of postoperative complications mainly surgical site infections.

The risk factors for iatrogenic ureteric injury include nature and indication of the abdominal or pelvic surgery, experience of the operating surgeon and patient related factors (pelvic adhesions from previous surgeries, history of pelvic radiation, enlarged uterus, pelvic malignancy, pelvic endometriosis, and anatomical abnormalities) (22, 23).

Ureteral injuries cause a significant mortality rate, therefore prompt diagnosis and then adequately care for the patient are of crucial importance. The three-quarters of ureteral injuries are of iatrogenic nature, that is, they occur as a result of an intervention in the abdomen or pelvis, whether it is a urological, gynecological and obstetrical, or general-surgical intervention. The risk of iatrogenic lesion is principally due to the close anatomic rapport between ureter, visceral organs and vascular structures. In fact, the distal third of the ureter is the most affected tract (51%), followed by the proximal and the middle third (30%, 19%, respectively) (24).

If the patient, his relatives or attending physician (in the case of hospitalized patient) provide information about recently performed procedure in the abdomen or pelvis, and the patient shows no clinical signs of the ureteral injury, then this is one of the possibilities we should surely think about, and the patient should be accordingly referred to the adequate diagnostics, whether it be the US as a preliminary examination or CT, IVU, anterograde urography and retrograde urography, on the basis of which, if the extralumination of contrast medium is diagnosed, it is possible to make a definitive conclusion.

Adequate and timely diagnostics is certainly one of the most important steps in the successful care of the patient. After the diagnosis, the treatment method is discussed on. This may include conservative methods, if it is a lesion of the ureter in the first degree, that is, the hematoma only. If it is a serious injury, methods of treatment that are in the selection include the surgical procedures or some of the INR techniques. The aim of our study was to show based on these two cases, that minor ureteral lesions can be very successfully treated with the interventional nonvascular radiology (INR) techniques and procedures, which are effective, significantly more comfortable and much cheaper. The latter is probably the most crucial for those patients in whom it is possible and justified to apply the INR as a method of treatment, because the fact that it is a iatrogenic injury means that there is a comorbidity in these patients, that is, it is quite possible that they have been exhausted with the previous interventions, and each quart of these patients means a great contribution to healing and reducing mortality and morbidity.

The first case described a patient that underwent resection of the sigmoid colon and partial resection of the bladder due to carcinoma of sigmoid colon. As a result of this intervention, there was a lesion of the ureter. Ureteral stents were placed by cystoscopy, but without significant effect, and therefore the patient was sent for treatment at the department of nonvascular interventional radiology. Firstly, the drainage catheter 6F was placed for the purpose of derivation of urine. After ten days, the modified ureteral endoprosthesis of 6F was placed, as a function of the internal-external derivation of urine. Since the extralumination of contrast medium or dilation of pyelocaliceal system was not detected in the follow-ups, the drainage bag was removed and endoprosthesis was left for the purpose of the internal derivation, and the satisfactory effects of treatment were achieved in the patient.

The second case involved a patient that gave birth by Caesarean section and on this occasion a ureteral injury occurred. This is quite a rare complication of cesarean section with the incidence 0.1-0.27% (25-27). After canalicular passing the location of injury, we placed the external-internal ureter-endoprosthesis for the purpose of derivation of urine. Also, since we detected the presence of peri-renal-ureteral pathology collection (urine), we performed firstly the puncture, and then we placed catheter 8F for the purpose of drainage. Follow-up radiography firstly showed partial and then complete regression of fluid collection, therefore the drainage catheter was removed. Since follow-up anterograde urography showed patency of endoprosthesis without dilation of pyelocaliceal system, the prosthesis was removed. Upon the removal of prosthesis, follow-up radiography showed patency of ureter without the extralumination of contrast medium and contrast medium lagging was not detected post-micaceously as well.

Finally it should be noted that, in recent years, the growth of the performance of endoscopic surgery (laparoscopy and ureteroscopy) leads to an increase in the incidence of ureteral injuries (28, 29), therefore we must consider the recommendations which may contribute to a reduction in the incidence of iatrogenic ureteral lesions and which include good knowledge of ultrasound anatomy of the abdomen and pelvis as well as possibly performing preoperative IVU, including placement of ureteral catheters for procedures to be carried out near the ureter (30).

CONCLUSION

Iatrogenic ureteral injuries are still an unsolved problem in abdominal surgery. Their management is controversial for the lack of guidelines. Radiologists may play a key-role in the diagnostic ureteral injuries and also in treatment. Presenting these cases, we have shown that minor ureteral lesions

can be successfully treated with nonvascular interventional radiology procedures that are comfortable for the patient.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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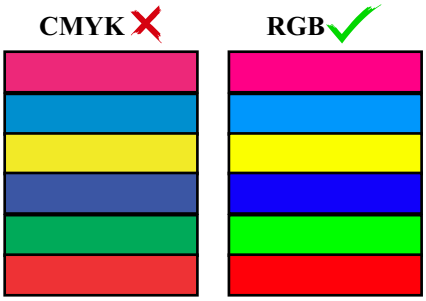

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P.O. Box 124
Tel. +381 (0)34 30 68 00 • Tfx. +381 (0)34 30 68 00 ext. 112
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